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Probiotic potential of *Bacillus* species isolated from freshwater fish *Anabas testudineus in Labeo rohita*

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ABSTRACT
The objectives of the present study were to evaluate the probiotic potential of *Bacillus* sp isolated from the gut of *Anabas testudineus*. Study reveals the antagonistic effect of the *Bacilli* against *Aeromonas Hydrophila* and *Vibrio harveyi* and protective effect and survival rate of *Carassius auratus* and *Xiphophorus maculates*. The *Bacilli* was incorporated to commercial fish diet with 41% crude protein to evaluate their effect on the growth performance in rohu (*Labeo rohita.*). One hundred and sixty rohu fingerlings with an average body weight of 6.10 g per fish were equally divided into two treatments of four replicates. T1-T4 was given basal diet (control), T5-T8 with *Bacillus sp* (initial number of 10^7 cells g diet-1). The experimental period was 60 days. The best growth rate, feed utilization and survival rate were noticed in treated group. Probiotics *Bacillus* sp has significantly enhanced fish growth and health, indicating the potential use of indigenous fish guts bacteria as feed probiotic in aquaculture.

Keywords: *Bacillus* sp, *Anabas testudineus, Carassius auratus Xiphophorus maculates, Labeo rohita, Aeromonas Hydrophila* and *Vibrio harveyi*

1. Introduction
During the last decades, antibiotics used as traditional strategy for fish diseases management but also for the improvement of growth and efficiency of feed conversion. However, the development and spread of antimicrobial resistant pathogens were well documented (SCAN 2003; Kim *et al.* 2004; Cabello 2006; Sørum 2006). There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of nonpathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens (FAO 2005). On the other hand antibiotics inhibit or kill beneficial microbiota in the gastrointestinal (GI) ecosystem but it also made antibiotic residue accumulated in fish products to be harmful for human consumption (WHO 2006). By the above reasons since January 2006 European Union ratified a ban for the use of all sub-therapeutic antibiotics as growth-promoting agents in animal production.
There is evidence that the alimentary tract of fish is a complex ecosystem, containing a large number of microorganisms. Microbial populations in the intestinal contents are much higher than those in the surrounding water. This indicates that the intestines provide favorable ecological niches for these organisms. It is known, mainly from studies of the intestinal microbiota of terrestrial species, that the resident bacterial population of the intestine influences the establishment of pathogenic microorganisms in the intestinal tract and have disease preventive effect (Huber *et al.* 2004). The GI microbiota of fish is characterized by high population density, wide diversity and complexity of interactions. While all major groups of microbes are represented, bacteria predominate. They are the main constituent of the gut microbiota in fish (Spanggaard *et al.* 2000; Pond *et al.* 2006). Numerous surveys of the bacterial flora in the GI tract of fish have been made during the last twenty years. The endogenous microbiota of freshwater fish species tends to be dominated by members of the genera *Aeromonas, Acinetobacter, Bacillus, Flavobacterium, Pseudomonas* representatives of the family *Enterobacteriaceae,* and obligate anaerobic bacteria of the genera *Bacteroides, Clostridium* and *Fusobacterium* (Sakata 1990;

Huber *et al*. 2004; Kapetanovic *et al*. 2005; Hovda *et al*. 2007; Kim *et al*. 2007). Various species of lactic acid bacteria (LAB) (*Lactobacillus, Lactococcus, Streptococcus, Leuconostoc*, and *Carnobacterium* spp.) have been also demonstrated to comprise part of this microbiota (Ringø and Gatesoupe 1998). Lactobacillus and bacillus are considered to be important and more dominant among the gut bacterial flora. Studies have shown that *Bacillus* species cause changes in the internal bacterial microflora composition and this prevents colonization of pathogenic organisms and helps the optimum utilization of feed (Sogaard & Jessen 1990).

The bacterial flora of the GI tract of fishes in general, represents a very important and diversified enzymatic potential. It is capable of producing proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which is important for digestion of proteins, carbohydrates, cellulose, lipids and chitin above all some pathogen inhibitory compounds (Bairagi *et al*. 2002; Gutowska *et al*. 2004). The enzyme producing microbiota can be beneficially used as probiotic supplements while formulating the fish diet, especially in the larval stages. It presents a scope for fish nutritionists to use the enzyme producing isolates as a probiotic in formulating cost-effective fish diets. However, much more research should be conducted to determine if the addition of such isolates to fish feeds do, in fact, provide some kind of benefit to the fish involved before advocating their use (Bairagi *et al*. 2002).

