Improvement of growth and hematological profile of juvenile silver barb *(Barbonymus gonionotus)* through dietary probiotic (*Bacillus* sp.) supplementation

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Abstract

A 75 days feeding trial was conducted to evaluate the influence of dietary supplementation of multi-strain (*Bacillus sp.*) probiotic (22×10^9 CFU/g) on growth and hematological parameters of *Barbonymus gonionotus*. A total 270 uniformed size (length 2.40±0.16 cm and weight 4.25±0.01g) fishes were equally and randomly distributed in nine (three treatments each with three replication) glass aquaria of 200L capacity. The control (T_1) group was fed with normal feed (Diet 1) and treatment T_2 and T_3 were fed a feed containing 1% (Diet 2) and 3% (Diet 3) probiotic respectively. Biometry and blood samples of experimental fishes were collected at the end of the experiment. Significantly ($p<0.05$) best growth performance such as length gain, weight gain, % weight gain, specific growth rate and best feed utilization parameters such as feed conversion ratio and protein efficiency ratio values were observed in fish fed with 1% probiotic supplemented diet than other treatments. Moreover, higher values in hemoglobin, hematocrit, red blood cell and white blood cell were observed in 1% probiotic treated group (T_2). Other hematological indices like mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration values supported the fact that fish fed with probiotic treated diet were healthier. The result suggests that supplementing diet with 1% probiotic could be effectively used as feed supplement for improving growth and health status of *B. gonionotus*.

Keywords: Fish, Mixed probiotic, Dietary supplement, Feed utilization, Immunity

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Introduction

Over the years, aquaculture has been expanded, diversified, intensified with advanced technologies and significantly contributing to the world's gross animal derived protein (FAO, 2018). The most important Challenges to aquaculture production are the lack of quality feed and disease outbreak (Alam and Guttormsen, 2019). Farmers use different chemicals and antibiotics in feed formulation and health management without knowing the appropriate dose (Kawser *et al.,* 2019). But the development of drug-resistant pathogens due to indiscriminate use of antibiotic in aquaculture is a growing concern (Gao *et al.,* 2012; Mog *et al.,* 2020). The residual effects of antibiotics can cause human health problems (Mog *et al.,* 2020) and may also lead to the loss of beneficial gastrointestinal microbiota (Jernberg *et al.,* 2010). As a result, it is necessary to search for alternative methods in aquaculture that could possibly maintain a microbiologically healthy environment for the animals and enhance production and economic profits.

Probiotics are safer alternatives to antibiotic and have much potential to increase fish production. Probiotics is known for its diversified functions in the field of fish feed technology (Dawood and Koshio, 2016). Probiotics alters the intestinal morphology, improves immune system and disease resistance (Xia *et al.,* 2018) and improves the growth performance of animals (Mohapatra *et al.,* 2014). Probiotics also enhance the survival rate (Wu *et al.,* 2014), water quality (Chi *et al.,* 2014) and exclude pathogen by producing extracellular inhibitory compounds, creating iron-limiting niche for the other pathogenic microorganisms (Travers *et al.,* 2011). Several enzymes can be produced in intestine by probiotics that can enhance intestinal digestibility and break down the indigestible compounds potentially present in the supplementary feed (Yi *et al.,* 2020). Thus, probiotics may improve the feed efficiency and reduce water pollution by lessening residual feed waste in water column (Peterson *et al.,* 2012). Probiotic supplement feed may prevent disease outbreaks by enhancing the immune system of fish and shrimp (Wang and Gu, 2010). In recent years, probiotics have been applied as an alternative to antibiotic in aquaculture is gaining more acceptances. Moreover, some of these studies have reported the possibility of improving fish growth, health and feed digestibility when probiotics are used. The study is therefore designed to find out the effect of probiotic in improving growth and immunity in *B. gonionotus*.

