Growth performance and disease resistance towards *Aeromonas hydrophila* in *Hemibagrus nemurus* (Valenciennes, 1840) fingerlings through probiotic feeding

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Abstract

A study was carried out to evaluate the probiotic activity of *Bacillus subtilis* G1 isolated from fermented pickles in growth performance and disease resistance of *Hemibagrus nemurus* fingerlings at Universiti Putra Malaysia. The probiotic was mixed in feed at doses of 0 (C, control), 3 x 10^9 (T1) 3 x 10^7 (T2) and 3 x 10^5 (T3) cfu g^{-1} and fed to the catfish fingerlings for nine weeks. Results showed that catfish fed a diet containing 10^7 cfu g^{-1} *B. subtilis* G1 had significantly higher percent weight gain (248.69 ± 3.31%), and better food conversion ratio (1.68 ± 0.03), than those of other treatments. Inhibitory activity of the probiotic *B. subtilis* G1 against fish pathogens *Aeromonas hydrophila* and *Streptococcus agalactiae* was evaluated by well diffusion agar method. Inhibition zones measured showed *A. hydrophila* and *S. agalactiae* were 16.13 ± 0.91 mm and 17.5 ± 1.84 mm, respectively, indicating strong inhibitory activity against the pathogens. Three weeks after the feeding trial, the fingerlings were challenged with 0.1 ml containing 10^6 cfu ml^{-1} of *A. hydrophila* by intra-peritoneal injection. After 14 days, the mortality rate of catfish was significantly lower in group T1 (30 ± 5.8%) compared to the control (C) group (56.7 ± 3.3%). The findings of this study proved that administration of *B. subtilis* G1 can improve growth and disease resistance in catfish.

Keywords: *Hemibagrus nemurus*, Probiotic, Growth performance, Disease resistance, *Aeromonas hydrophila*

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Introduction

Aquaculture sector has greatly been transformed to high technology activities for high market contribution to fulfill the domestic demand of high protein resources and export demand of fish products (Hamdan, 2011). In Malaysia, freshwater fish production is dominated by catfish, tilapia and various species of carps. *Hemibagrus nemurus*, the Asian redtail catfish is also identified as *Mystus nemurus* and locally known as baung (Rainboth, 1996). *H. nemurus* is a high price aquarium fish and commercially cultured as live food fish trade as it contains high nutritional values and tastes good (Chong *et al*., 2000).

However, disease has become a primary constraint to aquaculture growth and has caused severe impact on both the economic and socio-economic development in many countries (Subasinghe, 2005). Growth and survival of catfish fry to fingerlings varies greatly depending on the condition of the culture tank, stocking densities, food abundant and the incidence of infectious diseases. Bacterial disease is known to be the famous infections towards catfishes (Al-Dohail *et al*., 2009). The pathogens from genus *Aeromonas* were commonly found in freshwater fishes in Malaysia such *A. hydrophila* (69.6%), *A. caviae* (8.7%) and *A. sobria* (21.7%) (Freshwater Fisheries Research Centre, 2004). The use of probiotic bacteria has been suggested as an alternative method for growth and survival improvement, and, prevention and control of various diseases in aquaculture (Son *et al*., 2009; Chiu *et al*., 2010; Sun *et al*., 2010).

The use of probiotic is an alternative way to replace the use of antibiotic and other chemicals, which kill not just pathogens of the aquatic species, but also most of the beneficial bacteria in the water column (Sahu *et al*., 2008). Probiotics are defined as, "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved used of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" (Verschuere *et al*., 2000). Probiotics beneficially affect the host by producing inhibitory compounds, competing for adhesion site, nutrient and energy source, providing nutrients and enzymes for digestion, enhancing immune response, improving water quality, interacting with phytoplankton, and showing antiviral activity (Verschuere *et al*., 2000; Sahu *et al*., 2008; Son *et al*., 2009; Chiu *et al*., 2010; Sun *et al*., 2010).

Wide ranges of bacteria such as *Lactobacillus*, *Saccharomyces*, *Carnobacterium*, *Vibrio*, *Bacillus*, *Aeromonas* and *Pseudomonas* have

been applied as probiotics in aquaculture (Verschuere *et al*., 2000; Balcázar *et al*., 2006; Son *et al*., 2009; Chiu *et al*., 2010; Shakibazadeh *et al*., 2012). The genus *Bacillus* has been widely used in aquaculture as the bacterium produces endospores that are highly resistant to unfavourable environmental conditions such as extreme water temperatures. Some *Bacillus* species have shown inhibitory activity against various pathogens and also increases survival rate and growth performance of prawns and shrimps (Mujeeb Rahiman *et al*., 2010; Zokaeifar *et al*., 2012b). This study aimed to investigate the effect of probiotic *Bacillus subtilis* G1 isolated from fermented pickles (Zokaeifar et al., 2012a) on growth performance and disease resistance of *H. nemurus* fingerlings towards *A. hydrophila* infection.