In the light of the above, the present study on the isolation and evaluation of probiotic potential of *Bacillus* species isolated from freshwater fish *Anabas testudineus* was undertaken.

2. Materials and methods

The experimental fishes namely goldfish, *Carassius auratus* (0.9±0.1 g) and platy, *Xiphophorus maculates* (0.6±0.1 g) were procured locally and rohu, *Labeo rohita* (6.1±0.1 g) from carp hatchery of KUFOS. The fishes were allowed to acclimatize to the laboratory conditions for two weeks and then used for the experimental studies. They were fed 3% of their body weight with a commercial pellet feed, (The Waterbase, Nellore, India) containing crude protein (minimum 41%), crude fat (minimum 6%), crude fiber (minimum 3%) and moisture (maximum 11%).

A. Collection of *Anabas*

Fishes (*Anabas testudineus*) of size 25 g were collected from Panangad Lake, Ernakulum District, Kerala, using cast net and transported to the laboratory in polythene bags containing oxygenated water.

B. Isolation of *Bacillus sp* from gut

Fishes (*Anabas testudineus*) were stunned to death and washed several times with sterile saline to prevent contamination. The gut was removed by aseptic dissection and homogenized in a mortar and pestle by using 5 ml sterile 0.9% NaCl solution. This was added into 9 ml nutrient broth and incubated for 24 h at 30 ^{0}C followed by incubation at 45 ^{0}C for 10 min in a convection oven to activate the sporulation process. Ethanol (50% v⁄v) was added to a volume of 20 ml to each of the flasks which were incubated at 20 ^{0}C for 1 h. The contents were centrifuged at 10,000 g, the supernatants decanted and the resultant pellets incubated at 105 ^{0}C in a convection oven for 5 min. The dry pellets were reconstituted into 20 ml of sterile physiological saline and serially diluted to 10^{-4} in 10^{-1} increments. Thereafter, aliquots (0.1 ml) of each of the serial dilutions were spread onto soybean casein digest agar plates (Himedia). The plates were incubated for 24 h at 30 ^{0}C. Single colonies isolated from these plates, were purified and subjected to tests like Gram Stain, Morphology, Motility, Indole, VP, Citrate, Methyl red test, H_2S Production, Oxidase, Urease, Catalase, Starch utilization, Acid from glucose, Gas from glucose, Hemolysis and confirmed as *Bacillus* (Buchanan & Gibbons 1974).

C. *In vitro* inhibitory activity of *bacillus* isolates against fish bacterial pathogens

In vitro inhibitory activity of the isolate from fish gut was tested against *Aeromonas hydrophila* and *Vibrio harveyi* by cross streak technique. LB agar plates were prepared and inoculated with *Bacillus* isolate by a single streak of inoculum in the center of the petri dish. After 2 days of incubation at 37 °C the plates were seeded with pathogens by a single streak at a 90° angle to the isolate. The microbial interactions were analyzed by the observation of the size of the inhibition zone (Monthon & Songtham 2008).

D. Survival of *C. auratus* and *X. helleri* when grown with *Bacillus* isolate.

The fishes (*C. auratus* and *X. helleri*) were disinfected in 5 ppm KMnO4 solution for 15 min and twenty fishes each of *C. auratus* (0.9±0.1 g and 37.9±0.5 mm) and *X. helleri* (0.6±0.1 g and 36.1±0.4 mm) were introduced into series of glass aquaria of 50 l capacity i.e., $T_1 - T_6$ for *C. auratus*, and $T_7 - T_{12}$ for *X. helleri*. *C. auratus* and *X. helleri* were fed with pelleted feed at the rate of 5 and 3% of their body weight respectively, in 2 split doses daily.

A loopful of 24 h old culture of *Bacillus* sp was aseptically seeded into 10 ml Trypticase Soy broth (TSB) and incubated at 30±2 ^{0}C for 24 h. After incubation, this 10 ml broth culture was transferred to 500 ml TSB aseptically and reincubated at 30±2 ^{0}C for 48 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 20 ^{0}C. All the aquaria contained 30 l bore-well water and the cell suspension of *Bacillus* sp was added to the aquaria T_1, T_2, T_3, T_7, T_8 and T_9 so as to get a level of 10^7 cells/ml of rearing water. The aquaria, which received no bacterial inoculum served as control for *C. auratus* (T_4, T_5 and T_6) and *X. helleri* (T_{10}, T_{11} and T_{12}). The fishes were maintained in their respective tanks for 15 days and fed daily with pelleted feed. The faecal matter and other wastes were siphoned out on every 3rd day. Mortality, external signs of infections and behavioural abnormalities were recorded daily and the percentage survival (PS) was calculated (Abraham & Banerjee 2007).