B. gonionotus has been selected for the study because it has high market value due to its good taste, higher growth rate and simple management practices with high economic returns (Jahan *et al.,* 2020). Silver barb becomes a popular aquaculture candidate among the marginal fish farmers because it can tolerate a wide range of environmental condition and can be introduced in different culture system (Halim *et al.,* 2018). Though there are documented reports on use of probiotics in fish but

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information regarding the effects of probiotic on the growth and immune system of this candidate species is therefore scarce. Considering the economic as well as the biological importance, this study was designed to evaluate the effects of probiotic on the growth enhancement and hematological profile of *B. gonionotus.*

Materials and method

Collection and rearing of experimental fish

Healthy and homogenous size juvenile of the *B. gonionotus* fish (average length 2.40±0.16 cm and average body weight 4.25±0.01g) were collected from Reliance Aqua Farms, Mymensingh, Bangladesh and were transferred to the Fisheries Biology and Aquatic Environment, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur. Fingerlings were allowed to acclimatize to the laboratory conditions for two weeks. They were housed in large water tanks containing tap water provided with aeration. Fishes were fed with commercial crumble floating feed (32% protein, 8% Lipid, 14% Ash and 12% Moisture) up to satiation.

Experimental design

The experiment was conducted in a completely randomized design with three treatments, each with three replications. A total of nine rectangular glass aquaria (each of 200 L) were used for the rearing experiment. All the glass aquaria were cleaned with NaCl salt and clean water for the protection from germs. Then these glass aquaria were filled with clean ground water for running the experiment. The aquaria were served with artificial aeration. Randomly selected 30 fishes were stocked in each tank for feeding trial. Experimental diet with three different levels of probiotic including 0% (control, T_1), 1% (T_2) and 3% (T_3) probiotic were administered for 75 days for studying the growth and hematology of this fish. Water was changed once in every three days.

Experimental diet preparation

Three iso-nitrogenous diets were formulated to include 0% (Diet 1), 1% (Diet 2) and 3% (Diet 3) probiotic. Fishmeal, soybean meal, full fat soybean, rice bran, wheat bran, mustard oil cake, vitamin and mineral premix were collected from a local fish feed company. A commercial multi-strain probiotic (Pond Care) containing mainly *Bacillus sp.* (22×10^9 CFU/g) was collected from a well-recognized pharmaceutical company (Eskayef Pharmaceutical Limited, Bangladesh). Advantage of this probiotic was that they were able to survive in the extrusion process. All the ingredients were completely mixed and homogenous dough was prepared by adding water. The dough was passed through a meat chopper (Brand-Filizola) to obtain pellets of 2 mm diameter and sun-dried for 2 days. The pellets were then stored in plastic containers at refrigerator for further use. Ingredient's (%)

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composition and proximate composition of the basal diet is shown in Table 1.

Table 1: Ingredient composition of the basal diet and proximate chemical composition (on dry matter basis).

Name of ingredients	Composition (%)		
	Diet 1 (0%)	Diet 2 (1%)	Diet 3 (3%)
Fish meal	50	50	50
Probiotic (*Bacillus sp.*)	0	1	3
Soybean meal	10	10	10
Full fat soybean	10	10	10
Mustard oil cake	10	10	10
Wheat bran	15	14	12
Rice bran	4	4	4
Vitamin and mineral premix	1	1	1
Protein	32.4	32.5	32.4
Lipid	8.2	7.9	8.1
Ash	14.3	13.8	13.6
Moisture	12.4	13.4	12.6
Fiber	7.6	6.7	7.1

Water quality parameters

Water temperature, dissolved oxygen and pH of water were monitored according to APHA (1995) in all test tanks every day. The temperature measurement was done by a thermometer. Dissolved oxygen and pH were measured by DO meter (Pro20i, YSI, Paradise Scientific Company Limited, USA) and pH meter (pH1200, YSI, Paradise Scientific Company Limited, USA), respectively.

