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## Original Article

# The effects of different levels of Beta Plus on growth performance, microbial flora and blood parameters of Caspian trout, *Salmo caspius* (Kessler, 1877)

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**Abstract:** This study was conducted to evaluate the effects of Beta plus probiotic, a mixture of *Bacillus leicheniformis* and *Bacillus subtilis*, on the growth performance and intestinal microbial flora of Caspian trout (*Salmo caspius*). A basal diet was formulated and supplemented with Beta plus at 0, 0.5 and 1 g kg<sup>-1</sup>, leading to three experimental diets. Each experimental diet was randomly assigned to quadruple 1500 L tanks. The Caspian trout with an initial weight of 108.7 ± 1.8 g were randomly distributed in the experimental tanks. The results showed that inclusion of dietary Beta plus significantly increased the final weight and specific growth rate (SGR) of Caspian trout compared to those the control treatment. The feed conversion ratio (FCR) was also improved significantly after probiotic administration to the experimental fish. However, the body composition and blood parameters were not influenced by the probiotic inclusion. Total count of Gram positive and negative bacteria in the intestine of the fish increased by feeding on diet contained 1 g kg<sup>-1</sup> Beta Plus ( $P < 0.05$ ). In conclusion, administration of the probiotic Beta plus can improve the nutrient efficiency and growth performance of Caspian trout confirming the positive effect of a mixture of *Bacillus* spp.

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## Introduction

The Caspian trout, *Salmo caspius*, is a valuable commercial species and known as one of the nine brown trout species (Quillet et al., 1992). Caspian trout is distributed mainly at the western and southern cost of Caspian Sea (Kiabi et al., 1999) and showed a better growth and higher weight gain in compare to other brown trout populations (Sedgwick, 1995). The population of this species shows a steady decline over the past two decades and considered to be an endangered species in the southern of Caspian Sea (Kiabi et al., 1999).

Recent development in aquaculture industry may lead to introduction of the Caspian trout as a new candidate. This species can be cultured in cage, concrete pond and flow-through system (Kalbassi et

al., 2006; Sarvi et al., 2006).

Intensive production system may deteriorate water quality, increasing stress level and incident of disease (Losordo et al., 1999). These conditions can threat fish immune system leading to more vulnerability to bacterial infection (Liltved and Cripps, 1999; Irianto and Austin, 2002). In such a condition, antibiotics have been introduced as a solution to prevent disease and enhance growth performance in fish (Burr et al., 2005). However, an increase in antibiotic use may have negative side effects such as the development of antibiotic-resistant bacteria which makes the use of antibiotic growth promoters undesirable. In addition, some antibiotics make fish more susceptible to bacteria, viral, and parasitical disease (Gatlin, 2006). The

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public concern of antibiotic concentration in food has been resulted in the regulation of strict measures on antibiotic usage for farm animal treatment (Patterson and Bourkholder, 2003).

Probiotics are usually live microorganisms that may confer a health benefits on host (Fuller, 1992). In aquaculture, probiotics have been used to control diseases, enhance specific and non-specific immunity (McCracken and Gaskins 1999; Verschuere et al., 2000) providing nutrients and enzymatic functions (Wache et al., 2006), and improve water quality (Balcazar et al., 2006). The lactic acid bacteria, including *Bacillus* spp. are widely used as probiotics in humans and their application have led to health benefits against gastrointestinal disorders including diarrhea, inflammatory bowel disease, and lactose (Madsen, 2001). In addition, *Bacillus* and *Lactobacillus* have been supplemented as an immune-stimulator to enhanced the growth, innate immune responses, and disease resistance of a number of fish species (Sun et al., 2010; Geng et al., 2011; Geng et al., 2012). The lactic acid bacteria are also known to be present in the intestine of healthy fish (Balcazar et al., 2007).

BioPlusis a commercial probiotic composing naturally occurring bacteria strains including, *Bacillus subtilis* and *Bacillus licheniformis*, which improved growth parameters of rainbow trout larvae (Alizadeh et al., 2011). A mixture of *Bacillus subtilis*, *Bacillus licheniformis*, *Lactobacillus* spp. and *Arthrobacter* spp. improved immune system, growth and lower mortality against the pathogens of Cobia, *Rachycentron anadum*, (Geng et al., 2012). *Bacillus* sp. also changes gut microbial composition in rainbow trout, *Oncorhynchus mykiss* (Ramos et al., 2013)

Since some work is available on the effects of different probiotics on fish performance and health parameters, but a little information is available on the effect of a mixture of *Bacillus* sp. on those parameters in Caspian trout. Therefore, this study was conducted to assess the effects of Beta Plus as a probiotic on the fish performance, survival rate and microbial flora in the Caspian trout

Table 1. The percentage of ingredient used in the experimental diet on percentage dry matter weight basis.

