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The use of *Bacillus* probiotics in-feed improved stress resistance of *Trichopodus trichopterus* (Pallas, 1770) larvae

Hojatollah Jafariyan[1*], Javad Sahandi[2], Mehdi Taati[3], Khalil Eslamloo[4]

[1]*Faculty of Natural Resource, Gonbad Kavous University, Gonbad Kavous, Iran*

[2]*Faculty of Marine Life Science, Ocean University of China, Qingdao, China*

[3]*Faculty of Fishery and Environmental Science, Gorgan Agricultural Science and Natural Resource University, Gorgan, Iran*

[4]*Faculty of Science, Memorial University, Newfoundland and Labrador, Canada*

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ABSTRACT

Objective: To evaluate the effects of four different concentrations of *Bacillus subtilis* (*B. subtilis*) and *Bacillus circulance* (*B. circulance*) (1×10^4, 2×10^4, 3×10^4 and 4×10^4 CFU/g) on growth and resistance of three spot gourami (*Trichopodus trichopterus*) for a period of 30 days.
Methods: During this study, experimental fish were fed with supplemented diets. *Bacillus* probiotics with concentrations of 1×10^4 to 4×10^4 CFU/g were administered to improve the growth performance and larval resistance to challenge tests containing acidic pH and basic pH, ammonia and salinity tests.
Results: The addition of *B. subtilis* and *B. circulance* to the diet did not increase larval growth rate, but increased larval resistance against the challenge tests.
Conclusions: The results of this study investigated the positive effects of *B. subtilis* and *B. circulance* on *Trichopodus trichopterus* resistance time to the challenge test.

1. Introduction

The ornamental fish sector is a global component of international trade, fisheries and aquaculture development is one of the most economic and profitable areas of fish farming[1]. The total value of the wholesale ornamental trade was estimated at close to $ 15 billion dollars all over the world[2]. *Trichopodus trichopterus* (*T. trichopterus*) is a very hardy fish and also prolific and easy to breed[3]. It has been produced commercially in various color forms such as gold and opaline. The two main aspects that determined the trade and prosperity of the ornamental fish industry were health and nutrition of the fish[1]. The initial establishment of microflora in the larval stages depends, among other factors, on the microbiota associated with eggs and newly hatched larvae, microalgae and live prey introduced into the system and water of the rearing system[4-6]. Using bacteria was first preferred for decreasing harmful bacteria in the human digestive tract to improve human health[7]. Probiotics were defined as live microorganisms by Food and Agriculture Organization and World Health Organization and administrated in adequate amount to confer a health benefit on the host[8]. Most probiotics are supplied as supplements in diet, which has the ability to survive passage through the intestinal tract[9], and confer benefits by enhancing the host's response toward disease or by improving the quality of its ambient environment[10]. In recent years, efforts have been made to develop strategies for microbial control, to decrease the use of therapeutic chemicals and antibiotics[11], towards a more environmentally friendly and sustainable aquaculture. The use of probiotics for enhancing bio-growth parameters and improving disease-resistance ability have been well documented in aquaculture of fish for human consumption[5-7,12,13], but research on the effect of probiotics on ornamental fishes and their resistance ability are lacking. *Bacillus* spp. can act positively on cultured organisms by enhancing survival and growth[14]. Many studies indicated that growth performance and feeding efficiency of fish larvae were promoted by the use of *Bacillus* spp.[6,12,15]. The present study was conducted with the objective of supplementing *Bacillus subtilis* (*B. subtilis*) and *Bacillus circulance* (*B. circulance*) in the diet of *T. trichopterus* and evaluating its effect on host growth performance and toleration status toward environmental stress.

2. Materials and methods

2.1. Treatments and preparation of diet supplement

The probiotic bacterial strains, *B. subtilis* (1.075×10^9 CFU/

*Corresponding author: Hojatollah Jafariyan, Department of Fisheries, Faculty of Natural Resource, Gonbad Kavous University, Shahid Fallahi Street, Gonbad Kavous, Golestan, Iran.
Tel: +981722293401
E-mail: hojat.jafaryan@gmail.com

mL) and *B. circulance* (2.5×10^9 CFU/mL) used in this study were obtained from Protexin commercial product (London, England). The feed was commercial diet (Biomar, France) containing 54% protein, 18% lipid, 9.7% ash, 90% dry matter and 10% moisture. The probiotic supplements were prepared by gently spraying the bacterial suspension with required density on the diet and mixing it part by part to obtain final probiotic concentrations of 1×10^4, 2×10^4, 3×10^4 and 4×10^4 CFU/g respectively. Five treatments with three replicates were prepared (four experimental treatments and a control) as follow: control; T1 fed with diet containing probiotic bacteria at 1×10^4 CFU/g; T2 fed with diet containing probiotic bacteria at 2×10^4 CFU/g; T3 fed with diet containing probiotic bacteria at 3×10^4 CFU/g and T4 fed with diet containing probiotic bacteria at 4×10^4 CFU/g.

