EVALUATIONS OF PROBIOTIC BACTERIA (*BACILLUS* SPP.) AS DIETARY ADDITIVES ON GROWTH PERFORMANCE AND SURVIVAL RATE OF SIAMESE FIGHTING FISH (*BETTA SPLENDENS*)

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ABSTRACT

Effects of dietary administration of *Bacillus subtilis, B. pumilus and B. licheniformis* on growth performance and survival rate of Siamese fighting fish (*Betta splendens)*. were evaluated in the present study. The experiment was conducted for 8 weeks with 12 h light: 12 h dark cycles and performed in triplicate with 10 dietary groups (one control and nine modified diets) comprised of 45 fish in each group. The treatments consisted of three levels at an initial concentration of 1×10^6 $CFU.g^{-1}$ (T_1), 1×10^7 $CFU.g^{-1}$ (T_2) and 1×10^8 $CFU.g^{-1}$ (T_3) and one control, and were conducted every day. Siamese fighting fish (weight 0.1 ± 0.01 g) was fed with these diets and various growth indices and survival rate study were conducted for 8 weeks post-feeding. Survival percentage and condition factor of the diet containing 1×10^8 $CFU.g^{-1}$ of *B. subtilis* had the best performance and showed a significant difference from other treatments ($P< 0.05$). The results suggest that *Bacillus subtilis* supplemented at a dose rate of 1×10^8 $CFU.g^{-1}$ could significantly increase survival rate and is beneficial to *Betta splendens*.

KEYWORDS : Diet, Duncan's test, Growth index, Fish growth rate, Siamese fighting fish (*Betta splendens)*

INTRODUCTION

Probiotics are live microorganisms that improved growth performance, innate immune responses, and resistance against disease, so are widely used in aquaculture (Denev *et al*., 2009; Nayak, 2010).

Production of Siamese fighting fish (*Betta splendens* Regan, 1910) has been providing the highest income among exported ornamental fish in Iran. During the life span of fish, live diets such as rotifers, infusorians, water fleas, and mosquito larvae are mainly used. Propagation of the live diets mostly uses the wastes from avian and porcine farms that cause the occurrence of diseases and environmental impacts. In order to increase successive growth and survival of fish, artificial diets with improved nutrient utilization are important. Many methodologies were used to increase nutrient utilization, such as probiotics (Son *et al*., 2009; Yanbo and Zirong 2006). Therefore, this study was attempted to investigate the effect of different doses of *B. subtilis*, *B. pumilus* and *B. licheniformis* on the growth performance and survival percentage of Siamese fighting fish (*Betta splendens)*.

MATERIALS AND METHODS

All the three bacterial strains purchased from Protexin Co and were preserved in the laboratory and their purity was checked routinely during the entire course of study. The probiotic strains, *Bacillus subtilis, B. pumilus* and *B. licheniformis* were cultured on normal nutrient agar by spore staining (using the Shaeffer-Fulton method) with the spread plate technique and counted- (Marshall and Beers, 1967). The pellet feed was stored in a cool dry place until use. The basal diet was used as control diet (T0). Three probiotic supplemented diets, designated as T1, T2 and T3, were prepared with three levels of inclusion of *Bacillus* sp. (*B. subtilis*, *B. pumilus* and *B. licheniformis*), i.e. 1×10^6, 1×10^7 and 1×10^8 $CFU.g^{-1}$ diet, respectively. The survival of the supplemented bacteria

in the diet was assessed following storage at 4 ^{0}C and at room temperature (26 ^{0}C) on weekly basis for four weeks (Irianto and Austin, 2002). One gram of the diet was homogenized in 9.0 ml sterile saline solution, and serial dilutions down to 10^{-4} were prepared and 0.1 ml was spread onto triplicate plates of nutrient agar. The colonies were counted after incubation for 24 h at 30 ^{0}C. Based on the survivability data feeds were prepared on weekly basis to ensure high probiotic levels in the diet. The main feed contained fish meal (30%), soybean meal (20%), wheat gluten (12%), squid meal (5%) and wheat flour (20%), lecithin (2%), fish oil (1 %), soyabean oil (2.6%), mineral mixture* (0.05%) vitamin mixture** (0.25%), vitamin C (0.1%), fermented rice (0.2%) and fiber (5%).

