PROBIOTIC EFFECT OF A LIVE BACTERIAL ISOLATE IN NUTRITION OF AN INDIAN MAJOR CARP, ROHU (LABEO ROHITA)

Archana Sinha* and P.K. Pandey1
Central Institute of Fisheries Education,
Kolkata Centre, Salt Lake City, Kolkata-700 091, India

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ABSTRACT
Use of probiotics as an efficient feed supplement is explained by feeding isolated probiotic strain to an Indian major carp, rohu (Labeo rohita). Two Bacillus strains (one non spore forming and the other spore forming), isolated from the gut of the carp, confirmed their probiotic effect by agar overlay method and antagonistic assay against three pathogenic strains which were incorporated in feed (protein - 40%) at graded levels of 5 X 107 cells g-1 feed, 5 X 106 cells g-1 feed and 5 X 105 cells g-1 feed. Rohu fingerlings (Avg wt - 1.08 g), stocked in plastic circular troughs, were fed with the prepared feed @ 5% of body weight. Feeding trial was continued for 30 days, based on the biogrowth parameters and proximate composition of fish. The spore forming Bacillus incorporated at concentration of 5 X 106 cells g-1 feed recorded the highest growth rate while at concentration of 5 X 107 cells g-1 feed recorded the highest survival. There existed significant difference (P< 0.05) in growth rates between fish fed with spore forming Bacillus and non spore forming Bacillus as probiotic. However, the proximate composition analysis did not reveal significant differences between the fish fed with spore forming Bacillus and non spore forming Bacillus as feed probiotic. Supplementation of spore forming Bacillus in artificial diet improved the growth and feed conversion ratio of fish.

Key words: Bacillus, Biogrowth, Feed, Nutrition, Probiotic, Rohu.

INTRODUCTION
The success of modern aquaculture depends to a great extent on sound nutritional practices based on the knowledge of nutrients required by the species cultured. It is estimated that the total feed costs in culture accounts for 30 to 70% of the production cost, depending on the type of culture and the intensity of feeding. There is a limit to the maximum growth rate beyond which further increment is possible only through genetic manipulation or administration of growth promoters that act pharmacologically to improve metabolic and/or digestive process. The supplementation of diet with growth inducing substances has the potential to be profitable because of the improved growth rate or reduced culture period. A wide range of substances including hormones, antibiotics, nutrient mixtures and herbal products have been tested on farmed fish for their growth promoting potential when fed at graded levels. A recent innovation in this aspect is the use of probiotics in aqua feed.

Probiotics etymologically means ‘for life’. Probiotics has been defined “as microbial cells administered in such a way as to enter the gastro-intestinal tract and to be kept alive with the aim of improving health” (Gatesoupe, 1999). Most probiotics are supplied as live supplements, which must have the ability to survive passage through the intestinal tract (Fuller, 1992). The probiotic bacteria act by competitively excluding the pathogenic bacteria or produce substances that inhibit the growth of the pathogenic bacteria; providing essential nutrients to enhance the nutrition of cultured animals and providing digestive enzymes that enhance the digestion of cultured animals.

The constraints associated with feeding commercial probiotic preparations, mainly their ineptness to establish, colonize and proliferate in the gastro-intestinal tract of carps has reiterated the need of supplementing carp feed with a live probiotic strain isolated from the gastro-intestinal tract of a carp.
species. The present study was undertaken with the objective to monitor the effect of feeding rohu fingerlings with feed supplemented with live probiotic isolates, on its biogrowth parameters and proximate composition.

**MATERIALS AND METHODS**

**Experimental set-up:** The experiment was conducted for a period of 30 days in the Aquarium Laboratory of Central Institute of Fisheries Education, Kolkata Centre, Kolkata, India. The test fish, rohu fingerlings (Avg wt - 1.08 g), procured from a local fish farm, were first disinfected by placing in 5 mg L⁻¹ potassium permanganate (KMnO₄) solution and subsequently acclimatized for a period of fifteen days in plastic circular troughs of fifty liters capacity.

