Enhancement of growth performance and hematological changes in rainbow trout (Onchorhynchus mykiss) alevins fed with Bifidobacterium bacteria

Javad Sahandi1,2 • Hojatollah Jafaryan1 • Mehdi Soltani3 • Pouneh Ebrahimi4

1 Department of Fisheries, Faculty of Natural Resource, Gonbad Kavous University, Gonbad Kavous, Iran.
2 Department of Marine Environmental and Ecological Sciences, College of Marine Life Science, Ocean University of China, Qingdao, China.
3 Department of Aquatic Animal Health and Diseases, Faculty of Veterinary, University of Tehran, Tehran, Iran.
4 Department of Chemistry, Faculty of Basic Sciences, Golestan University, Gorgan, Iran.

Abstract
The study of probiotic application as an important rearing strategy was started more than 30 years ago and most of these studies were carried out to increase growth and survival of larvae. Effect of Bifidobacterium animalis PTTC-1631 and B. lactis PTTC-1736 as probiotic supplement has been studied on growth performance and hematological changes on rainbow trout, Oncorhynchus mykiss alevins with initial body weight of 0.583 ± 0.197 g. The commercial diet was supplemented with graded levels of probiotics (1×107, 2×107 and 3×107 CFU g−1 dry feed) to obtain 3 sets of experimental diets (T1, T2, and T3 respectively) and fed four times a day for 60 days. T1 alevin showed the best growth performance in terms of specific growth rate, weight gain, metabolic growth rate, feed conversion ratio and survival rate. The highest red and white blood cell concentrations were noticed in fish fed T2 and T3 diets, respectively, no significant difference was observed in hemoglobin content. T1 showed the significant elevation of serum biochemical parameters and reduction of cortisol level. The results of present study might suggest likely positive effects of probiotic supplements with concentration of 1×107 CFU g−1 dry feed on growth and hematology on rainbow trout alevins.

Keywords: Rainbow trout; alevins; probiotic; blood; Bifidobacterium; feed.
limitations of vaccination and chemotherapy (Panigrahi et al. 2005). Despite its wider acceptance in aquaculture, many questions remain unanswered about its influence on fish physiology. Recently, some studies have looked into the some kinds of probiotics increased immune response and growth performance in rainbow trout (Panigrahi et al. 2010; Ramesh et al. 2015). The administration of probiotic in adequate amounts confers health benefit on the host in reduction of mortality (Jafaryan et al. 2007; Gupta et al. 2014). However, due to various applications of probiotics, there is an attempt to screen and identify new probiotic strain with specific characteristic to be appropriate for growth performance. The use of Bifidobacterium sp. as probiotic has been recognized and accepted (Charteris et al. 1998; Gill et al. 2001), but very few studies were published. Also, there is no report about effect of B. animalis PTCC-1631 and B. lactis PTCC-1736 on growth and hematology of rainbow trout. Bifidobacterium are genus of gram-positive bacteria that ferment various carbohydrates mainly to lactate and acetate. These bacteria are able to produce biogenic compounds such as bioactive peptides and fatty acids. These components were shown to be possible mechanism for their health enhancing properties (Gobetti et al. 2010). The present study investigated effect of dietary B. animalis PTCC-1631 and B. lactis PTCC-1736 as probiotic on growth performances and hematological profile of rainbow trout alevins.

2 | METHODOLOGY

2.1 | Experimental design

The present experiment was carried out for 60 days. The rainbow trout (Onchorhynchus mykiss) alevins were obtained from a private Coldwater fish farm (Amol, Mazandaran- Iran). Alevins were transferred with nylon bags (one part water and two parts oxygen) to the laboratory. They were acclimatized to the laboratory condition for two weeks during which they were fed commercial diet. After acclimatization 30% of total fish were weighed to determine the initial body weight (mean ± SE) initial weight, 0.583 ± 0.197 g). A total of 480 alevins were randomly divided in 4 treatments (3 experimental treatments and a control), each with three replicates. Fish rearing system was consisting of 16 self-cleaning 20-L plastic tanks that filled with 15 liters of water and the alevins were stocked a density of 2 fish per liter. The experiment was conducted in temperature (17.66 °C) and photoperiod (12 h light and 12 h darkness) controlled condition. Each tank had permanent water exchange (2 L min) and aerated with air stone. The water quality parameters were found to be in the range of temperature (17.66 ± 1.33 °C), pH (7.63 ± 0.08), TDS (2.01 ± 0.13 mg L⁻¹), alkalinity (240 mmol L⁻¹) and total hardness (391.6 mg L⁻¹) throughout the experiment period.

