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Effect of Probiotics on the growth and survival rate of Indian major carp *Labeo rohita*

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Abstract: In this study the effect of Probiotics preparation *(Lactobacillus sp and Bacillus sp)* was investigated against fish bacterial pathogen *Aeromonas hydrophila* and also evaluates their effectiveness on the growth and survival rate in fresh water fish *Labeo rohita. In vitro* antagonism test of the probiotics was performed by using well diffusion method. The inhibition zone of 9 mm was observed against *A. hydrophila.* For *in vivo* evaluation 500 *Labeo rohita* fish fingerlings with an average body weight of 8.00 g/fish were equally divided into 4 treatment tanks with 300L capacity, initially fishes in all tubs fed with probiotics combination for 42 days except control. After that they were challenged by *A. hydrophila* feed twice within 7 days. The results regarding the weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) noticed in probiotic treated tanks. The fish challenged with *A. hydrophila* in control tub showed 80% mortality rate, the probiotics treated tub showed below 12 % mortality rate respectively. So, it was concluded that the use of probiotics significantly enhanced the growth and survival of the fish *Labeo rohita*.

Keywords: *Probiotics, Labeo rohita, in vitro Antagonism, Fingerlings, Mortality rate*

1. INTRODUCTION

Aquaculture is one among the fastest growing food producing systems, which has been emerged recently as an industry and now it is possible to supply protein rich food throughout the world. Disease management is one of the major constraints in the successful development of aquaculture. It can be done by two methods such as prophylaxis and curing method. The prophylactic and therapeutic control of the bacterial diseases is based on the oral administration of antibiotics; however, such a treatment may cause the development of resistant bacteria (Aoki *et al.*, 1980). Use of expensive antibiotics for controlling disease has widely been criticized for their negative impact like residual accumulation, development of drug resistance and immune suppression, thus resulting in reduced consumer preference for fish food treated with antibiotics (Anderson, 1992).

In addition, antibiotics can affect the normal microflora of digestive tract which is beneficial to host and may be inhibited by treatment (Aly *et al*., 2008). In this respect, use of probiotic bacteria is a new approach gaining acceptance in aquaculture to control potential pathogens (Gomez-Gil *et al*., 2000; Kim and Austin 2008).

Probiotics are products designed to deliver potentially beneficial bacterial cells to the microbiotic ecosystem of humans and other animals. The use of commercial probiotics in fish is relatively ineffective unable to survive or remain viable at high cell density in the intestinal environment of fish during the active growth phase of the fish (Moriarty, 1996). Hence, there is elegant logic in isolating putative probiotics from the host in which the probioticis intended for use. Such strains should perform better because they have already adhered to the gut

wall of the fish and, thus, are well-adapted to compete with pathogens for nutrients.

Several bacteria have been used as probiotics in the aquaculture and they can be either delivered directly into the water, or via pelletized dry food (Gomez-Gil et al., 2000). Strains of Lactic Acid Bacteria and *Bacillus* sp are the most common microbes employed as probiotics. Lactic acid bacteria (LAB) are known microorganisms that have probiotic properties. They can produce inhibitory compounds such as lactic acid, hydrogen peroxide, diacetyl, acetaldehyde and bacteriocin. These compounds are able to inhibit the growth of harmful microorganisms (Gatesoupe, 1999; Ringo and Gatesoupe, 1998). *Bacillus* sp. has been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*., 2001; Barbosa *et al*., 2005). Several research articles demon-strate the benefits of using *Bacillus* to improve fish growth performance, survival, immunity and disease resistance in aquaculture (Farzanfar, 2006).

The present study aimed to investigate the effect of probiotics such as *Lactobacillus* sp and *Bacillus* sp on the growth and survival rate of Indian major carp *Labeo rohita* with the presence of the fish pathogen *Aeromonas hydrophila*.

2. MATERIALS AND METHODS

2.1 Isolation of probiotics: Curd is the best source for *Lactobacillus* sp. Under the aseptic conditions curd was serially diluted in 9 ml of sterile peptone piluents. Bacteria were enumerated from the diluted curd sample, 0.1ml of dilute was spread on sterilized MRS medium (HiMedia, India). The plates were incubated in incubator at 37°C. The *Lactobacillus* colony formed on the MRS agar plates was identified using morphological and biochemical characteriza-tion. *Bacillus* sp isolated from intestine of healthy *Labeo rohita*. They were brought to the laboratory alive and sacrificed. The abdomen surfaces were thoroughly scrubbed with an alcohol (70% ethanol) and aseptically dissected to remove the intestines, The Intestines were crushed by homogenizer and dissolved in 5 ml of 1.5% NaCl per fish and the diluted 1.5% NaCl were heat shocked on water bath at 80 °C for 20 min followed by cold shock with normal tap water immediately. Then the sample solution was spread on plates using spread plate technique on nutrient agar and incubated at 37°C for 24 hours. Isolates were identified based on their morphological and biochemical characteristics.