2.2. Experiment fish and design

T. trichopterus with (50.0 ± 0.8) mg initial body weights were obtained from a private ornamental fish farm (Golestan, Iran) and were stocked at a density of 3 larvae per liter in twenty fiberglass tanks with a capacity of 15 L. Every day, thirty percent of tank water was changed. Fish were fed four times daily (6:00 and 12:00 am, 18:00 and 24:00 pm). The fish were monitored for mortality daily and the dead ones were immediately removed and recorded. At the end of experiment, total fish samples were anesthetized with 100 mg/L of *Eugenia caryophyllata* extract and were weighed (± 0.01 mg) by a digital scale (Kern model, Germany), and total length was measured with a caliper (± 0.1 mm). The experiment was run for 30 days. The evaluated parameters were calculated by the equations presented below:

Specific growth rate (SGR) (%/day) = $100 \times [(\text{Ln}W_f - \text{Ln}W_i)/T]$

Feed conversion ratio (FCR) = $\text{TFI}/(W_f - W_i)$

Food conversion efficiency (FCE) (%) = $[(W_f - W_i)/\text{TFI}] \times 100$

Condition factor = $100 \times (W/TL^3)$

Average daily growth (%) = $100 \times [(W_f - W_i)/(W_i) \times T]$

Weight gain (WG) (mg) = $W_f - W_i$

Growth conversion efficiency (GCE) = (SGR/RFI) × 100

where, W_f means final weight (mg); W_i means initial weight (mg); T means duration of study (day); TFI means total feed intake (mg); TL means total length (mm); RFI means relative feed intake (mg).

2.3. Challenge test

At the end of feeding trial, three fish from each replicate were captured and transferred to the challenge tanks for acidic pH value, basic pH value, salinity and ammonia challenge tests separately according to method of Jafaryan *et al*. and the time of fish tolerance were recorded in second until all fish died[7].

2.3.1. pH challenge

For this trial, three fish from each replicate were randomly captured and transferred to the prepared tanks. Acidic (pH = 2) and basic (pH = 12) challenges were run and pH value was monitored with portable pH meter (Metrohm, Switzerland).

2.3.2. Salinity challenge

The salinity challenge test was carried out by adding 20 g/L commercial salt (without iodine) into rearing fresh water. Three fish from each replicate were then randomly captured and placed into the brackish water.

2.3.3. Ammonia challenge

This challenge was carried out to evaluate probiotic effect on fish resistance toward ammonia exposure. For this trial, three fish were captured from each replicate randomly and transferred into water with 5 mg/L ammonia.

2.4. Statistical analysis

The significant difference in growth rates and resistance parameters among the different experimental treatments was calculated by a One-way ANOVA followed by Duncan's multiple range test to examine which of them varied significantly. In all statistical tests, $P = 0.05$ was taken as level of significance (SPSS verson 19 software).

3. Results

At the end of feeding trial, final body weight ranged from (330.83 ± 108.03) to (349.51 ± 122.62) mg with no significant difference among dietary treatments. The SGR in fish fed with dietary treatments was significantly higher than that of the control fish. The larvae fed with 3×10^4 and 4×10^4 CFU/g supplemental *B. circulance* and *B. subtilis* had a higher SGR than those fed with diets supplemented with 1×10^4 and 2×10^4 CFU/g (Figure 1).

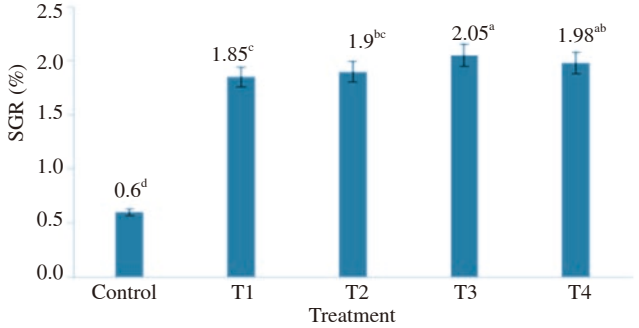

Figure 1. Effect of dietary *B. circulance* and *B. subtilis* on SGR.
Values sharing the same superscript letter were not significantly different determined by Duncan's test ($P > 0.05$).