Fish sampling

Fishes from each aquarium were collected at the end of the rearing experiment and anesthetized with MS-222, and length and weight were recorded. Blood sample of 10 fishes from each tank from the caudal vein with a syringe were collected in a tube containing EDTA (BD Microtainer®, UK) and preserved for hematological analyses (Bricknell *et al.,* 1999).

Growth performance

The growth performances during the experimental period were calculated according to the following formula:

Length gain (%)= (Average final length- Average initial length/Average initial length)×100

Weight gain (%) = (Final weight - Initial weight/Initial weight)×100

Specific growth rate (SGR) = 100 × (ln W_2- lnW_1)/T; Where W_1 and W_2 are the initial and final weights of the larvae and T is the time in days

Feed conversion ratio (FCR) = Feed fed/Fish weight gain

Protein efficiency ratio (PER) = weight gain (g)/protein offered (g)

Fish survival (%) = 100 × (final number of fish / initial number of fish)

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Blood glucose estimation

Blood glucose was measured by using a digital blood glucose kit (GLUCOLAB). From each tank, 3 fish randomly selected and blood sample was collected. Then one drop blood sample was set on the strip of GLUCOLAB Auto-coding blood glucose test meter and readings were recorded. The values were expressed in mmol/L.

Hemoglobin estimation

Hemoglobin estimation was done by using a digital EasyLife® Hb meter. One drop of blood sample collected from each tank was applied on the test strips connected to the EasyLife® Hb meter and values were recorded. The values were expressed in g/dl.

Hematocrit's value (Packed cell volume)

Packed cell volume (PCV) was determined by Wintrobe's hematocrit tube. Collected blood samples were inserted into dry Wintrobe's hematocrit tube with anticoagulant by Pasteur pipette exactly up to 0 or 100 marks. Care was taken to avoid any air bubble formation in the tube. The tubes were placed in a special rack in vertical position for 1 hour. The tubes were then spun in hematocrit centrifuge for 5-7 min at 4000 rpm for determining the hematocrit value (PCV=Packed Cell Volume). After centrifuge, tubes were removed and three layers were observed. At the top plasma layer, in the middle leucocytes layer and the lower layer, erythrocyte layer known as PCV or hematocrit value. The volume at the peak of the red column (i.e. erythrocyte layer) was measured from the bottom of the tube (if the RBC line at an angle then it was measured from the center point) and expressed in %. Packed cell volume (PCV) was measured by using the following formula:

$$\text{Hematocrit percentage} = (100 / \text{total sample length}) \times \text{RBC length}$$

Blood cell count

Blood samples were immediately analyzed for the estimation of numbers of erythrocytes (RBC $\times 10^6/mm^3$) and leucocytes (WBC $\times 10^4/mm^3$) were using the Neubauer hemocytometer after examining at 4X and 10X magnifications using a research microscope (OLYMPUS-CX21, Japan). The formulas are as follows:

$$\text{RBC's per cu mm} = \frac{\text{number of RBC counted} \times \text{dilution}(200) \times 4000}{\text{area counted} \times \text{depth of fluid}}$$

$$\text{WBC's per cu mm} = \frac{\text{number of WBC counted from 4 large squares} \times \text{dilution}(10)}{\text{volume}(0.4)}$$

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Mean cell volume (MCV)

The mean cell volume indicates the average red cell volume in blood sample. It is expressed in μm3. MCV was a calculated by using the following formula:

$MCV = (PCV \div RBC) \times 10$

Mean cell hemoglobin (MCH)

MCH represents the absolute amount of hemoglobin in the average red cell in blood sample. It is expressed as picograms (pg) per cell. The MCH was calculated from the Hb and the RBC using the following equation:

$MCH\ (pg) = (Hb / RBC) \times 10$

Mean cell hemoglobin concentration (MCHC)

MCHC is expressed in %. It was calculated from the Hb and the PCV using the following equation:

$MCHC = (Hb / PCV) \times 100$

Statistical analysis

Prior to statistical analysis, all data were tested for normality and homogeneity using Shapiro-Wilk test and Levene test, respectively. Statistical analysis was performed using the ANOVA, and significant differences among means were determined by the Least Significant Difference (LSD) option of the package at $p<0.05$. The standard errors of treatment means were also estimated. All statistics were carried out using SPSS 16.0 software.