2.2 Isolation of fish pathogens: In order to isolate the potential pathogenic bacteria, recently dead fishes were collected from the kadayanallur fish market, Tirunelveli, Tamil Nadu in sterile polyethylene bags and brought to the laboratory for further analysis. Samples were collected from the infected portions like skin and from the internal organs such as eye, kidney, intestine and liver of the diseased *Labeo rohita* for further isolation and identification of pathogenic bacteria. One gram of each sample from muscle, liver, gills and intestine were taken from the diseased fish using sterile scalpel under aseptic condition. It was homogenized with sterile distilled water using homogenizer. Then the homogenized sample was serially diluted 1ml was taken from each dilution and spread plate technique was carried out for the enumeration of total heterotrophic bacterial count using sterile nutrient agar medium. The plates were incubated at 37^0C for 24 - 48 hrs. The biochemical Characterization were carried out to identify the fish pathogens following Bergey's Manual of Bacteriological classification.

2.3 Antagonistic activity of probiotic bacteria against *A. hydrophila* (Disc diffusion method): Selected probiotics strains were inoculated into 100 ml of Zobell marine broth individually. The probiotics strains (*Bacillus subtilis, Lactobacillus*) were centrifuged at 5000 rpm for 10-15 minutes and then the clear supernatant was obtained, then impregnated on to sterile 6mm disc an antibacterial activity was assayed following the disc diffusion assay on Muller Hinton agar medium inoculated with 0.1ml of *A. hydrophila*, (10^6 cfu/ml). Control disc filled with sterile Zobell marine broth and

standard antibiotic chloramphenicol disc 100µg were performed as negative control and positive control respectively. The inhibition zone was measured from the border of the disc to edge of the clear zone.

2.4 Preparation of Probiotic Bacteria Feed: The preparation of probiotic bacteria (*Lactobacillus sp* and *Bacillus sp*) was carried out with inoculating the cultures in TSB (Tryptic Soy broth) and incubated at 37° C for 48 h, then centrifuged at 5000 rpm for 30 min. After centrifugation, the bacteria were washed twice with sterile saline and the final suspension concentration was adjusted to approximately 10^6 CFU ml-1of saline. The saline, containing the probiotic bacteria (*Lactobacillus sp* and *Bacillus sp*) was added to the commercial feed to give as a diet. The diets were transferred to plastic bags and stored in a refrigerator.

2.5 Experimental Setup: Healthy *Labeo rohita* fingerlings were obtained from private fisheries farm in Manimuthar, Tirunelveli District, Tamilnadu, India and it was transported to the laboratory in well aerated polythene bags. They were allowed to acclimatize the laboratory condition for 2 weeks and then used for experimental studies. Experiments were carried 300 L tanks filled with fresh, clean and unchlorinated ground water, and each tank was cleaned daily by siphoning fish faces and remaining feed with 75% of total water volume, then refilled to fixed volume again.

The water temperature ranged between (28 ±2º C during experimental period). Totally 500 *Labeo rohita* fingerlings with an average body weight of 8.0 g/fish were equally divided into 4 treatments. The fingerlings were fed with 3% of their body weight on experimental diets twice a day for 42 days. Every third day, one third of water was partially changed in each tank. The mean temperature (28 ± 1.50c), dissolved oxygen (7.4 ±0.6mg/l) and total ammonia (0.5 ± 0.2mg/1) were recorded.

2.6 Growth analysis: To study the growth of fish fed with different diets, different growth parameters and survival rates were estimated. During the feeding trial, survival was recorded and fingerlings were weighed every fortnight interval to determine weight gain, specific growth rate (SGR) and feed conversion ratio (FCR). Weight gain, SGR and FCR were calculated by the following equations, Weight gain (g) = Final live weight – Initial live weight**,** Specific Growth Rate (%/day) = In Final weight – In initial Weight/ Time (days) X 100, Food Conversion Ratio (FCR) = Dry food Consumed (g)/weight gain (g)**,** Survival = No. Of fish introduced/ No of fish survived X 100.

2.7 Challenge Test with Pathogens: After the feeding trial (probiotic feeding), pathogenic diet (*A. hydrophila* + feed) was prepared by blending feed and the pathogenic *A. hydrophila* by the method which was followed for the preparation of probiotic basal diet as described earlier. The fishes in all the treatment groups were fed with pathogenic *A. hydrophila* (3% of total body weight – $10^{7 \pm 1}$cells g - 1) except the control group. All groups were kept under observation for 7 days. The moribund and freshly dead fish were recorded.