Despite significant differences in SGR, other growth and nutritional parameters such as WG, FCR and FCE showed no significant difference between experimental groups in comparison with the control (Table 1).

Likewise, no significant difference was observed in GCE and average daily growth between experimental groups in comparison with control ($P > 0.05$) (Table 1).

There were significant differences between groups in condition factor ($P < 0.05$) (Figure 2). Values for control and T1 were similar and no significant difference was observed; likewise T3 and T4 showed similar results with each other, but T2 did not show significant difference with other groups ($P > 0.05$).

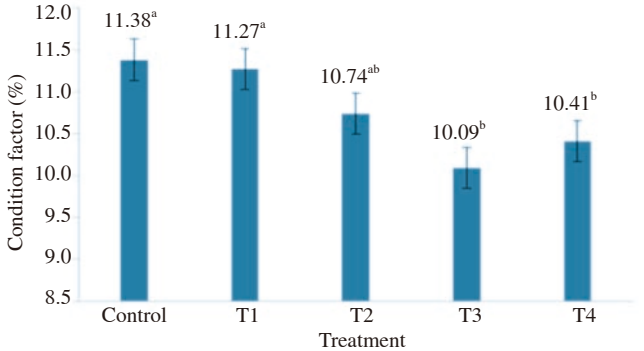

Figure 2. Effect of dietary *B. circulance* and *B. subtilis* on condition factor.
Values sharing the same superscript letter were not significantly different determined by Duncan's test ($P > 0.05$).

Table 1

Growth response of gourami larvae fed with diets supplemented with graded levels of *B. circulance* and *B. subtilis*. mean ± SD.

Parameters	T1	T2	T3	T4	Control
Initial body weight (mg)	50.000 ± 0.800	50.000 ± 0.800	50.000 ± 0.800	50.000 ± 0.800	50.000 ± 0.800
Final body weight (mg)	349.510 ± 122.620[a]	340.760 ± 117.740[a]	330.830 ± 108.030[a]	332.580 ± 105.280[a]	334.750 ± 120.950[a]
WG (mg)	284.750 ± 120.950[a]	299.510 ± 122.620[a]	290.760 ± 117.740[a]	280.830 ± 108.030[a]	282.580 ± 105.280[a]
FCR	0.920 ± 0.510[a]	0.920 ± 0.480[a]	1.010 ± 0.970[a]	1.080 ± 0.560[a]	1.080 ± 1.020[a]
FCE (%)	155.560 ± 54.580[a]	151.670 ± 52.400[a]	147.250 ± 48.080[a]	148.030 ± 46.860[a]	148.990 ± 53.830[a]
GCE (%)	0.017 ± 0.004[a]	0.017 ± 0.003[a]	0.017 ± 0.004[a]	0.017 ± 0.003[a]	0.017 ± 0.005[a]
Average daily growth (%)	19.960 ± 8.170[a]	19.380 ± 7.840[a]	18.720 ± 7.200[a]	18.830 ± 7.010[a]	18.890 ± 8.060[a]

Values in the same row with same superscripts are not significantly different ($P > 0.05$).

The results of challenge tests were presented in Table 2. Feeding of supplemented diet containing 1×10^4 and 4×10^4 CFU/g *B. circulance* and *B. subtilis* resulted in the highest resistance time against the acidic challenge ($P < 0.05$).

Table 2

Resistance response (time of fish tolerance) of gourami larvae fed with diets supplemented with graded levels of *B. circulance* and *B. subtilis* to challenge tests. s.

Treatments	Physical and chemical stress (challenge)			
	Acidic exposure (pH = 2)	Basic exposure (pH = 12)	Ammonia (5 mg/L)	Salinity (20 g/L)
Control	468.33[c]	787.33[b]	576.23[d]	796.68[d]
T1	644.67[a]	933.00[a]	668.23[b]	928.67[a]
T2	537.67[b]	999.00[a]	590.67[d]	841.67[c]
T3	528.67[bc]	967.00[a]	744.33[a]	866.00[bc]
T4	615.67[a]	930.67[a]	636.33[c]	887.33[b]

Means in the same column sharing the same superscript letter were not significantly different determined by Duncan's test ($P < 0.05$). The significant differences between experimental groups were determined by One-way ANOVA.

Significant differences were observed in experimental groups in comparison with control. Challenge tolerance time was similar between all experimental groups but significantly different from control in the basic pH challenge. The supplemented diet containing 3×10^4 CFU/g *B. circulance* and *B. subtilis* resulted in significantly different resistance time in comparison with other groups during the ammonia challenge ($P < 0.05$). During the salinity challenge, the resistance time significantly enhanced by supplemented diet containing 1×10^4 CFU/g *Bacillus* sp.