Experimental diet	Amount
Soybean meal	15
Fish meal	42
Meat meal	15
Corn	10
Wheat	7
Fish oil	5
Molasses	2
DL-methionin	0.84
NaCl	0.4
Lysine	0.76
Premix	2
Nutrient composition of the experimental diet percentage (dry weight)	
Moisture	6.5
Crudeprotein	43
Crude fat	12
Crude ash	10
Energy (Kcal/kg)	3650

## Materials and methods

**Experimental system and animal:** This study was carried out at an experimental facility of rainbow trout farm located in the northern part of Iran (Savadkoh, Mazandaran Province, Iran). The Caspian trout juveniles were propagated at a nearby reproduction facility and adapted to a commercial diet (Chineh. Company, Iran) and new environment for a week prior the experiment. After adaptation period, the fish with initial weight of  $108.7 \pm 1.8$  g were divided randomly into twelve 1500 L tanks, with a stocking density of 25 individuals per tank. The experiment was lasted 8 weeks. The used probiotic i.e., Beta Plus (BP), composes *Bacillus leicheniformic* (DSM 5749), *Bacillus subtilis* (DSM 5750) and betaien with a commercial name of Beta Plus (Biochem Co., Germany).

A basal diet was formulated with estimated gross energy and protein levels of  $12.56 \text{ MJ kg}^{-1}$  and  $433 \text{ g kg}^{-1}$ , respectively (Table 1). Three levels of BP (0,

Table 2. Growth performance in Caspian trout, *Salmo caspius*, feeding on different levels of Beta Plus (BP; g/kg) over eight weeks experimental period. All values are means of four replicates (tanks)/ treatment  $\pm$  SD (Standard Deviation).

Parameters	Diets		
	Control	BP 0.5	BP 1
Initial weight (g)	110.11 $\pm$ 7	107.14 $\pm$ 4	108.78 $\pm$ 5
FCR	1.55 $\pm$ 0.17 <sup>b</sup>	1.54 $\pm$ 0.88 <sup>b</sup>	1.37 $\pm$ 0.05 <sup>a</sup>
Final weight (g)	297.3 $\pm$ 0.83 <sup>b</sup>	298.5 $\pm$ 3.6 <sup>b</sup>	323.9 $\pm$ 2.3 <sup>a</sup>
SGR	1.16 $\pm$ 0.96 <sup>b</sup>	1.17 $\pm$ 0.13 <sup>b</sup>	1.57 $\pm$ 0.06 <sup>a</sup>
Mortality	0	0	0

Different superscript letters show significant differences.

0.5 and 1.5 g kg<sup>-1</sup> prebiotic diet) were added to the basal diet to prepare three experimental diets. All ingredients were finely ground, mixed and pelletized. A pellet maker with 4 mm diameter die was used for producing the diets. All three experimental diets were air-dried at 50°C at the same time and stored at -20°C.

**Experimental procedure:** Fish were weighed on the 1<sup>st</sup> and the last day of the experiment. They were hand fed with the experimental diets at the rate of 1.2-1.6% of biomass, three times a day (8.00, 13.00, and 18.00 h). The experimental diets were randomly assigned to three treatments including 12 tanks i.e. four replicates per diet. Water quality parameters were monitored daily to ensure being in appropriate range. The water temperature and pH were 9-11°C and 7.3-7.9, respectively, during the experiment. The oxygen concentration was always above 8 mg L<sup>-1</sup>. In addition, water flow was 12 L min<sup>-1</sup> for each tank.

On day 56, all fish were weighed and five specimens were randomly sampled from every experimental tank. They were sacrificed using an overdose of clove essence solution and analyzed their body composition. Three specimen were randomly selected from each tank for bacterial count. For analyzing blood parameters, three fish were randomly sampled from each tank before weighing and immediately anaesthetized using clove essence solution and one mL of blood was collected by caudal vein puncture using syringes containing 3 mg of Na<sub>2</sub>EDTA. Then, they were stored at 4°C after gently shaken for further examination.