2.2 | Bacterial strains and preparation

Bifidobacterium animalis PTCC-1631 and B. lactis PTCC-1736 were obtained from the Persian Type Culture Collection (Tehran-Iran). Bifidobacterium species were cultivated separately in MRS plate and incubated at 37 °C for 24–48 hrs. The cell free supernatants (CFS) for each bacterial species were obtained separately according to optimal density (OD) method, reading against Mc-Farland turbidity standard solution using Bio-chrome spectrophotometer (Libera-S22) at 625 nm wavelength (Dehghan 2012). In this procedure, Mc-Farland turbidity standard was used as reference to adjust turbidity of bacterial suspensions so that the number of bacteria will be within given range to standardize microbial testing. Three concentrations of 1×10⁷ (T₁), 2×10⁷ (T₂) and 3×10⁷ (T₃) CFU g⁻¹ dry feed of probiotics were employed with equal-proportional amounts.

2.3 | Preparation of diets

A commercial diet (BEYZA, Pars province- Iran) containing 50% crude protein, 14% crude fat, 9.7% crude ash and 4300 Kcal/kg digestible energy was supplemented with graded levels of probiotics (1×10⁷, 2×10⁷ and 3×10⁷ CFU g⁻¹ dry feed) to obtain 3 sets of experimental diets and the control (without probiotic). To prepare experimental diets bacterial colonies were scratched from the surface of plates and mixed with physiological solution (0.9%, w/v), to prepare an equal volume for all treatment sets and sprayed into the diet and mixed part by part. Feed with different concentrations of probiotic were prepared every week and kept in refrigerator and manually administered for four times daily, during 60 days of study. Alevins were fed according to 4–8% of body weight and daily record was kept of feed offered.

2.4 | Examination of growth performance

At the end of the experiment total alevins from each tank were captured for measurement of growth performance as per the following formulae.

\[
\text{Weight gain (WG)} = \text{final weight} - \text{initial weigh (Tacon 1990)}
\]

Specific growth rate for weight (SGR %) = (\(\text{Ln final weight of fish} - \text{Ln initial weight of fish}\) ×100/days of feeding trial (Helland et al. 1996))

Metabolic growth rate = ((weight gain (g)) / ([([W_{ini}-\text{nal}\times1000]^{0.8} + W_{final} / 1000)^{0.8}/2)]/study duration (Dabrowski et al. 1986)

Feed conversion ratio (FCR) = food intake (g) / living weight gain (g) (Helland et al. 1996)
2.5 | Hematological parameters

Alevins were starved for 24 h, then five alevins from each tank were randomly sampled, thereby total of fifteen fish were taken from each treatment. For evaluation of blood parameters each sample of fish were analyzed separately and for biochemical parameters pooled samples of five fish were used for each replicate (three pooled sample for each treatment). Blood was drawn with an insulin siring from caudal vein of individual fish after anaesthetization with 100 mg L clove flower powder. Blood samples were got in heparinized (20 µl) vial of 1.5 ml capacity and following parameters were measured: Red (RBC) and White Blood Cells (WBC) were counted under Neubaur hemocytometer (Bullis 1993). Hemoglobin content was determined after cyanmethemoglobin method (Drobkin 1945). Hematocrit value was measured after Svetina et al. (2002). Following blood cell indices were assessed (Campbell and Ellia 2007):

Mean corpuscular volume (MCV) = (Hct×10)/RBC (fl)
Mean corpuscular hemoglobin (MCH) = (Hb×10)/RBC (pg)
Mean corpuscular hemoglobin concentration (MCHC) = (MCH/MCV) × 100 (g/dL)

2.6 | Blood serum biochemical changes

Blood samples which were collected without anticoagulant were incubated in an upright position at room temperature for 30 min to allow clotting. After incubation time samples were centrifuged for 15 min at 5000 rpm. The obtained serum were used to estimate the biochemical changes as follow: total protein (g/dL), albumin (g/dL), lipase (U/L), amylase (U/L), alkaline phosphatase (U/L), ALT and AST (U/L) were determined using Pars Azmoon Kit (Tehran-Iran) according to instruction of producer. Also cortisol content in serum samples were determined using Monobind ELA assay Kit (USA) according to instruction of producer.

2.7 | Statistical analyses

The differences in growth rates and parameters among the different treatments were calculated by employing one way ANOVA followed by Duncan’s multiple range test by using SPSS (version 19).

3 | RESULTS

After 60 days, mean weight varied significantly among groups, T2–T3. The mean weight of each treatment was significantly higher than the control and T3. The lowest FCR was observed in T2 and T1 respectively. The values of WG and SGR in all groups, fed with probiotic at all concentrations were significantly higher than those of the Control and T3. However, mean values of WG, MGR and SGR were significantly different among treatments. These results showed that different concentrations of B. animalis PTTC-1631 and B. lactis PTTC-1736 composed of equal concentration in each treatment and increased growth performance on rainbow trout alevins (Table 1).