(***Mineral mixtures:** 1 kg of feed contained 30 mg iron, 20mg zinc, 25 mg manganese, 5mg copper, 5mg iodine and 0.2 mg selenium.

****Vitamin mixtures:** 1 kg of feed contained 4000 IU vitamin A, 2000 IU vitamin D3, 50mg vitamin E, 10 mg vitamin K, 20 mg thiamine, 20 mg riboflavin, 20 mg pyridoxine, 200 mg calcium panthothenate, 150 mg niacin, 2 mg biotin, 5 mg folic acid, 0.2 mg vitamin B_{12}, 400 mg inositol and 200 mg ethoxyquin.)

Juvenile fish were obtained from a private farm of Qazvin Province, Iran. The fish were acclimatized indoors, in tanks (60 cm diameter × 30 cm height) with water temperature of 28.5 ± 0.3 ^{0}C, and fed with the control (unmodified) diet for 7 days before starting the experiments. The fish of 0.1 ± 0.01 g initial weight and 33.80 ± 0.04 mm initial length were randomly distributed into 30 aquaria (18 × 19 × 34 cm), 15 fish per aquarium with a porous white cubic box (6 × 16 × 22 cm) for reducing aggressive stress between fish . The experiment was conducted for 8 weeks with 12h light/12h dark and performed in triplicate with 10 dietary groups (one control and nine modified diets) comprised of 45 fish in each group. The treatments consisted of three levels of bacillus sp. at an initial concentration of 1× 10^6 CFU.g^{-1} (T_1), 1× 10^7 CFU.g^{-1} (T_2) and 1× 10^8 CFU.g^{-1} (T_3) and one control ie. unmodified diet. Fish were not fed on the sampling day. Culture conditions were 26 ÚC, 12: 12 h light: dark photoperiod, and distilled water with or without the addition of 5 g/L of common non-iodized cooking salt. The daily rations were supplied four times per day at 4-h intervals (08: 00, 12: 00, 16: 00, and 20: 00 h), gentle aeration was added to facilitate even food distribution, and 80% of the water was replaced daily. Weight and length of the fish were measured individually.

The water quality during the experimental period was as follows: temperature 27.55 ± 0.33 °C, pH 7.22 ± 0.06, dissolved oxygen 4.15 ± 0.06 mg. L^{-1}, conductivity 0.45 ± 0.01 mS. cm^{-1}, total alkalinity 89.74 ± 1.22 mg. L^{-1} $CaCO_3$, total hardness 110.45 ± 0.55 mg. L^{-1} $CaCO_3$, free carbon dioxide 1.66 ± 0.05 mg. L^{-1}, nitrate 0.042 ± 0.004 mg. L^{-1}, nitrite 0.0035 ± 0.0001 mg. L^{-1}, total ammonia nitrogen 0.022 ± 0.003 mg. L^{-1} and phosphorous 0.026 ± 0.002 mg. L^{-1}.

Data were expressed as ± standard deviation of mean in triplicate observations. One-Way Analysis of Variance was used for evaluating growth performance parameters. Significant differences between means were ranked using Duncan's multiple range tests at 95% significance level (differences were considered to be significant at values of $P < 0.05$). All statistics were performed using SPSS for Windows version 16 (SPSS, Chicago, USA).

RESULTS AND DISCUSSION

The data on weight gain and total length presented in Table 1 reveals that highest body weight gain and total length were observed in *Bacillus subtilis* supplemented diets followed by *B. pumilus* and least in *B. licheniformis.* Further T3 (1 × 10^8 CFU.g^{-1}) showed significantly ($P < 0.05$) highest weight gain and total length among the three levels of the probiotics and among the three probiotics at this level *Bacillus subtilis* proved better than *B. pumilus* and *B. licheniformis*. Supplementation of other two strains showed non significant increase in body weight and total length at all the three levels.