Materials and methods

Diet preparation

The probiotic bacteria, *B. subtilis* strain G1 (GenBank accession number HQ731482), which has identified with 100% similarity as *B. subtilis* subsp. *spizizenii* NRRL B-23049^T, was grown in TSB for 24 h using shaking incubator at 29°C. The cultures were then centrifuged at 3000 rpm for 15 min and the pelleted bacteria were collected and re-suspended in normal saline solution (NSS). The concentration of the suspension was calculated to the colony-forming unit (cfu) using spread-plate technique and also optical density (OD) value using biophotometer at 600 nm. Commercial feed (Starfeed, Malaysia) was used as basal diet and *B. subtilis* suspension were soaked with the feed as described by Robertson *et al*. (2000) to give a final concentration of 3×10^9 cfu g^{-1} (T1), 3×10^7 cfu g^{-1} (T2), and 3×10^5 cfu g^{-1} (T3). No soaking of probiotic with feed for control diet (C). The feed were then oven-dried at 35°C for 2 hours. One gram of each prepared feed type was sampled to determine the *B. subtilis* concentration by spread-plate technique using mannitol-egg yolk-polymyxin agar (MYP agar, Difco, USA). The feed preparation was done once a week in order to maintain the concentration of the probiotic bacteria inside the feed.

Probiotic administration to catfish

Catfish *H. nemurus* fingerlings with size of 7±1 cm were purchased from a private farm in Perlok, Pahang. Fish were acclimatized for one week and were fed with unaltered commercial pellet. Fish were randomly sampled and weighed, and then placed in 100 L glass aquarium containing 70 L sterilized water. Each aquarium was equipped with a top water-filter. Experiment was conducted in a completely randomized design, with four treatment groups consisted of T1, T2, and T3, for fish administrated feed mixed with probiotic and C for feed without probiotic. Each

treatment was conducted in three replicates contained 30 individuals of fish per aquarium. Fish were fed twice daily at *ad libitum* for nine weeks. Fish from each aquarium were weighed once every week until the end of the trial. The growth parameters and survival of fish were calculated as below:

Weight gain (g) =Final weight (g) – Initial weight (g)

Percent weight gain (%)= ([Final weight (g)–Initial weight (g)]/Initial weight (g)) ×100

Specific growth rate (%)= ([ln Final weight– ln Initial weight] / Days) ×100

Feed conversion ratio, FCR=Feed intake (g)/ [Final weight (g) –Initial weight (g)]

Survival rate (%)= ([Initial stocking– Dead fish]/Initial stocking) ×100

Water quality management

Water quality was monitored weekly. Temperature and pH were measured by a YSI pH and Temperature meter (YSI, USA), respectively. Dissolved oxygen (DO) was measured by YSI DO and Temperature meter Model 57 (YSI, USA) and ammonia-nitrogen was measured by an Ammonia-Nitrogen LaMotte Tes Tab® Reagent Test Kit (LaMotte, USA). The fish aquaria were daily cleaned from feces by the top water-filter and the water were weekly changed by 100% after sampling.

Assessment of antibacterial activity by agar well diffusion method

Two freshwater pathogens, *A.hydrophila* and *S. agalactiae*, which were obtained from the culture collection maintained at Aquatic Animal Health Unit of the Faculty of Veterinary Medicine, UPM, were used to evaluate the antagonistic ability of the probiotic (*in vitro*). Probiotic *B. subtilis* G1 was grown in TSB at 29°C for 24 h. After incubation, the bacterial suspensions were removed by centrifugation (3000 rpm, 15 min) and the culture supernatant was used in this experiment. An exact 0.1 ml of 24 h cultured of *A. hydrophila* (or *S. agalactiae*) grown in TSB were spread onto TSA plate and air-dried for about 10 min. Then, five wells were punched into the agar by using 6 mm diameter cork borer. A 0.1 ml of *B. subtilis* supernatant were added into the four wells as replications and the other well with uninoculated TSB as control. Experiment was conducted in duplicate. After 24h incubation at 29°C, diameters (in mm) of inhibition zone around the wells were recorded (Mujeeb Rahiman *et al*., 2010).