Table 2 represents hematological parameters and differential counting of red and white blood cell respectively. The highest RBC and WBC count within 60 days were observed in T1 and T2 respectively. There were no significant difference between experimental groups and the control in hemoglobin level and similarly MCV and MCH (P > 0.05).

Table 3 represents fish blood serum biochemical changes and differential determining of total protein, amylase and alkaline phosphatase respectively that found in rainbow trout supplemented various concentrations of B. animalis PTTC-1631 and B. lactis PTTC-1736. The highest content of total protein and alkaline phosphatase level within 60 days were observed in T1. No significant difference was observed in albumin content and ALT content between treatments (P > 0.05). Control group showed the highest content of cortisol.

4 | DISCUSSION

One of the main objectives of developing larval rearing strategies is the establishment of feeding regimen with probiotic supplementation that will resulted in optimal growth, survival and health. High growth performance of T1 group fed diet containing the lowest concentration of Bifidobacterium in comparison with other experimental groups might showed that intestinal layers is suitable for...
given low concentration of *Bifidobacterium* to adhe- 
sers and grow, in addition high concentration of probiotics 
necessarily did not cause better growth (Swain et al. 
1996; Sahandi et al. 2012).

**TABLE 2** Hematological profile of alevins fed diets supple- 
mented with *B. animalis* PTTC-1631 and *B. lactis* PTTC-1736

| Parameters          | Treatment          | Control  | T1       | T2       | T3       |
|---------------------|--------------------|----------|----------|----------|----------|
| Hematocrit (%)      |                    | 41.33 ±  | 30.33 ±  | 22.66 ±  | 30.33 ±  |
|                     |                    | 3.17a    | 3.84b    | 2.60b    | 1.45b    |
| Hemoglobin (g/ 
L)      |                    | 5.139 ±  | 6.145 ±  | 5.765 ±  | 5.581 ±  |
|                     |                    | 0.21     | 1.60     | 1.66     | 0.74a    |
| RBC (10 \(^6 \) 

cell/mm\(^3\)) |                    | 1.53 ±  | 0.99 ±  | 0.90 ±  | 1.63 ±  |
|                     |                    | 0.07ab   | 0.31b    | 0.12c    | 0.12b    |
| WBC (10 \(^3 \) 

µL)      |                    | 34.61 ±  | 36.37 ±  | 59.10 ±  | 43.56 ±  |
|                     |                    | 5.71c    | 4.17c    | 10.48a   | 4.703b   |
| MCV (fl)            |                    | 268.35 ± | 368.25 ± | 254.71 ± | 186.199 ±|
|                     |                    | 10.07    | 120.68   | 23.97    | 6.02c    |
| MCH (pg)            |                    | 33.71 ±  | 65.74 ±  | 63.787 ± | 34.091 ± |
|                     |                    | 2.85     | 11.95    | 16.46    | 4.06c    |
| MCHC (g/ 
L)         |                    | 12.65 ±  | 20.14 ±  | 24.51 ±  | 18.295 ± |
|                     |                    | 1.42c    | 4.38b    | 4.41a    | 1.97b    |

*Data are expressed as mean (± SE). Means in the same row 
sharing the same superscript letter and NS are not significantly 
different determined by Duncan’s test (P > 0.05)*

**TABLE 3** Blood Serum biochemical changes of alevins fed 
 diets supplemented with *B. animalis* PTTC-1631 and *B. lactis* 
PTTC-1736

| Parameters          | Treatment          | Control  | T1       | T2       | T3       |
|---------------------|--------------------|----------|----------|----------|----------|
| Total protein (g/ 
 
/dL)    |                    | 4.18 ±  | 6.60 ±  | 5.31 ±  | 4.39 ±  |
|                     |                    | 0.24b    | 0.60a    | 0.54a    | 0.40a    |
| Albumin (g/dL)      |                    | 2.97 ±  | 3.60 ±  | 3.55 ±  | 3.14 ±  |
|                     |                    | 0.28     | 0.39     | 0.45     | 0.42a    |
| Alkaline phos- 
phatase (U/L) |                    | 678.75 ± | 838.12 ± | 522.25 ± | 508.87 ± |
|                     |                    | 100.51a  | 102.41a  | 25.91b   | 61.51c   |
| Amylase (U/L)       |                    | 738.50 ± | 913.12 ± | 837 ±  | 743 ±  |
|                     |                    | 76.20b   | 46.26a   | 49.89ab  | 26.99b   |
| AST (U/L)           |                    | 22.62 ±  | 6.25 ±  | 18.75 ±  | 21.12 ±  |
|                     |                    | 5.49     | 0.83b    | 3.20a    | 4.18b    |
| ALT (U/L)           |                    | 306.87 ± | 271.87 ± | 381.25 ± | 261.12 ± |
|                     |                    | 79.65    | 32.61    | 28.95    | 27.78c   |
| Lipase (U/L)        |                    | 14.87 ±  | 45.87 ±  | 29 ±  | 19.37 ±  |
|                     |                    | 1.65c    | 3.14a    | 7.78b    | 1.46c    |
| Cortisol (µg/dL)    |                    | 18.80 ±  | 5.05 ±  | 6.55 ±  | 5.62 ±  |
|                     |                    | 0.22a    | 0.11b    | 0.08b    | 0.11b    |