3. RESULTS

3.1 Isolation of probiotics: Total plate count of intestine of fish and curd sample were enumerated as 4.2 x 10^4 CFU ml-1 and 3.8 x 10^4 CFU ml-1 respectively. Twenty four different morphological appearances were isolated and streaked separately on TSA plates to check their purity. The isolated colonies were further purified by streak plate method using sterile nutrient agar medium. The initial analysis suggests that the isolates belong to the order Eubacteriales as per the Bergey's Manual of Determinative Bacteriology (Holt, 2000). Among the isolates AP2 and AP12 confirmed as *Lactobacillus* sp and *Bacillus* sp respectively based on the morphological and biochemical characterization (Table 1 and Table 2).

3.2 Isolation of *Aeromonas hydrophila:* The *Aeromonas hydrophilia* was isolated from the infected fish based on their clinical symptoms and biochemical Characterization. The results of the quantitative estimation of microbial count in muscle, gills, liver and intestine of diseased *Labeo rohita* were

recorded. The highest microbial load of 6.3±1.8 x 10^7 CFU ml-1 was observed in muscle tissue. A higher percentage of *A. hydrophila* (16%) was observed in the muscle of the diseased *Labeo rohita*. *Aeromonas hydrophilia* was confirmed based on the morphological and biochemical characteristics of the isolates following Bergey's Manual of classification (Holt *et al.*, 1998) and the results were recorded (Table 2).

Table 1: Morphological Characterization of selected probiotics strain AP2 and AP12

S. No	Morphological Characters	*Lactobacillus* sp	*Bacillus* sp
1	Morphology	rod	rod
2	Motility	-	+
3	Gram Staining	+	+
4	Colony morphology on Selective Medium	On MRS Agar White, smooth irregular colony	On nutrient agar, colonies are circular, smooth round, waxy, slight yellow to white, mucoid produces

Note: + Positive; - Negative

Table 2: Biochemical characterization of selected probiotic strains AP2 and AP12

Biochemical Characters	*Lactobacillus* sp	*Bacillus* sp
Indole	-	-
Methyl Red	-	-
Voges Proskauer Test	-	+
Citrate utilization	+	+
Nitrate reduction	-	+
Urease	+	-
Catalase	-	-
Oxidase	-	+
L-Arabinose	-	+
Fructose	+	-
Glucose	+	-
Lactose	+	-
D-Arabinose	-	-
Maltose	+	-
Rhamnose	+	-

Note: + Positive, - Negative

3.3 Antagonistic activity of probiotic bacteria against *A. hydrophila:* Further study concerning antagonism against *A. hydrophila*. The antagonistic effect of *Lactobacillus* sp. AP2 and *bacillus* sp. AP12 against the fresh water fish pathogen, *Aeromonas hydrophila* evaluated by disc diffusion method. The isolates AP2 and AP12 displayed strong antagonistic activity 9 mm zone of inhibition was observed against *A. hydrophila*.

3.4 Growth Performance: Growth performance was tabulated in Table 3 showing that the final weight, weight gain, specific growth rate and Feed conversion ratio of *Labeo rohita* increased significantly. When lots were fed with a diet containing mixed bacteria these values are significantly low in all other test fish group. In single probiotic feed experiments CF + AP2 and AP12 shows highest growth while comparing to other single probiotics treatment. Control group (CF) shows decreased values while comparing to other groups (Figure 1).

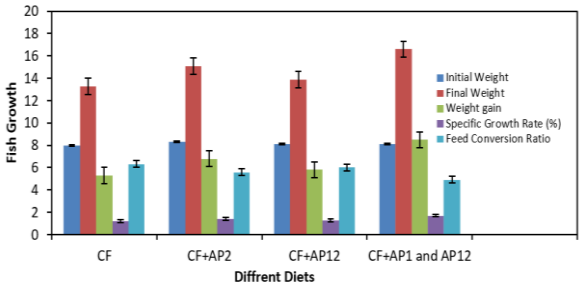

Fig. 1: Average Growth parameters of (*Labeo rohita)* for 42 days of tank culture, under the influence of *probiotics strains* (AP2 and AP12). (Values are average of 125 fishes).

3.5 Challenge Test: The mortality rate 12% was recorded *on treatment tank with combined diet.* The mortality rate of *Labeo*

rohita which fed on a diet containing other probiotic treatment lot was ranged between 10-20% including single probiotic treatment. The control group (CF) shows 80% mortality, revealed 10% of the fish were observed with diseased symptoms (Figure 1).

Table 3: Biochemical characterization of *A. hydrophila* isolated from infected fish.