Results

Water quality parameters

Water quality parameters (temperature, dissolved oxygen and pH) were monitored during the experiment period. Temperature and pH values were almost uniform in the treatments and dissolved oxygen values increased in treatments where probiotics were used (Table 2). All the parameters were within the range of *B. gonionotus* culture.

Table 2: Water quality parameters (Mean ± SD) during the study period.

Parameters	Treatments		
	T_1 (Control)	**T_2 (1%)**	**T_3 (3%)**
Temperature (ºC)	25.17± 1.28	25.00± 1.25	25.10± 1.30
pH	7.45± 0.79	7.18± 0.34	7.23± 0.27
Dissolved oxygen (mg/L)	5.23± 0.51	6.57± 0.19	5.62± 0.61

Growth parameters

The results of growth performance of *B. gonionotus* fingerlings fed diets either supplemented with or without probiotic, for a culture period of 75 days, are summarized in Table 3. Significantly ($p<0.05$) better growth performance was obtained from probiotic treated treatments than the control treatment. Significantly ($p<0.05$) best length gain, weight gain, % weight gain, SGR (%/day) were obtained from 1% probiotic containing group (T_2). Different level of probiotic has no effect on fish survivability.

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Table 3: Growth performance of *B. gonionotus* fed with different experimental diets for 75 days*

Parameters	Treatments	1 day	15 day	30 day	45 day	60 day	75 day
Length (cm)	T_1	2.40±0.09^a	2.67±0.19^c	2.97±0.14^c	3.40±0.09^c	3.87±0.14^c	4.50±0.09^c
	T_2	2.40±0.16^a	3.15±0.04^a	4.66±0.06^a	6.94±0.09^a	9.14±0.38^a	11.07±0.29^a
	T_3	2.40±0.12^a	2.94±0.10^b	3.74±0.13^b	5.16±0.19^b	6.32±0.05^b	7.52±0.12^b
Weight (g)	T_1	4.25±0.32^a	5.37±0.35^{ab}	5.87±0.32^c	6.68±0.09^c	7.78±0.12^c	9.14±0.08^c
	T_2	4.25±0.09^a	6.12±0.23^a	8.15±0.23^a	12.44±0.37^a	17.29±0.60^a	22.06±0.25^a
	T_3	4.25±0.09^a	5.55±0.12^{ab}	7.32±0.19^a	9.24±0.27^b	11.50±0.15^b	14.08±0.29^b
LG (%)	T_1		11.01±3.87^b	11.64±2.46^c	14.45±2.60^c	13.70±1.24^c	16.48±4.10^c
	T_2		31.68±7.82^a	47.87±3.69^a	49.12±2.01^a	31.55±3.80^a	21.26±1.76^a
	T_3		22.58±2.25^c	27.42±7.86^b	37.96±5.07^b	22.63±4.55^b	19.05±1.93^a
WG (%)	T_1		26.27±1.49^c	9.47±2.79^c	14.10±7.92^b	16.48±1.49^c	17.54±0.87^{ab}
	T_2		43.96±2.35^a	33.30±7.13^a	52.85±6.42^a	38.91±0.69^a	27.70±3.74^a
	T_3		30.77±4.98^b	31.92±5.74^b	26.31±3.46^b	24.59±5.31^b	22.40±1.24^{ab}
SGR (%/day)	T_1		1.55±0.08^c	0.60±0.17^c	0.87±0.46^b	1.02±0.09^c	1.08±0.05^{ab}
	T_2		2.43±0.11^a	1.91±0.35^a	2.82±0.28^a	2.19±0.03^a	1.63±0.19^a
	T_3		1.78±0.25^b	1.84±0.29^b	1.56±0.18^b	1.46±0.28^b	1.35±0.07^{ab}
Survival (%)	T_1		100	100	100	100	100
	T_2						
	T_3						

*Values expressed as mean ± standard deviation (SD) (n = 3). Means in the same row with different superscripts are significantly different ($p < 0.05$).