*Data are expressed as mean (± SE). Means in the same row 
sharing the same superscript letter and NS are not significantly 
different determined by Duncan’s test (P > 0.05)*

Probiotics produce extra cellular enzymes which help the 
alevins in digestion (Jafaryan et al. 2007). So it can be 
suggested that administration of *B. animalis* PTTC-1631 
and *B. lactis* PTTC-1736 in feed of alevins resulted in en-
hancing of nutrient utilization and improving of growth 
including low FCR, high SGR and FCE. Gram-positive bac-
teria, secreted a wide range of exon-enzymes (Jafaryan 
et al. 2007), so better enzyme activities obtained with supple-
dimented diet improved digestibility (Ramesh et al. 
2015), and caused fast maturation of digestive system 
(Waché et al. 2006) which might in turn explain the better 
growth performances. In addition probiotics can break 
down proteins and carbohydrates (Farzanfar 2006) whereby 
it could improve absorption of broken nutrients. There- 
fore, it can be suggested that distribution of 
*Bifidobacterium* bacteria on rainbow trout alevins result-
ed in enhanced digestion of feed and improved growth 
performance (Gupta et al. 2014; Ramos et al. 2013).

The effect of probiotic on blood parameters have been 
studied in a number of studies (e.g. Rawling et al. 2009; 
Merrifield et al. 2010). In present study white blood cell 
count were increased in experimental groups in compari-
sion with the control and this was an inevitable result 
because probiotics effect on immune system which might 
appear in white blood cell density (Panigrahi et al. 2005) 
and may increase phagocytosis and cytotoxic activity (Pic-
chietti et al. 2007). The use of *Micrococcus luteus* on Nile 
tilapia resulted in increasing of RBC level (Kumat et al. 
2008; Abdel-Rahman 2009; Nayak et al. 2010) and it’s 
similar with our finding. Major hematological changes in 
experimental groups were observed in T1 groups which 
showed statistically noticeable rise of total numbers of 
MCHC and WBC (P < 0.05). Total protein increased signifi-
cantly in fish fed supplemented diet containing probiotics 
with the lowest concentration (P < 0.05). The present 
findings confirmed by those of Marzouk et al. (2008); 
Zhou et al. (2010) and Chelladurai et al. (2013) who re-
ported that total protein level were significantly in-
creased by using probiotics. The use of 1 × 10\(^7\) CFU g\(^-1\) 
*Bifidobacterium* was increased the ALP content and it is probably 
related to increasing of nutrient absorption of alevins 
(Kumar et al. 2006; Marzouk et al. 2008). Also Panigrahi 
et al. (2010) reported that use of *Lactobacillus rhamnosus* 
significantly elevated ALP in rainbow trout after 30 days. 
The use of *Bifidobacterium* significantly increased the 
lipase and amylase and its might be occurred by the mu-
cellular activity of inoculated probiotic bacteria. Balcazar 
et al. (2006) suggested that probiotics have beneficial 
effect on digestive operation because probiotic strains synthe-
size extracellular enzymes such as proteases, amylases 
and lipases (Ramesh et al. 2015). Therefore, nutrients are 
absorbed more efficiently when the feed was supple-
manted with probiotics (El-Haroun et al. 2006). Stress is 
one of the main causes affecting growth with cortisol be-
ing primary mediator for stress response in fish (Vijayan 
et al. 2003). Its means that any stress in fish would be 
ed to secretion of cortisol as its confirmed by Barton 
et al. (1985) before in rainbow trout and Coho. In present
study experimental treatments showed the lowest cortisol level in comparison with the control. Similar results were reported by Ramos et al. (2015) and Rollo et al. (2006) on sea bass alevins. Also this result was consistent with survival rate in different groups. Survival rate in experimental groups were significantly higher than the control and this finding was in agreement with Gatesoupe (1994), Robertson et al. (2000), and Nikoksalat et al. (2001). The results of this study showed that dietary B. animalis PTTC-1631 and B. lactis PTTC-1736 of 1x10^7 would give rise to positive effects on hematological factors of the rainbow trout alevins, and improvement of growth performance and reduction in feed conversion ratio. Also the use of *Bifidobacterium* as new probiotic strains in aquaculture caused the decreasing of cortisol and increasing of rainbow trout survival rate in comparison with the control.

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CONTRIBUTION OF THE AUTHORS

JS primary data collection; HJ research supervision; JS, MS & PE manuscript preparation.