The experiment was conducted in seven distinct experimental groups, T₀, T₁, T₂, T₃, T₄, T₅ and T₆ in triplicates in twenty one uniform sized plastic circular troughs of fifty liters capacity each. The ambient temperature of the aquarium laboratory was generally in the range of 16–36 °C and the laboratory was well ventilated so as to allow sufficient fresh air. The troughs were disinfected with potassium permanganate solution (20 mg L⁻¹), thoroughly cleansed with freshwater and filled with bore well water (forty liters) and continuous aeration was provided throughout the experiment. After taking the length and weight of each individual, each trough was stocked with eighteen numbers of fingerlings.

**Preparation of control diet:** The selected ingredients for preparing control diet, T₀ (40% protein) is presented in Fig 1. The finely grounded ingredients were thoroughly mixed and dough was prepared by adding required amount of water. The dough was sterilized for fifteen minutes in autoclave and put into a hand pelletizer to make 1.00 mm pellets. The pelleted feed was sun-dried and ground before storing in air tight bottles for further use.

**Preparation of experimental diet**

**Maintenance of probiotic strains:** Tryptose soya broth medium (TSB) (Hi-Media) was used for the growth and maintenance of the two isolated Bacillus strains, one non spore former and the other spore former. The two Bacillus strains, isolated from the gut of Cirrhinus mrigala, were identified for their probiotic effect by agar overlay technique using a pathogenic Aeromonas strain (received from CIFE, Mumbai) and were subsequently confirmed for their probiotic status by antagonistic assay by cross streak method against three pathogenic strains of Aeromonas, Pseudomonas and Edwardsiella (received from CIFE, Mumbai). The two Bacillus strains were routinely grown on Tryptose soya agar (TSA) at 30 ± 2 °C for 24-48 h, maintained by fortnightly transfer on agar slants and stored at room temperature.

**Preparation of bacterial cell suspensions:** The Bacillus strains (one non spore former and the other spore former) maintained on TSA slants were streaked onto TSA plates and incubated at 30 ± 2 °C for 24-48 h to get young discrete colonies. Two or three young colonies were aseptically picked, transferred to 10 ml TSB and incubated at 30 ± 2 °C for 24 h. These 24 h old cultures were then transferred to 1000 ml TSB broth and re-incubated at 30 ± 2 °C for 48 h. The cells were harvested by centrifugation at 6000 rpm for 20 min at 6 °C in a cooling centrifuge (C - 23, Remi). The cell pellets were washed twice by centrifugation with sterile normal saline solution (NSS) and finally resuspended in 50 ml sterile NSS and used immediately. A portion of the cell suspension was suitably diluted up to 10⁻⁷ in sterile NSS and the number of cells/ml of suspension was determined by spread plating on TSA plates after incubation at 30 ± 2 °C for 48 h.

**Preparation of feed:** The test feed T₁, T₂, T₃, T₄, T₅ and T₆ were prepared by incorporating the two Bacillus strain suspensions at graded levels of 5 X 10⁷ cells g⁻¹ control feed, 5 X 10⁶ cells g⁻¹ control feed and 5 X 10⁵ cells g⁻¹ control feed (Table 1). The probiotic cell suspensions were added in the control feed.
diet after the dough was autoclaved and subsequently cooled, before pelletizing.

**Feeding:** The fishes of each group were fed with the respective diets for a period of thirty days. The fishes were fed twice daily at the rate of 5 % of body weight per day. The daily allowance was divided in equal proportion and fed at 08.00 hr and 17.00 hr.

**Growth evaluation:** The growth performance was evaluated by taking the weight and length of rohu fingerlings after a period of thirty days. The growth performance of the fishes was calculated based on the data collected during the experimental period using the following formula:

- **Weight gain (%)** = \( \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \)
- **Food Conversion Ratio (F.C.R.)** = \( \frac{\text{Dry weight of the feed given}}{\text{Wet weight gain}} \)
- **Survival (%)** = \( \frac{\text{No. of fish at the end of the experiment}}{\text{No. of fish at the start of the Experiment}} \times 100 \)
- **Specific growth rate (S.G.R.)** = \( \frac{\text{In Final average net weight} - \text{In Initial average net weight}}{\text{Days of culture}} \times 100 \)
- **Condition Factor (C.F.)** = \( \frac{\text{Weight}}{\text{(Length)}^3} \times 100 \)

**Biochemical analysis of fish tissue:** The proximate composition of fish tissue in all the treatment groups at the end of thirty days were analysed for moisture, crude protein, fat and total ash. Chemical composition was determined using the following procedure: Dry matter after drying at 100 oC for 24 hours, ash by combustion at 550 oC for 12 hours, crude protein (N X 6.25) by Kjeldahl method after acid digestion and fat by diethyl ether extraction (Snedecor and Cochran, 1967).