Challenge test

Experiment was conducted with four treatment groups consist of T1, T2, and T3, for fish fed with probiotic and C for fish fed control diet (without probiotic). Each group comprised 10 catfish per 10 L aquarium. Fish were fed *ad libitum* twice daily with their respective diets for three weeks. Then, all four groups

were injected intraperitoneally with 10^6 cfu ml^{-1} of *A. hydrophila* suspension (0.1 ml $fish^{-1}$). The experiment was mortalities were counted daily up to 14 days.

Statistical analysis

Data on growth parameters and susceptibility were statistically analyzed using one-way analysis of variance (ANOVA) and Duncan Multiple Range Test was applied to identify the significant differences among means. All statistical analysis was performed using SPSS, version 16.0.

Results

Growth performance

Table 1 shows final weights, weight gains and percent weight gain (PWG) of fish treated with probiotics (T1, T2 conducted in three replicates. Fish were continuously fed with their respective diets during the challenge period and and T3) were significantly higher than the fish without probiotic treatment (C). Within the three probiotic treatment groups, fish fed diet containing 10^7 cfu g^{-1} *B. subtilis* G1 (T2) showed significantly higher PWG compared to the fish fed T1 and T3 diets. Fish fed T1 and T2 diets were observed significantly having better FCR values as compared to the control and fish fed T3 diet. There were no significant differences in SGR and survival of fish among treatments. However, a tendency of slightly higher value of SGR was observed in the group of fish fed with probiotic.

Table 1: Effect of *Bacillus subtilis* G1 on growth performance of *Hemibagrus nemurus* fingerlings.

Diet	C	T1	T2	T3
Initial weight (g)	6.03 ± 0.03^a	6.07 ± 0.03^a	6.07 ± 0.03^a	6 ± 0.06^a
Final weight (g)	18.1 ± 0.44^b	20.17 ± 0.27^a	21.1 ± 0.32^a	19.73 ± 0.54^a
Weight gain (g)	12.03 ± 0.41^b	14.1 ± 0.31^a	15.03 ± 0.29^a	13.77 ± 0.52^a
PWG (%)	198.47 ± 5.8^c	232.16 ± 5.15^b	248.69 ± 3.31^a	229.18 ± 7.65^b
SGR (%)	1.73 ± 0.03^a	1.90 ± 0.03^a	1.98 ± 0.01^a	1.89 ± 0.04^a
FCR	1.9 ± 0.04^a	1.76 ± 0.04^b	1.68 ± 0.03^b	1.81 ± 0.04^{ab}
Survival (%)	83.33 ± 1.93^a	85.56 ± 1.11^a	84.44 ± 1.11^a	84.44 ± 1.11^a

Values (means±SE) in the same row with different superscript are significantly different ($p< 0.05$). PWG: percent weight gain; SGR: specific growth rate; FCR: food conversion ratio; C: control diet (without *B. subtilis* G1); T1: diet + 10^9 cfu g^{-1} *B. subtilis* G1; T2: diet + 10^7 cfu g^{-1} *B. subtilis* G1; T3: diet + 10^5 cfu g^{-1} *B. subtilis* G1

Water quality parameters

During the experimental period, the weekly reading of water temperature, pH, DO and NH_3-N of the rearing water were ranged from 27.7 to 28°C, pH 6.1 to 6.3, 6.2 to 6.6 mg L^{-1}, and 2 to 2.8

ppm, respectively (Table 2). There was a significant difference in the NH_3-N values where T1 and T2 had lower concentrations than the control. The DO of T1 was significantly higher than T3 which may be due to the aeration power.

Table 2: Water quality of *Hemibagrus nemurus* culture water.