4. Discussion

The results of the present study showed that dietary treatments increased the SGR of larvae during experiment but no significant difference was observed in final body weight. Similarly, Boyd *et al*.[16] reported that adding commercial probiotics did not have any significant effect on growth parameters of channel catfish, and addition of bacteria into the rearing system of halibut larvae (*Hippoglossus hippoglossus* L.) did not increase the growth of larvae[17]. It is possible that these probiotics produced substance during antagonistic process against each other that inhibited the growth and adherence of them or other microbiota and the used concentrations were not effective. According to de Vrese and Marteau, mechanism and function of probiotics depended mainly on the interactions between probiotic species and microbiota of the host or with immunocompetent cell of the intestinal mucus[18]. However, the growth of rainbow trout (*Oncorhynchus mykiss*) was significantly increased by feeding a dietary supplement of *Bacillus* spp.[12]. The significant difference in SGR refers to different effect of probiotics during experiment. The loading of probiotics during the experiment was different and it was investigated by Makridis *et al*.[17]. The beneficial effects of dietary supplements like probiotics have been recorded in a wide range of animal models including fish. The innate immune system was the only defense weapon of invertebrates, and a fundamental defense mechanism of fish and the main parameters of the innate system were commonly divided into physical parameters, cellular and humoral factors[19]. In the present study, increase in tolerance time against acidic (pH 2), basic (pH 12), ammonia (5 mg/L) and salinity (20 g/L) challenge was observed. Experimental groups fed with supplemented diet containing *B. circulance* and *B. subtilis* showed higher resistance time against the challenge tests in comparison with the control. The use of probiotics improved host digestion and it was well studied by Jafaryan *et al*.[20]. The improvement of digestion leads to increase in protein, vitamin, minerals and other nutrients absorption and it causes increasing of host resistance. Fietto *et al*. reported that the use of *Saccharomyces cerevisiae* and *Saccharomyces boulardii* as probiotic enhanced host resistance against thermal and acidic pH stresses[21]. Also the use of same probiotic increased rainbow trout larvae resistance against salinity challenge (10, 15 g/L)[22]. Significant increase in the resistance of larvae of *Oncorhynchus mykiss* fed with probiotics as well as high protection against thermal and hypoxia challenges was recorded by Tukmechi and Bandboni[23], and results of this study also indicated that addition of *Bacillus* into the diet had effects on fish resistance toward different challenges. Probiotics may protect through a recuperation of mucosal barrier function when disturbed and may stimulate mucus production[24,25]. The same results about hypoxia, thermal and salinity challenges in rainbow trout were reported by Kitao and Yoshida[26]. The species composition of the intestinal microflora of fish larvae can be influenced at an early stage of development, when few, if any, bacteria are present in the larval gut, by addition of specific bacterial strains to the live food or the water[6]. The microbial balance of fish biomotor as well as digestion has effects on all physiological operations in the fish body. Probiotics can be inoculated onto fish skin and gill surfaces as well as digestive tract and stimulate local cells for the best operation[27]. There are other studies that used probiotic which caused improvement of fish survival like rainbow trout[12,13]. Similarly Ako *et al*. has reported enhancement of the resistance to physical stress in larvae of *Mugil cephalus* fed with bioencapsulated *Artemia* nauplii[28]. Probiotics have positive effects as reported before; similarly Kumar *et al*. fed *Labeo rohita* with feed containing *B. subtilis* and reported significant survival rate after challenge with *Aeromonas hydrophila*[29]. Challenge with basic pH causes significant resistance in experimental groups fed with supplemented diet in comparison with the control ($P < 0.05$). Despite of low concentration of *B. circulance* and *B. subtilis,* the higher survival time was noted for T1 which was supplemented with 1×10^4 CFU/g. Similar results were observed in salinity challenge. The improvement of animal resistance after using probiotics was reported by Fuller[9]. Similar findings have been reported in many fish species including rainbow trout by Irianto and Austin[30], which used probiotics to control furunculosis. The

use of probiotics is a new concept in aquaculture and the present study has not only highlighted significantly improved growth and survival of *T. trichopterus* larvae with the dietary use of probiotic in comparison to unsupplemented diets, but also demonstrated even greater success in increasing resistance against physicochemical challenges when applied probiotics synergistically. So researchers have to focus on different aspects of probiotics and suggest different concentration.

In summary, the results of the present study showed that diet supplemented with *B. subtilis* and *B. circulance* did not show any significant effect on gourami larvae growth parameter except SGR and condition factor, but did increase resistance time against physical and chemical challenges.

Conflict of interest statement

We declare that we have no conflict of interest.

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