E. Probiotic supplemented diet preparation

Commercial pellet feed (The Waterbase, Nellore, India) was used as the basal diet for the supplementation of probiotic. The isolate was inoculated in TSB and incubated at 30 °C for 48 h and then centrifuged at 5000 rpm for 30 min. After centrifugation, the bacteria were washed twice with sterile saline and the final concentration of suspension was adjusted to 10^{10} cells /ml in saline. The saline, containing the probiotic bacteria was added to the commercial feed to give an initial number of 10^7 cells/ g diet and dried at 40 ^{0}C under sterile conditions for 6 h. The prepared feed was stored at -20 ^{0}C and

the probiotic supplemented diet for daily feeding was kept at 4 ^{0}C. The commercial feed sprayed with sterile diluent alone served as the control diet. (Aly *et al.*, 2008 b).

F. Feeding experiment

Fishes (*Labeo rohita*) were divided into eight equal groups, so that each 100 l culture tank had 20 fishes each and all the tanks were provided with aeration. The untreated basal diet served as control (T1,T2,T3and T4) and The base diet mixed with probiotic *Bacillus sp* was the treatment (T5,T6,T7 and T8). The fishes were fed twice daily at the rate of 3% (avg. of $10^7 \pm 1$ cells/g) of body weight, 6 days a week for 60 days. The water temperature in the experimental tanks ranged from 27 to 30 °C.

G. Determination of nutritional effects

The fishes in each treatment were counted and weighed on termination of the experiment. The growth parameters and feed utilization were calculated as follows. Feed conversion ratio (FCR), specific growth rate (SGR), and protein efficiency ratio (PER) were estimated using the following formulae:

Feed conversion ratio (FCR) = $F1 (B2 + B_{dead} – B1)^{-1}$

Where F1, B1 and B2 are the feed intake, the biomass at the start and end of the experiment and B_{dead} is the biomass of the dead fish.

$$PER= [(BW_F - BW_I)/AP] \times 100$$

Where BW_F is the final mean weight, BW_I is the initial mean weight, and *AP* is the amount of protein fed.

$$SGR= 100 (In W2 – W1)/ T$$

Where *t* is the period of culture in days, ln *W*1 is the natural logarithm of the weight of the fish at the beginning of the experiment, and ln *W*2 is the natural logarithm of the weight of the fish at end of the experiment (*W*1 and *W*2 are in g).

H. Statistical analysis

One way analysis of variance (ANOVA; Systat. 10.0 software, SPSS Inc, Chicago, Il, USA) was used to determine whether significant variation between the treatments existed. All tests used a significance level of $p = 0.05$. Data are reported as mean ± standard error.

3. Results

A. Isolation and identification of *Bacillus* sp

Nine isolates belonging to the genus Bacillus were isolated by the procedure described. All are identified as same species by total protein profiling (Laemmli, 1970). The bio chemical characters of the bacillus sp are given in the table 1 below.

Table 1: biochemical characteristics of isolated fish gut *Bacilli*

Characteristic feature	Isolate
Gram Stain	+
Morphology	Rod
Motility	+
Indole	-
VP	-
Citrate	+
Methyl red test	+
H_2S Production	-
Oxidase	-
Urease	-
Catalase	+
Starch utilization	+
Acid from glucose	-
Gas from glucose	-
Hemolysis	β
Nitrate reduction test	+
Gelatin hydrolysis test	-
Casein hydrolysis test	+
Identified as	***Bacillus sp***

B. *In vitro* inhibitory activity of *bacillus* isolates against fish bacterial pathogens

The probiotic bacteria exhibited *in vitro* antagonistic activity against the pathogenic *A. hydrophila* and *V. harveyi* and the zone of inhibition were found to be 7.5 mm and 3.8 mm respectively on LB agar plates.

C. Survival of *C. auratus* and *X. helleri* when grown with *Bacillus* isolate.

Survival rates of *C. auratus* and *X. helleri* when grown with *Bacillus sp* (2.15×10^7/ml) isolated from fish gut is presented in Table 2. The isolate was found harmless to the fishes as no clinical signs or mortalities were noticed during the probiotic treatments. The addition *Bacillus* sp in to the fish-rearing medium markedly reduced the mortality rate of both *C. auratus* and *X. helleri*. The results show that the *Bacillus sp* confers benefit to *C. auratus* and *X. helleri* when administered as probiotics in water.