Parameter	C	T1	T2	T3
Temperature (°C)	27.83 ± 0.07^{a}	27.83 ± 0.07^{a}	27.8 ± 0.06^{a}	27.93 ± 0.09^{a}
pH	6.27 ± 0.03^{a}	6.17 ± 0.03^{a}	6.13 ± 0.03^{a}	6.17 ± 0.07^{a}
DO (mg L^{-1})	6.47 ± 0.03^{ab}	6.57 ± 0.03^{a}	6.5 ± 0.06^{ab}	6.3 ± 0.1^{b}
NH_3-N (ppm)	2.73 ± 0.07^{a}	2.13 ± 0.07^{b}	2.07 ± 0.07^{b}	2.4 ± 0.23^{ab}

Values (means±SE) in the same row with different superscript are significantly different (p<0.05). C: control diet (without *B. subtilis* G1); T1: diet + 10^9 cfu g^{-1} *B. subtilis* G1; T2: diet + 10^7 cfu g^{-1} *B. subtilis* G1; T3: diet+ 10^5 cfu g^{-1} *B. subtilis* G1

Antibacterial activity of probiotic

B. subtilis G1 showed strong antibacterial activity against *A. hydrophila* and *S. agalactiae* with inhibition zones of 16.13±0.91 and 17.5±1.84, respectively (Table 3).

Table 3: Antagonistic activity of *Bacillus subtilis* G1 strain against pathogens.

Strain	Diameter of inhibition zones (mm)	
	A. hydrophila	*S. agalactiae*
B. subtilis G1	16.13 ± 0.91	17.5 ± 1.84

Values (means ± SE) are means inhibition zone of 8 replicates
Inhibition zones: <12.0 mm (Resistant); 12.0-16.0 mm (Intermediate); >16.0 mm (Susceptible) including the well (6 mm)

Challenge test

After three weeks of feeding, the catfish were infected with *A. hydrophila* for two weeks in order to determine the disease resistant of the fish after being fed with probiotics. Fig. 1 showed, 30±5.8% mortality of fish fed T1 diet (10^9 cfu g^{-1} *B. subtilis* G1) was significantly lower (p<0.05) than the fish fed with control diet (56.7 ± 3.3%). Death occurred after 4 days of challenge for all groups except for group T1 which occurred after 5 days of challenge.

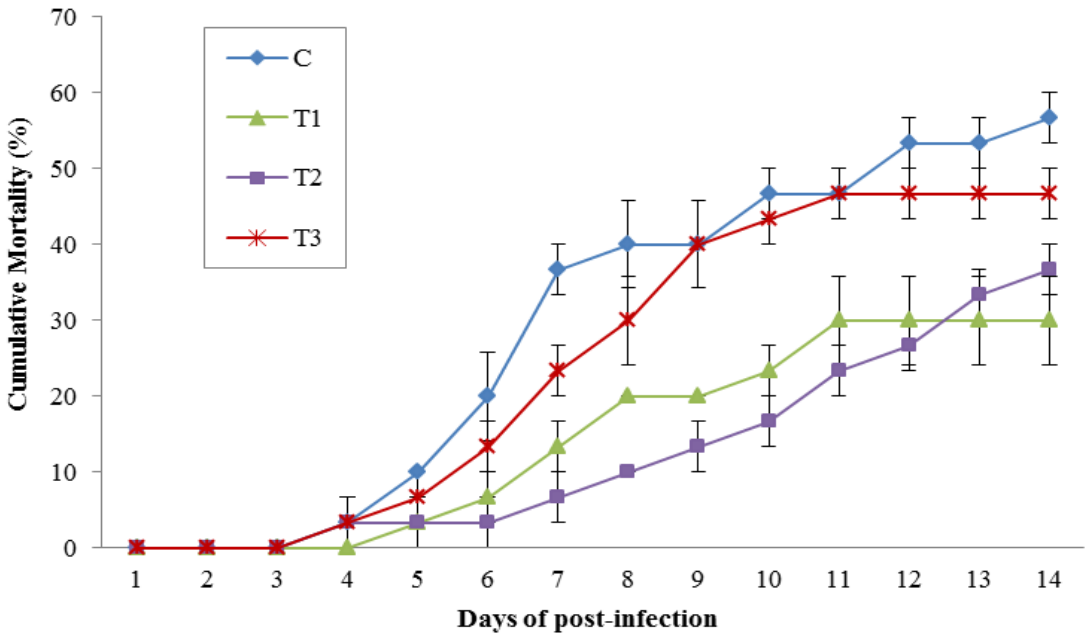

Figure 1: Cumulative mortality of catfish (*H. nemurus*) fingerlings fed probiotic over 14 days post-infection with pathogen *A. hydrophila*. C, fish fed without probiotic; T1, fish fed 10^9 cfu g^{-1} *B. subtilis* G1; T2, fish fed 10^7 cfu g^{-1} *B. subtilis* G1; T3, fish fed 10^5 cfu g^{-1} *B. subtilis* G1