Biochemical Characters *(Aeromonas hydrophila)*	Results
Gram Staining	-
Motility	+
Oxidase	+
Catalase	+
Starch Hydrolysis	+
Gelatin Hydrolysis	+
Huge & Leifson tests	+
NaCl(1%)	+
Indole	+
Methyl Red	+
Voges Proskauer Test	+
Arginine	+
Lysine	+
Ornithine	-
Urease	-
H_2S Production	+
TSI	Alkaline slant with Acid butt

Note: + Positive, - Negative

Table 4: Average Growth parameters of (*Labeo rohita)* for 42 days of tank culture, under the influence of *probiotics strains* (AP2 and AP12). (Values are average of 125 fishes).

Parameters	CF	CF + AP2	CF + AP12	CF + AP2 and AP12
Initial Weight	**8.0±0.1**	**8.3±0.1**	**8.1±0.3**	**8.1±0.4**
Final Weight	**13.3±0.1**	**15.1±0.1**	**13.9±0.3**	**16.6±0.2**
Weight gain	**5.3±0.05**	**6.8±0.2**	**5.8±0.05**	**8.5±0.6**
Specific Growth Rate (%)	**1.21±0.01**	**1.42±0.04**	**1.28±0.03**	**1.70±0.15**
Feed Conversion Ratio	**6.32±0.01**	**5.59±0.1**	**6.03±0.03**	**4.92±0.1**
Survival (%)	**80%**	**15%**	**19%**	**12%**

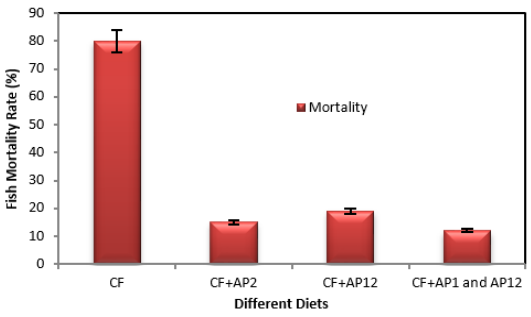

Fig. 2: Mortality rate of (*Labeo rohita)* for 42 days of tank culture, under the influence of *probiotics strains* (AP2 and AP12). (Values are average of 125 fishes).

4. DISCUSSION

Probiotics are the beneficial microorganisms and their products provide health benefits to the host animal. They are found useful in aquaculture as growth promoters, disease controlling agents and in some cases, replacing the use of antimicrobial compounds. In the present study, addition of *Lactobacillus sp* and *Bacillus sp* incorporated in the diets of *Labeo rohita* resulted in better growth performance and survival. Survival and growth of fishes mainly depend on the food quality quantity and immunity as well as body size *(*Pandian, 1967).

In the present study results were recorded when the experimental fish were fed on diets (CF, CF+AP2,CF+AP12, CF+AP2 and AP12). Among all the experimental diets, significant weight gain and survival were observed in diet CF+AP2 and AP12 (8.5 ± 0.6 g), followed by diet CF+AP2 (6.8±0.2g), and CF+AP12 (5.8± 0.05g). By referring Table 4 it clearly understood that treated probiotics CF+AP2 and AP12 (*Lactobacillus sp* and *Bacillus sp)* shown significant gains in weight. Correspondingly it also revealed that an increase in efficiency to control pathogenic infection. The present findings were similar to those of Abed *et al*, 2009 and Parthasarathy and Ravi (2011) shows the combination of the two probiotic bacteria (*Micrococcus luteus* & *Pseudomonas sp*) is said to be given adverse antagonistic effect against *A.hydrophila* in freshwater fish Nile tilapia, *Oreochromis niloticus* and *Catla catla* respectively. Moreover, *Enterococcus faecium-ZJ4* was found to enhance the growth-performance of Nile tilapias, *O. niloticus* (Wang et al, 2008), and *Silurtes glanis* (Bogut *et al*, 2000).

The application of photosynthetic bacteria and *Bacillus sp.* as a mixture of probiotics in Nile tilapia and observed better results in their growth performances. Similarly Wang, (2007) also reported significant growth rate (1.65%) and daily weight gain (0.0384g) in *Penaeusvannamei* fed with high concentrations of photosynthetic *Bacillus* incorporated diet.

5. CONCLUSION

In conclusion, the results of this study showed that probiotics *(Lactobacillus sp and Bacillus sp)* feeding can modulate the intestinal microflora and significantly enhance survival and growth performances of *Labeo rohita*. The subsequent challenge experiment using *A. hydrophila* demonstrated significant effect of probiotics *(Lactobacillus sp and Bacillus sp)* by increasing survival of *Labeo rohita*. The results also indicate that the effects of probiotic plays a major role in disease control strategies, growth promotion and enhancement of immune system of fish, and hence it could be recommended to fish farmers in fish farming.

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