Table 2: Survival of platy, *Xiphophorus maculates* and goldfish, *Carassius auratus* when grown with Bacillus *sp* ($2.15x10^7/ml$). Each value (mean ± SD) is a mean of four replicates.

Days of immersion	Survival (%) of *X. maculates*		Survival (%) of *C. auratus*	
	Probiont	control	Probiont	control
0	100	100	100	100
5	100	98	100	98
10	100	95	100	95
15	100	89	100	90

D. Growth performance

Results (Table 3) show that the weight gain (4.13 g), specific growth rate, SGR (0.3741% per day), Feed Conversion Ratio, FCR (14.52) and Protein Efficiency Ratio, PER (1.63) of *L. rohita* increased significantly when fed a diet containing mixed bacteria. The growth performance was found to be poor in control (weight gain 1.21 g, SGR 0.1423 % per day, FCR 47.60, PER 0.57) when compared to test groups.

Table 3: Growth performance of *L rohita* in feeding trials with *Bacillus sp* (10^7cells/g). Each value (mean ± SD) is a mean of four replicates.

Treatment	Weight gain	% weight gain	FCR	PER	SGR
control	1.21	21.72	47.6	0.57	0.1423
Test	4.13	67.70	14.52	1.63	0.3741

4. Discussion

Probiotics are micro-organisms with health benefit to the host. They are used in aquaculture as means for disease control and as supplementary nutrients. In the present study, the probiotic potential of bacilli isolated from the gut of the *Anabas* was studied.

From the study, it was found that the *Bacilli* sp have a probiotic effect in vitro against *A. hydrophila* and *V. harveyi* possibly by the production of bacteriocin-like substance. There are several reports on the benefits of adding desirable Gram-positive and Gram-negative bacteria into the rearing water (Austin *et al.*, 1995; Spanggaard *et al.*, 2000; Abraham *et al.*, 2001). *Lactobacillus* sp. (Ringo and Gatesoupe, 1998) and *Bacillus* sp. (Sugita *et al.*, 1998) from fish gut have been reported to produce antibacterial substances. Vichai and Penkhae (2006) reported that *Bacillus* sp isolated from hepatopancreas of black tiger shrimp were found active against four shrimp pathogenic *Vibrio* spp. This proved that the probiotic bacteria used in the present study is safe for *L. rohita.*

From Table 3, it is obvious that an increase in probiotic level in the feed resulted in increase in growth rate. The probiotic treatment resulted in higher SGR (Table 3) with values 0.3741% being significantly higher as compared to control groups (0.1423%). Changes in growth rate (GR) showed similar fluctuations as with SGR. Moreza *et al*. (2011) also evaluated the effects of *Bacillus licheniformis and Bacillus subtilis* in rainbow trout and observed increased SGR and decreased FCR in treatments receiving probiotic. In the present study, significantly lower FCR values were recorded in the probiotic fed groups as compared to control. Changes in protein efficiency ratio (PER) as a result of probiotic level in the diet resulted in fluctuations in protein to meat conversion rate. This can be related to the specific characteristic of *Bacillus* spp. to

produce protease enzymes in digestive tract of many animals that improve the quality of digestion and absorption of protein compounds (Irianto and Austin, 2002). As mentioned earlier, the *Bacillus* spp. Are capable of secreting enzymes such as protease and can result in faster digestion of the protein components in the diet and convert them to simpler peptides and amino acids by peptilytic and proteolytic enzymes (Sogaard & Jessen 1990; Bairagi *et al*. 2002; Gutowska *et al*. 2004) thus enabling the fish to digest and absorb protein in the food more efficiently. In addition, the ability to produce vitamin B such as biotin and B12 in this group of bacteria could be considered as another factor in optimizing the rate of metabolism in fish (Farzanfar, 2006).

It could be concluded that the indigenous fish gut bacteria provided benefit to the culture fishes in terms of survival, pathogen protection and increasing growth potential and thus fulfilled the major requirements of being effective probiotics. However the principles behind their uses remain sound and their full potential needs to be explored further more and works are needed to assess the probiotic effects on growth and immune responses in fishes, including Indian major carps and other ornamental fishes.

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