Discussion

Bacillus is commonly used as probiotic in aquaculture. This species was found to have improved growth performance, immunity and disease resistant of some shrimp, prawn and fish. Although several studies have been done on the effectiveness of probiotics in aquaculture, the exact mechanism of action is still not well understood. Zokaeifar *et al*. (2012a) isolated *B. subtilis* strain G1 from fermented pickles (garlic), molecularly identified and characterized as potential probiotic for shrimp culture. This *B. subtilis* G1 strain was previously applied in shrimp culture with a salinity of 20 ppt. In the current study, it was carried out in Dietary administration of *B. subtilis* G1 to *H. nemeurus* fingerlings significantly improved final weight, weight gain, PWG and FCR of the fish in the present study. According to Lovell (1989), weight gain usually considered the most important measurement of the quality of experimental feeds. Different concentration of the probiotic may contribute to the fish growth. Studies showed significant increases in PWG of fish fed with diet 10^7 cfu g^{-1} of *B. subtilis* G1, suggesting the optimal concentration of *B. subtilis* G1 in diets. In previous study by Zokaeifar *et al*. (2012b), mixture of *B. subtilis*, strains G1 and L10 in diet demonstrated higher weight gain and SGR in *Litopenaeus vannamei* culture at dose 10^8 cfu g^{-1}.

This prove that a higher concentration of probiotic may not lead to a better growth performance (Son *et al*., 2009) and too low of probiotic concentration was not enough to trigger the probiotic effect to the host. Low FCR value of fish in the present study proved that feeding with probiotic is good to control fish feeding and feed cost as the probiotic makes the feed high in quality. Improvement of growth in the fish could be attributed by other mechanisms such as increasing of digestive enzyme activity or ability of probiotic to out-compete with other bacteria for space and nutrients (Verschuere *et al*., 2000; Zokaeifar *et al*., 2012b).

Previous study by Zokaeifar *et al*. (2012b) showed no effect of probiotic on the water quality of shrimp culture. However, in the present study showed some improvement in the rearing water of catfish fed with probiotic diet, with lower NH_3-N concentration compared to the rearing water of catfish fed with control diet, even though the concentration itself was high (above 2 ppm). This was due to the weekly change of the water, while in the previous study the water was changed twice weekly thus the effect of the probiotic to the rearing water was not much affected. The probiotic help to reduce the NH_3-N concentration by oxidizing ammonia to nitrite and nitrate (Verschuere *et al*., 2000; Sahu *et al*., 2008), thus prevents growth of pathogens, enhanced mineralization of organic matter in water and sediment and removal of undesirable waste compounds (Zhou *et al*., 2009).

The survival rate of fish in the present study was high as *H. nemurus* is known to be a hardy fish. However, the fingerlings were sensitive to extreme temperature changes which caused stress and susceptible to bacterial infection. Zokaeifar *et al*. (2012a) showed the maximum antibacterial activity was observed at 1% NaCl against two marine pathogens, *Vibrio harveyi* and *V. parahaemolyticus*. The present study showed that *B. subtilis* G1 was capable of inhibiting some of freshwater pathogens such as *A. hydrophila* and *S. agalactiae*. Therefore, fish that were infected with *A. hydrophila* for two weeks and fed with 10^9 cfu g^{-1} of *B. subtilis* G1 showed significantly lower mortality compared to the control. Probiotic bacteria have a great impact on immune system of cultured aquatic animals as non-specific immune modulators which would strengthen the antibody level and the activity of macrophage (Verschuere *et al*., 2000; Balcázar *et al*., 2006; Sahu *et al*., 2008). These can enhance disease resistance of the aquatic animals. Stimulation of the immune system involved increasing phagocytosis, antibacterial activity (Balcázar *et al*., 2006) and lysozyme activity (Nayak, 2010). Liu *et al*. (2012) suggested that resistance against pathogens is

correlated with increased alternative complementary pathway activities (ACH_{50}) and lysozyme activities of fish fed diet containing *B. subtilis*.

Effects of probiotic on growth and disease resistance are dependent on species of the aquatic organism, feeding duration and dosage, origin of the probiotic strain, different defence mechanism of fish to different pathogens, and different pathogenicity of pathogens (Son *et al*., 2009; Standen and Abid, 2011). Differences in the gut microbiota and physiology of fish and shrimp, or different fish species may affect the results (Gisbert and Castillo, 2011). In conclusion, the *B. subtilis* G1 improved growth of *H. nemurus* fingerlings by increasing weight gain, improved water quality and increased resistance against *A. hydrophila* infection.

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