**Statistical analyses:** One way ANOVA with replication was followed to test the level of significance among the experimental diets and critical difference calculated to examine which of the treatments varied significantly (Snedecor and Cochran, 1967).

**RESULTS AND DISCUSSION**

**Growth parameters:** The percent body weight gain, S.G.R. and F.C.R. values, survival and percentage change in condition factor of the different experimental groups recorded on the termination of the experiment is given in Table 2. The highest weight gain and S.G.R. was recorded in fishes fed with spore forming Bacillus at a concentration of 5 X 10^6 cells g^-1 feed and the lowest in fishes fed with the non spore forming Bacillus at the minimum level. The values of F.C.R. were correspondingly high in non spore forming Bacillus supplemented feed and the lowest in spore forming Bacillus supplemented feed. The lowest value of survival was recorded in fishes fed with control diet and the highest value recorded in fishes fed with spore forming Bacillus at a graded level of 5 X 10^7 cells g^-1 feed. The weight gain (%), S.G.R. and F.C.R. values of experimental fishes, fed with spore forming Bacillus at concentrations of 5 X 10^7 cells g^-1 and 5 X 10^6 cells g^-1 feed, varied significantly (P<0.05) from the rest. However, the differences in the survival and percentage change in condition factor of groups, fed with different experimental diets, were statistically non-significant (P>0.05).

The experimental set up was same for all groups and fishes were fed on the same basal diet with variations only in the nature and concentration of the probiotic strain. The changes recorded in the growth, survival and F.C.R. of the fishes could be mainly attributed to the influence of the probiotic strains. The values of percent body weight gain, S.G.R., F.C.R. and survival were very encouraging in fishes fed with spore forming Bacillus as dietary supplement and differed significantly from fishes fed with control diet and non spore forming Bacillus as dietary supplement. The results of the study are in direct conformity to Ghosh et. al (2002) who fed rohu spawn with feed supplemented with an isolated fish intestinal probiotic, Bacillus circulans and observed direct effect on improvement in growth rate, weight gain and survival. Bairagi et. al (2004) fed rohu fingerlings with isonitrogenous (35% crude protein approximately) and isocaloric (18.37 kJ g^-1) experimental diets formulated/inoculated with fish intestinal bacteria B. subtilis and B. circulans exhibited excellent growth response, feed conversion ratio, protein efficiency ratio, apparent net protein utilization and enzyme activity. The addition of spore

<table>
<thead>
<tr>
<th>Feed</th>
<th>Probiotic Bacteria</th>
<th>Concentration of cells g^-1 of control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_1</td>
<td>Spore forming Bacillus</td>
<td>5 X 10^7</td>
</tr>
<tr>
<td>T_2</td>
<td>Spore forming Bacillus</td>
<td>5 X 10^6</td>
</tr>
<tr>
<td>T_3</td>
<td>Spore forming Bacillus</td>
<td>5 X 10^5</td>
</tr>
<tr>
<td>T_4</td>
<td>Non-spore forming Bacillus</td>
<td>5 X 10^7</td>
</tr>
<tr>
<td>T_5</td>
<td>Non-spore forming Bacillus</td>
<td>5 X 10^6</td>
</tr>
<tr>
<td>T_6</td>
<td>Non-spore forming Bacillus</td>
<td>5 X 10^5</td>
</tr>
</tbody>
</table>

**TABLE 1:** Supplementation levels of bacterial isolates in experimental diets
Biochemical composition: The change in the biochemical composition of the fish tissue in terms of moisture, protein, lipid and ash content during the experiment is given in Table 3. The moisture and ash content were generally lower at the end than at the beginning of the experiment. There was in general increase in the protein content of the fish with the maximum increase observed in fishes fed with spore forming Bacillus at a concentration of 5 X 10⁶ cells g⁻¹ feed. The lipid content was however found to be lower at the end of the experiment than at the beginning. The biochemical composition change of the fish tissue in terms of moisture, protein, lipid and ash content of fishes fed with spore forming Bacillus and non spore forming Bacillus as feed probiotic at varied concentrations, were however, statistically non-significant (P>0.05).