Feed Utilization parameter

The feed utilization data including Feed Conversion Ratio (FCR) and Protein Conversion Ratio (PER) are presented in Figure 1. In case of feed utilization parameters significantly the lowest means the best FCR was observed in T_2 treatment where 1% probiotic was used than the T_3 (3%) and the control group. The best PER value was also observed in T_2 (1%) treatment than the other treatments.

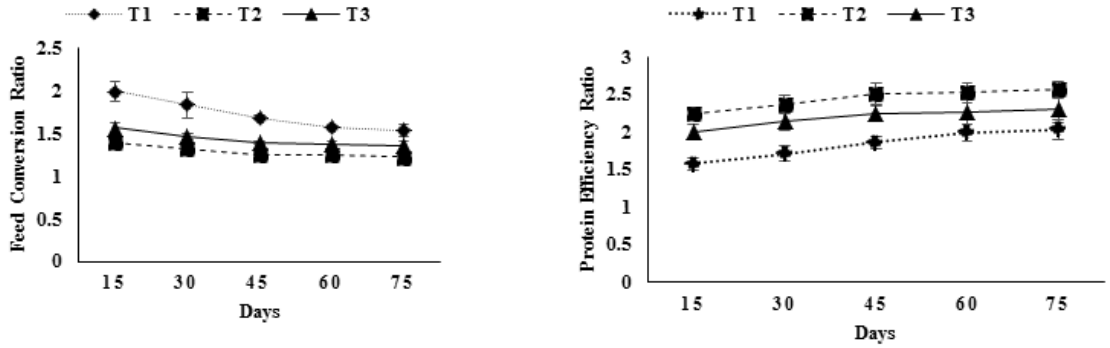

Figure 1: Feed utilization (FCR and PER) of *B. gonionotus* fed with different experimental diets for 75 days. The error bars represent means ± standard deviation (SD) (n = 3). Means with different superscripts are significantly different ($p<0.05$).

Effects of Probiotic on hematological parameters

Different hematological parameters were measured at the end (75 days) of the experiment (Fig. 2). The blood glucose is the primary source of energy of an animal. The blood glucose levels were significantly decreased in

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treatment T_2 and T_3 compared to control group (T_1). Level of Hg (g/dl) significantly increased after probiotic treatment in T_2 (1% probiotic) compared to T_3 (3% probiotic) and T_1 (control). Hematological parameter including PVC, RBC and WBC significantly increased with the increasing level of probiotic in feed. Other hematological indices including MCH and MCHC significantly increased in probiotic treated treatments (T_2 and T_3) compared to control group (T_1) but there was no difference in MCV was found among the treatment means.

