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

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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 × 10^7, 2 × 10^7, 3 × 10^7 and 4 × 10^7 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 × 10^7 to 4 × 10^7 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 and is a popular aquarium fish species. The trade and prosperity of the ornamental fish industry were health 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 × 10^8 CFU/
Feed conversion ratio (FCR) = \( \frac{TFI}{W_f} \)

where, \( W_f \) means final weight (mg); \( Wi \) means initial weight (mg); \( TFI \) means total feed intake (mg); \( TL \) means total length (mm); \( RFI \) means relative feed intake (mg).

### 2.2. Experiment fish and design

*T. trichopterus* with \( (50.0 \pm 0.8) \) 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 carpyphylla* 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) \((\text{%/day}) = \frac{100 \times [\ln W_f - \ln W_t]}{T} \)
- Feed conversion ratio (FCR) = \( \frac{TFI}{W_f} \)
- Food conversion efficiency (FCE) \((\%) = \frac{[W_f - W_i]}{TFI} \times 100 \)
- Condition factor = \( 100 \times \left( \frac{W_f}{WTL} \right)^{\frac{3}{2}} \)
- Average daily growth \((\%) = 100 \times \left( \frac{W_f - W_i}{W_f} \times T \right) \)
- Weight gain (WG) (mg) = \( W_f - W_i \)
- Growth conversion efficiency (GCE) = \( \frac{SGR}{RFI} \) × 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 = 12) challenges were run and pH value was monitored with portable pH meter (Metrohm, Switzerland).

#### 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 version 19 software).

### 3. Results

At the end of feeding trial, final body weight ranged from \((330.83 \pm 108.03) \) to \((349.51 \pm 122.62) \) 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 \( 3 \times 10^4 \) and \( 4 \times 10^4 \) CFU/g supplemental *B. circulance* and *B. subtilis* had a higher SGR than those fed with diets supplemented with \( 1 \times 10^3 \) and \( 2 \times 10^3 \) CFU/g (Figure 1).

![Figure 1. Effect of dietary *B. circulance* and *B. subtilis* on SGR.](image1)

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 (\( P > 0.05 \)).

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.](image2)
Marteau, mechanism and function of probiotics depended mainly during antagonistic process against each other that inhibited the larvae [17]. It is possible that these probiotics produced substance (any significant effect on growth parameters of channel catfish, increased the SGR of larvae during experiment but no significant 4. Discussion

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

| 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                                 | 787.33                         | 576.23          | 796.68            |
| T1         | 644.67                                 | 933.00                         | 668.23          | 928.67            |
| T2         | 537.67                                 | 999.00                         | 590.67          | 841.67            |
| T3         | 528.67                                 | 967.00                         | 744.33          | 866.00            |
| T4         | 615.67                                 | 930.67                         | 636.33          | 887.33            |

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 $3 \times 10^3$ CFU/g $B.\ \textit{circulance}$ and $B.\ \textit{subtilis}$ 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 $1 \times 10^4$ CFU/g $\text{Bacillus sp}$. |

### Table 1

Growth response of gourami larvae fed with diets supplemented with graded levels of $B.\ \textit{circulance}$ and $B.\ \textit{subtilis}$. 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 | 340.760 ± 117.740 | 330.830 ± 108.030 | 332.580 ± 105.280 | 334.750 ± 120.950 |
| WG (kg)                     | 284.750 ± 120.950 | 299.510 ± 122.620 | 290.760 ± 117.740 | 280.830 ± 108.030 | 282.580 ± 105.280 |
| FCR (%)                     | 0.920 ± 0.510  | 0.920 ± 0.480 | 1.010 ± 0.970  | 1.080 ± 0.560   | 1.080 ± 1.020    |
| FCE (%)                     | 155.560 ± 54.580 | 151.670 ± 52.400 | 147.250 ± 48.080 | 148.030 ± 46.860 | 148.990 ± 53.830 |
| GCE (%)                     | 0.917 ± 0.004  | 0.917 ± 0.003 | 0.917 ± 0.004  | 0.917 ± 0.003   | 0.917 ± 0.005    |
| Average daily growth (%)    | 319.960 ± 8.170 | 319.380 ± 7.840 | 318.720 ± 7.200 | 318.830 ± 7.010 | 318.890 ± 8.060  |

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

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 ($\text{Hipoglossus hippocampus} \text{ L.}$) 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 ($\text{Oncorhynchus mykiss}$) was significantly increased by feeding a dietary supplement of $\text{Bacillus spp}$.13. 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 $B.\ \textit{circulance}$ and $B.\ \textit{subtilis}$ 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 $\text{Saccharomyces cerevisiae}$ and $\text{Saccharomyces boulardii}$ as probiotic enhanced host resistance against thermal and acidic pH stresses[21]. Also the use of same probiotic increased rainbow trout larva resistance against salinity challenge (10, 15 g/L)[22]. Significant increase in the resistance of larva of $\text{Oncorhynchus mykiss}$ 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 $\text{Bacillus}$ 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 Kito 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 $\text{Magil cephalus}$ fed with bioencapsulated $\text{Artemia nauplii}[28]$. Probiotics have positive effects as reported before; similarly Kumar et al. fed $\text{Labro rohita}$ with feed containing $B.\ \textit{subtilis}$ and reported significant survival rate after challenge with $\text{Aeromonas hydrophila}[29]$. 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 $B.\ \textit{circulance}$ and $B.\ \textit{subtilis}$, the higher survival time was noted for $T1$ which was supplemented with $1 \times 10^4$ CFU/g. Similar results were observed in salinity challenge. The improvement of animal resistance after using probiotics was reported by Fuller[30]. Similar findings have been reported in many fish species including rainbow trout by Irianto and Austin[31], 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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