Red blood cell and white blood cell counts were estimated following the method of Schalm et al. (1975). Haemoglobin (Hb) and Haematocrit (Ht) concentrations were determined based on Barros et al. (2002).

**Chemical analysis:** The food samples were collected and pooled at regular intervals during the experimental period and grounded using a 1 mm screen grinder before analyses. Dry weight of food and fish body were measured for dried samples for 24 hrs at 103°C (ISO 6496 1983). Ash content was determined by incineration in a furnace for 4 hrs at 550°C (ISO 5984 1978). Crude protein (N $\times$ 6.25) was measured by the Kjeldahl method after acid digestion according to ISO 5983 (1979). Lipid was extracted with petroleum ether in a Soxhlet apparatus. Energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000, Fa. IKA- Analysentechnik, Weikersheim, Germany).

**Fish performance:** Weight gain was determined by the difference between initial and final body weights. Feed conversion ratio (FCR) was calculated as follow: FCR = feed consumed (g) / wet body weight gain (g). Specific growth rate (SGR) was calculated as follows and expressed as a percentage: SGR = 100 (Ln W<sub>final</sub> - Ln W<sub>initial</sub>)  $\times$  days<sup>-1</sup>. The calculations were based on the dry weight of the diets.

**Statistical analysis:** Data are presented as means of each treatment  $\pm$  standard deviation (SD). Normality of data after transformation (ASIN) and homogeneity of variances were tested using

Table 3. Bacterial counts in the Caspian trout, *Salmo caspius*, feeding on different levels of Beta Plus over eight weeks experimental period. All values are means of four replicates (tanks)/ treatment  $\pm$  SD.

Bacterial community	Diet		
	Control	BP 0.5	BP 1
Gram positive*	$2.3 \times 10^4 \pm 45^b$	$2.6 \times 10^4 \pm 50^b$	$6.8 \times 10^5 \pm 40^a$
Gram negative**	$2.4 \times 10^4 \pm 62^b$	$2.5 \times 10^4 \pm 23^b$	$3.6 \times 10^5 \pm 25^a$

Different superscript letters show significant differences.

Table 4. Body composition in Caspian trout, *Salmo caspius*, feeding on different levels of Beta Plus over eight weeks experimental period. All values are means of four replicates (tanks)/treatment  $\pm$  SD.

Parameters	Diets		
	Control	BP 0.5	BP 1
Dry mater	$26.54 \pm 2.33$	$26.51 \pm 2.54$	$26.56 \pm 2.77$
Protein	$15.82 \pm 1.32$	$15.79 \pm 1.40$	$15.80 \pm 0.88$
Fat	$7.18 \pm 0.89$	$7.19 \pm 0.78$	$7.21 \pm 0.96$
Ash	$3.11 \pm 0.11$	$3.09 \pm 0.17$	$3.07 \pm 0.14$

Table 5. Body composition in Caspian trout, *Salmo caspius*, feeding on different levels of Beta Plus over eight weeks experimental period. All values are means of four replicates (tanks)/treatment  $\pm$  SD.

Parameters	Diets		
	Control	BP 0.5	BP 1
Hematocrit (%)	$39.77 \pm 1.00$	$41.33 \pm 2.08$	$41.89 \pm 1.52$
Hemoglobin (gr/dL)	$13.60 \pm 0.36$	$13.10 \pm 0.72$	$13.87 \pm 0.40$
White blood cells ( $\times 10^3/L\mu$ )	$11.52 \pm 1.37$	$11.99 \pm 2.65$	$12.55 \pm 2.58$
Red blood cells ( $\times 10^6/L\mu$ )	$0.68 \pm 0.26$	$0.71 \pm 0.37$	$0.78 \pm 0.65$

Levene's F test. One-way ANOVA was used to determine the effects of BP levels on fish performance, blood parameters and bacterial count using the SAS software. The means were compared by a Tukey's post hoc test. For all statistical analyses, each tank was considered as the experimental unit.

## Results

There were no mortality observed throughout the study. The results revealed a positive impact of BP on the growth performance of Caspian trout. Inclusion of  $1 \text{ g kg}^{-1}$  probiotic improved final weight compared to control one ( $P < 0.05$ ). However, lower level of BP supplementation ( $0.5 \text{ g kg}^{-1}$ ) did not have a significant effect on final weight (Table 2).