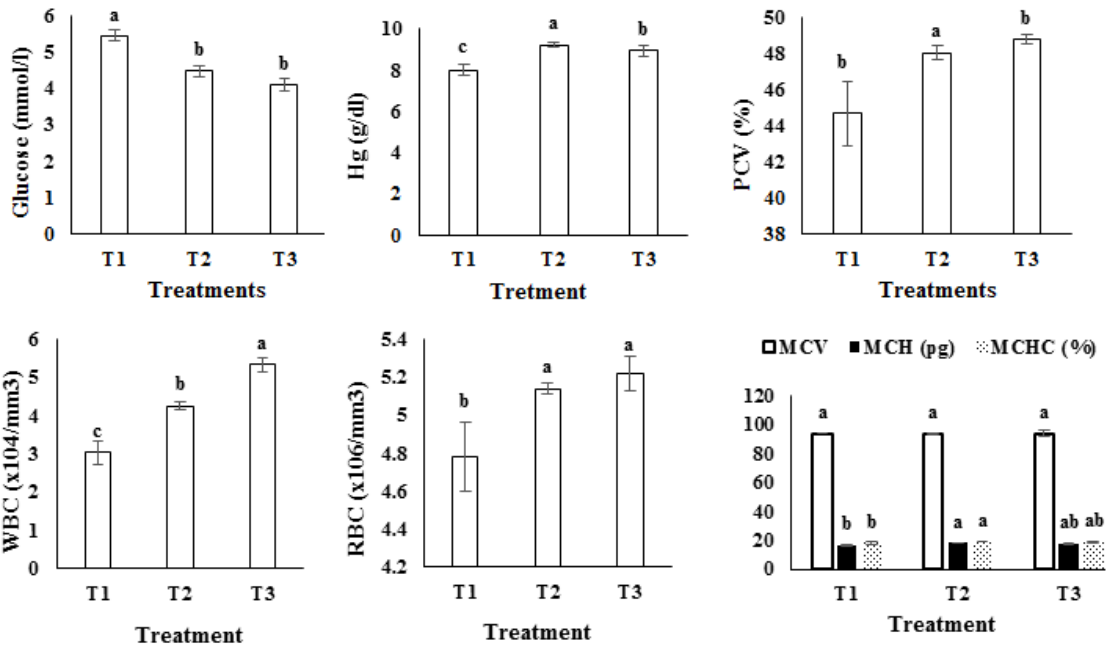

Figure 2: Hematological parameters of B. gonionotus in different treatments fed with different experimental diets for 75 days. Values expressed as mean±standard deviation (SD) (n=3). Means with different superscripts are significantly different (p<0.05).

Discussion

Probiotics are considered as eco-friendly, safe alternatives to antibiotics in aquaculture because probiotics not only help in fighting against pathogens but also improve feed utilization, and growth of fish (Huynh *et al.,* 2017). Probiotics were found to improve feed conversion efficiency and growth in farmed aquatic species (De *et al.,* 2014). In the present study, inclusion of 1% probiotic in diet improved growth and feed utilization in silver barb. Likewise, grass carp (*Ctenopharyngodon idella*) fingerlings and the Indian major carp *Labeo rohita* fed dietary *Bacillus* and *Lactobacillus* species displayed marked growth improvements (Wang, 2011). The enhanced growth rate of fish might be due to the increase in digestive enzyme activities induced by probiotics (Sumon *et al.,* 2018). Extracellular digestive enzymes such as protease, amylase, trypsin, and lipase secreted by *Bacillus sp.* may also have contributed to the growth performance of host (Chowdhury *et al.,* 2020; Maas *et al.,* 2021). Additionally, better growth

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performance and nutrient efficiency could possibly be related to lower stressor levels in fish fed the probiotic diet. Decreased cortisol levels have been reported by Carnevali *et al.* (2006) when fish was fed a diet supplemented with *Lactobacillus delbrueckii*. The author claimed that the decreased cortisol levels affected the transcription of two genes, insulin like growth factor (IGF-1) and myostatin (MSTN), both of which regulate growth performance.

FCR and PER was used in this study to determine the effectiveness of feed. The best FCR and PER values observed with 1% probiotic supplemented diet suggested that, probiotic used can decrease the amount of feed necessary for animal growth and reduces production cost. Probiotics containing *Bacillus* bacteria produces vitamins and diet detoxification and/or degradation of indigestible components; this is most probably related to the production of proteolytic and peptidolytic enzymes by the bacteria found in the probiotic, which hydrolyze macromolecular compounds to peptides and amino acids (Irianto and Austin, 2002). Similarly, Chowdhury *et al.* (2020) also reports a decrease in FCR in probiotic treated striped catfish *Pungasiadon hypophthalmus* but Reyes-Becerril *et al.* (2012) did not find significant change in FCR in Pacific red snapper (*Lutjanus peru*). It is perhaps because factors like probiotic and fish species, age, dosage and duration of probiotic intake, environmental factors like temperature, etc. can make changes in the result of using probiotics (Ramos *et al.,* 2013).