Application of the probiotic, *Bacillus* sp., significantly increased survival rate ($P< 0.05$) in all treatments when compared with the control diet (Table 1). A significant increase in survival rate was found in diet supplemented with *B. subtilis* (96.7 ± 0.2%) compared with *B. pumilus* (93.8 ± 1.8%) and *B. licheniformis* (80.9 ± 1.9%) at 1×10^8 CFU.g^{-1}. However, no significant difference ($P > 0.05$) was found between *B. pumilus* and *B. licheniformis* in survival rate and also no significant difference was observed between the doses of various treatments with *B. licheniformis* (Table 1). Supplementation of probiotics affects FCR variably. There were no significant difference in FCR between control group and other treatment group of all the strains at 1×10^6CFU.g^{-1} wheras at other treatments (1×10^7 and 1×10^8 CFU.g^{-1}) significant differences were observed (excluding *B. licheniformis*) in FCR . Highest FCR (2.02±0.1) was observed in control and lowest (1.70 ± 0.06) FCR, in *B. subtilis* containing diets at 1×10^8 CFU.g^{-1}. It was clear from this study that the application of probiotic, *Bacillus* sp. via the diet had beneficial effects on the survival rate and growth of Siamese fighter (*Betta splendens)* with significantly highest effect of *B. subtilis*. Similarly, Jafaryan *et al.* (2008) found that *Bacillus* supplementation to the diet significantly increased growth performance of *Acipenser nudiventris* larvae. Improvement in growth performance by dietary supplementation with *Bacillus* sp. has been reported in many aquatic species by a number of workers (Aly *et al.* 2008; Gonçalves *et al.* 2011; Balcázar *et al.* 2007; Zhang *et al.* 2010; and Cha *et al.* 2013).

Bacillus subtilis has been shown to produce digestive enzymes such as amylase, protease and lipase which enrich the concentration of intestinal digestive enzymes (Gupta and Dhawan 2012). The bacteria could also have improved digestive activity via synthesis of vitamins and cofactors or via enzymatic improvement (Gatesoupe, 1999). Gullian *et al.*(2004) demonstrated a significant improvement in growth in shrimp inoculated with *Bacillus* sp. whilst El-Dakar and Goher (2004) found that enhanced growth was generally obtained in shrimp fed diets with *B. subtilis* inclusion.

Table 1: Final body weight, total length and net weight gain, survival rate and FCR of Siamese fighting fish (Bettasplendens) fed the experimental diets containing three different Bacillus sp. for 8 weeks.

	Treatment	FBW (g)	TL(cm)	NWG (g)	Survival (%)	FCR
T0	Control	0.69 ± 0.15	1.58 ± 0.11	0.59±15	70.6±0.8^{a}	2.02±0.1^{a}
T1	*B. subtilis*	0.73 ± 0.29^{a}	1.63 ± 0.32^{a}	0.63 ± 0.29^{a}	76.4±1.9^{a}	1.87±0.1^{a}
	B. pumilus	0.72 ± 0.33^{a}	1.59 ± 0.11^{a}	0.62 ± 0.33^{a}	74.6±1.8^{a}	1.91±0.04^{a}
	B. licheniformis	0.69 ± 0.41^{a}	1.58 ± 0.43^{a}	0.59 ± 0.41^{a}	71.6±2.8^{a}	1.98±0.1^{a}
T2	*B. subtilis*	0.85 ± 0.19^{a}	1.68 ± 0.37^{a}	0.75 ± 0.19^{a}	84.7±0.9^{b}	1.81±0.1^{b}
	B. pumilus	0.78 ±	1.63 ± 0.55^{ab}	0.68 ± 0.23^{ab}	78.5±1.6^{ab}	1.83±0.08^{ab}
	B. licheniformis	0.71 ± 0.35^{b}	1.59 ± 0.33^{b}	0.61 ± 0.35^{b}	75.9±2.8^{ab}	1.95±0.08^{a}
T3	*B. subtilis*	0.98 ± 0.29^{a}	1.75 ± 0.43^{a}	0.88 ± 0.29^{a}	96.7±0.2^{b}	1.7±0.06^{b}
	B. pumilus	0.89 ± 0.33^{b}	1.68 ± 0.24^{b}	0.79 ± 0.33^{b}	93.8±1.8^{b}	1.81±0.09^{b}
	B. licheniformis	0.75 ± 0.41^{c}	1.62 ± 0.11^{c}	0.75 ± 0.41^{c}	80.9±1.9^{a}	1.93±0.1^{ab}

FBW: Final Body Weight; TL: Total Length; NWG: Net Weight Gain

Values are means of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different ($P < 0.05$).

T1: 1 × 106; T2: 1 × 107; T3:1 × 108 CFU.g-1

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