The use of probiotics as a dietary supplement in aquaculture of cold water food fishes like turbo, rainbow trout, salmon and cod (Storm and Ringo, 1993; Gatesoupe, 1994, 1997; Gildberg et. al, 1995; Austin et. al., 1995; Robertson et. al., 2000) have shown promising results, which emphasizes its necessity for use as a dietary supplement in feed of commercially important temperate water fishes like carp. Body composition varies from species to species and within the same species is influenced by age, sexual maturity and time of spawning, feeding conditions and chemical constituents of diet. There was an increase in protein content in all the experimental fishes. This could be attributed to the fact that probiotics are directly involved in nutrient uptake or provide nutrients or vitamins (Ring and Gatesoupe, 1998). The probiotic bacteria produce enzymes in the gut, which increases the digestive efficacy of the host animal thereby promoting protein digestion and growth (Lipton, 1998).

### Table 2: Weight gain, Survival, S.G.R., F.C.R. and change in C.F. of different experimental groups fed with probiotic feed at the end of the experimental period

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Weight gain (g)</th>
<th>Survival (%)</th>
<th>S.G.R. (%)</th>
<th>F.C.R. (%)</th>
<th>Change in C.F. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>30.306 ± 0.01</td>
<td>47.2 ± 3.96</td>
<td>0.88 ± 0.16</td>
<td>2.970 ± 0.2</td>
<td>-2.37 ± 13.14</td>
</tr>
<tr>
<td>T₁</td>
<td>44.019 ± 0.01</td>
<td>70.835 ± 5.8</td>
<td>1.215 ± 0.03</td>
<td>2.039 ± 0.01</td>
<td>-1.78 ± 1.48</td>
</tr>
<tr>
<td>T₂</td>
<td>59.002 ± 0.01</td>
<td>61.085 ± 7.9</td>
<td>1.545 ± 0.01</td>
<td>1.745 ± 0.1</td>
<td>-1.92 ± 26.76</td>
</tr>
<tr>
<td>T₃</td>
<td>41.180 ± 0.01</td>
<td>61.085 ± 7.9</td>
<td>1.149 ± 0.01</td>
<td>2.134 ± 0.2</td>
<td>1.02 ± 21.95</td>
</tr>
<tr>
<td>T₄</td>
<td>34.187 ± 0.01</td>
<td>66.67 ± 4.5</td>
<td>0.980 ± 0.03</td>
<td>2.732 ± 0.4</td>
<td>-0.01 ± 9.52</td>
</tr>
<tr>
<td>T₅</td>
<td>34.968 ± 0.01</td>
<td>66.67 ± 3.9</td>
<td>1.000 ± 0.01</td>
<td>2.650 ± 0.0</td>
<td>8.345 ± 5.41</td>
</tr>
<tr>
<td>T₆</td>
<td>29.426 ± 0.01</td>
<td>61.085 ± 7.9</td>
<td>0.858 ± 0.16</td>
<td>3.326 ± 0.3</td>
<td>2.28 ± 11.02</td>
</tr>
</tbody>
</table>

Values are mean ± SE of four groups per treatment. Values in the same column with different superscripts are significantly different (P<0.05).
carcass protein and dietary lipid which further substantiates the present study. Ghosh et al. (2001), working on rohu fingerlings, confirmed higher protein accretion and lipid depletion in the carcass when fed with feed supplemented with probiotic bacterial amylase. Ghosh et al. (2004) found increased protease activity and decreased lipid digestibility in rohu fingerlings fed with probiotic Bacillus circulans supplemented diet. Bairagi et al. (2004) reported higher protease activity in the intestines of rohu when fed with feed supplemented with an intestinal probiont, B. circulans. The increment in protease activity with an increase of dietary protein corroborates the findings of Gadadhar et al. (1997) in rohu.

Thus, dietary supplementation of rohu fingerlings with spore forming Bacillus had a marked positive influence on its weight gain, growth rate, survival and F.C.R. Among the concentration of probiont tested, 5 X 106 cells g-1 feed proved the most effective, inducing the best growth and F.C.R. The results clearly evince that supplementing the normal diet of carp fingerlings with spore forming Bacillus as probiotic, possess tremendous promise and potentiality.

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