However, some reports show no significant benefits of using probiotics as growth promoters. Use of *Bacillus licheniformis* and *Bacillus subtilis* as feed additive had no significant positive impact on growth rate of juvenile rainbow trout (Merrifield *et al.,* 2010), and tilapia (Shelby *et al.,* 2006). Merrifield *et al.* (2010) also reported slight improvement in growth performance in rainbow trout after 10-week treatment with *Enterococcus faecium*. This indicates that different probiotic strain may interact with the host in a different way and different period of administration (Bomba *et al.,* 2002). Along with growth efficiency, it is also important to know the safety level of probiotics. During the study period no sign of stress or mortality among the treatments were observed and all fish were healthy and activity.

An analysis of results of present study reveals that probiotic plays a vital role in increasing health status of fish. Hematological variables are commonly used as indicators of physiological condition in fish (Mohapatra *et al.*, 2014). In the present study, we observed differences in glucose, hemoglobin, hematocrit, red blood cells count, white blood cells count and hematological indices (MCHC, MCH and MCV). Some studies reported alterations on the hematological variables after feeding with *Bacillus* sp. (Telli *et al.,* 2014) or not (Soltan and El-Laithy, 2008). In this study, fish fed with probiotics showed

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lower blood glucose levels compared to control group. Similar findings have been reported by Mohapatra *et al.* (2014) where *Labeo rohita* fed with probiotic supplemented diet had lower blood glucose levels which might be due to the capability of probiotics to reduce the effects of stressors. The increase of glucose in blood is a sign of stress in fish (Simoes *et al.,* 2012). Probiotics is reported to increase insulin secretion, enhance fish tolerance to stress and normalized immune response (Tapia-Paniagua *et al.,* 2014).

There was higher hemoglobin, Hct % and RBC levels in the fish fed with probiotic treated diet. Reda and Selim (2015) also observed increase in hemoglobin and RBC levels in blood of Nile tilapia supplemented with probiotic. This is because probiotic bacteria enhance the iron absorption by releasing organic acids in the gut. This may increase the availability of iron and hemoglobin production (Dahiya *et al.*, 2012).

A significant increase in the white blood cell (WBC) count was perhaps due to the presence of foreign substances and direct stimulation of immunological defense. This also helps in the removal of cellular debris of necrosed tissue at a faster rate (John, 2007). Higher WBC count can also be correlated with higher antibody production which helps in survival. As in the current study, some authors found increased WBC count in the probiotic treated fish compared to non-probiotic fed fish (Ayoola *et al.*, 2013; Silva *et al.*, 2015). The rises in RBC, WBC counts indicate the immunostimulant effects of this probiotic (Quan and Gill 2002), which can improve fish immune system and potential disease resistance. The red cell Indices like MCV, MCH and MCHC values supports the fact that fish fed the probiotic diet were healthier, as also reported by Gabriel *et al.* (2004).

In the present study, better growth and health status was observed in 1% probiotic supplemented diet because high proportion (3%) of probiotic in the experimental fish feed may have affected the intestinal environment. Lower proportion (1%) for the given probiotic settle, grow and also lead into harbor a great number of microbial cells of host intestine. Increase in growth and hematological profile may be associated with probiotic proportion in the gut flora is probably due to competitive exclusion of other bacteria. It can strongly confirm the idea of out-competing the other bacteria by colonization of probiotic in intestine. It can be certainly suggested that the more probiotic cells in diets and host intestine necessarily does not result in the more improved growth and survival. Better growth, as observed in T_2, may establish better health conditions in *B. gonionotus* and therefore increased hematological values.

Conclusion

In the present study, it is logical to conclude the addition of probiotics in the regular diets significantly increased the growth and survival. Hematology

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parameters were significantly higher in fish fed diet supplemented with the probiotic, than the control diet. It can be deduced from this research that 1% probiotic can be incorporated in *B. gonionotus* diet, to enhance fish health, survival, and growth performance.

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