Also FCR and SGR were improved due to feeding

on  $1 \text{ g kg}^{-1}$  probiotic diet ( $P < 0.05$ ). However the lower levels of the probiotic did not affect those parameters (Table 2).

Dietary administration of BP affected bacterial count in the intestine of Caspian trout (Table 3). Both Gram positive and Gram negative counts were increased significantly by addition of  $1 \text{ g kg}^{-1}$  of BP to the diet ( $P < 0.05$ ). However, lower level of BP supplementation ( $0.5 \text{ g kg}^{-1}$ ) did not induce any change in bacterial count (Table 3).

Dietary BP did not influence body composition of Caspian trout over eight weeks experimental period (Table 4). Likewise, blood parameters of Caspian trout were not affected by supplementation of BP (Table 5). Nevertheless, there was a trend toward a greater red and white blood cells concentrations and

hematocrit in treatment fed 1 g kg<sup>-1</sup> BP diet.

## Discussion

The present study showed that supplementation of two *Bacillus* species improve growth performance including final weight, biomass gain, SGR and FCR in Caspian trout. A large number of previous works also showed positive effects of different dietary probiotics on fish growth performance and feed efficiency of rainbow trout (Bagheri et al., 2008; Alizadeh et al., 2011; Ramos et al., 2014), African catfish (*Clarias gariepinus*) (Al-Dohail et al., 2009), Gilthead sea bream (*Sparus aurata*) (Suzer et al., 2008) and zebra fish (*Danio rerio*) (Sedaghat et al., 2012). The beneficial effect of the probiotic on growth may be related to its effect on the microbial flora of intestine. The mechanisms by which probiotic bacteria stimulate growth rate are not yet clearly elucidated. Administration of *Bacillus* group may change the proportion of *Bacillus* count in the intestine thereby leading to a larger count of Germ positive bacteria. Lactic acid bacteria inhibit specific fish pathogens by production of bacteriocins (Thompson et al., 1999; Ebrahimi et al., 2012). The positive effects of *B. Subtilis* and *B. licheniformis* on the immune system and resistance to some pathogens were also reported in *Salmo salar* (Austin et al., 1992).

A better growth performance of Caspian trout in the current study may be also attributed to a lower proportion of Gram negative bacteria to Gram positive ones. The promotion of *Lactobacillus* population can limit pathogenic bacteria colonization (Ringo and Gatesoupe, 1998; Thompson et al., 1999; Verschuere et al., 2000). Low level BP administration (0.5 g kg<sup>-1</sup>), in this study, did not change growth parameters in Caspian trout that was in agreement with those of rainbow trout (Ramos et al., 2014). Enzyme secretion ability of *Bacillus* spp. such as protease may increase with proliferation of bacteria population (Fuller and Perdigon, 2003). This condition can elevate digestive enzyme activity (Askarian et al., 2011), thus leading to a better nutrient digestion achieving

a better growth.

The results also showed that presence of probiotic in the diet affect the gut microbiota of fish. The similar observation was also made by Balcazar et al. (2006) and Merrifield et al. (2010) in salmonids. The present experiment suggests that supplementation of BP modulates gut microbiota and through that significantly improves growth performance of juvenile Caspian trout. In addition, multi-species probiotics seems to be more effective than single-strain probiotics, because different strains are more likely to survive and find their specific niche in the gut as suggested by Bezkorovainy (2001).

Blood analysis revealed that the measured blood parameters were not affected by the dietary probiotic supplementation. This finding is similar to that of Dotta et al. (2011) who observed that probiotic supplementation did not influence the red blood cell and hematocrit in Nile tilapia, *Oreochromis niloticus*. In contrast, other results show that probiotic diet increases hematological parameters in African catfish, *Clarias gariepinus*, (Ayoola et al., 2013). The different results may indicate that some other parameters such as experimental condition, fish species and diet composition can influence the impact of probiotic on the hematological response.

In conclusion, the results demonstrated that the administration of BP as a dietary probiotic can improve growth performance and feed efficiency in the Caspian trout by promotion of Gram positive bacterial growth that may indicate growth improvement. Based on the findings, supplementation of BP at a level of 1.5 g kg<sup>-1</sup> diet is suitable for feeding of Caspian trout to support fish growth and health.

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