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Effect of probiotic supplemented feed on growth performance of molly fish (*Poecilia sphenops*) in Recirculating aquaculture system

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Abstract
Due to the alarming occurrence of antibiotic resistance pathogens there is a constant need for an effective alternate way for protection against disease and increased fish production. In this study growth of Molly fish with dietary supplementation of probiotic in an innovative recircularting aquaculture system was studied. In 30 days feeding experiment, the initial and final length of fishes fed with *Lactobacillus delbrueckii* LABT1 supplemented diet were 0.8±0.03 and 4.2±0.04 cm, showing a length gain of 3.4±0.01cm whereas fishes grown without probiotic diet had initial and final length of 0.8±0.02 and 2.85±0.04 with length gain of 2.05±0.02. The initial and final weights of fishes fed with probiotic were 0.9±0.02 and 3.02±0.02 g with a weight gain of 2.12±0.0 g. The specific growth rate of control fishes and *L. delbrueckii* LABT1 fed fishes were 5.5±0.64 and 7.9±0.52% with their survival of 100%. Also in the test experiment the fenugreek was grown using fish effluent showed significantly higher plant length. Hence it was concluded that supplementation of probiotic can be used for improving growth performance of fish in recircularting aquaculture system.

Keywords: molly, fish effluent, Fenugreek, probiotic diet, performance

Introduction
Ornamental fish is one of the important items among the various types of commercially important fishes marketed nationally and internationally. The production and trade of ornamental fish is a profitable alternative in the aquaculture sector. Despite the economical importance of this sector, the nutritional information for ornamental fish is scarce and often few or even no data of the nutritional requirements is available (Pour *et al.*, 2014) [1]. *Poecilia sphenops* commonly known as "Black Molly" is an omnivorous ornamental fish. It is highly adaptable and extensively thrives in many different environmental and ecological conditions as a model organism for the studies of ecology, evolution and behavioral due to their viviparous in nature and easy availability (Patel *et al.*, 2017) [2].

Numerous advances in animal productivity have been made through the use of nutritional supplements and the incorporation of antimicrobial compounds into livestock feed. Although undeniably effective in increasing animal productivity and reducing food costs, the future application of antibiotics to animal feed may be reduced because of academic and public scrutiny (Flint *et al.*, 2009) [3]. Knowledge of probiotics has increased; currently it is known that these microorganisms have an antimicrobial effect through modifying the intestinal microbiota, secreting antibacterial substances (bacteriocins and organic acids), competing with pathogens to prevent their adhesion to the intestine, competing for nutrients necessary for pathogen survival, and producing an antitoxin effect. The lactic acid-producing bacteria have been the focus of much interest (Cruz *et al.*, 2012) [4].

In conventional aquaculture systems, intermittent disposal of the considerable amounts of nutrient rich water leads to high water consumption as well as surface and groundwater pollution. Recircularting aquaculture system (RAS) can be considered a sustainable innovative agricultural production system, where the fish tank water is recycled for plant cultivation and regular exchange of water is not necessary (Gravel *et al.*, 2015) [5].

Most prospective probionts isolated from fish raise potential safety issues and effectiveness as a probiotic, pose a potential health risk to humans and other animals and the cost of undertaking the regulatory approval process for these indigenous species may prove to be a major obstacle for commercialization (Welker and Lim, 2011) [6].

In order to meet the increasing demand for medicinal plants in indigenous systems of medicine as well as in pharmaceutical industries many medicinal plants are need to be cultivated commercially, but soil salinity and other forms of pollutants represent serious threats to plant production. In this context fenugreek is used as anti-diabetic, anti-fertility, antimicrobial, anti-parasitic, hypo-cholesterolaemic, antileptic, anti-bronchitis, carminative, aphrodisiac, analgesic, antipyretic anticancer antioxidant (Nisha and Rao, 2018)) [7]. Hence this study aimed to isolate probiotic bacteria from milk and analyzing its effect on improving the growth performance of molly fish in Recirculartingaquaculture system and utilized the fish effluent for Fenugreek cultivation.

2. Materials and Methods

2.1 Isolation and identification of Probiotic strain

A lactic acid bacterium isolated from milk collected from local shop in Sivakasi, Virudhunagar (Dist.), Tamilnadu, India was identified the basis of microscopic and biochemical analysis given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) [8]. This bacterium was grown in MRS liquid medium (Hi media, Mumbai, India) at 37 °C for 24-48 h to achieve viable count of about 10^9 cells per ml.

2.2 Antagonistic activity of probiotic strain

Agar well diffusion method was used to detect antagonistic activities of probiotic isolates. The pathogenic strains obtained from laboratory bacterial stocks preserved in Dept. of Microbiology, SFR College for Women, Sivakasi, Virudhunagar, India were spread plated on surface of mueller hinton agar plate (Hi media, Mumbai, India). Well of 4 mm diameter were cut into these agar plates and 50 µl of cell free supernatant of lactic acid bacteria was poured into one well. MRS broth was added in another well as negative control. The plates were incubated aerobically at 37 °C for 24 h. After incubation the plates were examined for zones of inhibition.

2.3 Preparation of feed

Soybean meal (80 g) was taken in powder form as main ingredients and other ingredients like milk powder (60 g), Wheat bran (20 g), and rice bran (20 g) were added and mixed well. Agar powder (4 g) was added as binding agent with water and the homogenate mixture was boiled, cooled at room temperature. Probiotic supplement (0.8 g), vitamin mixture of vitamin B complex (1 g) and vitamin E (1 g) were added and kept under refrigeration for 12 h. The mixture was squeezed over polythene sheet and dried at room temperature for 48 hours. The collected dietary ingredients were grounded and sieved. Using hand operated pelletizer about 2 mm size feeds were prepared, sundried and stored in air tight container. The control fish feed was prepared in similar composition replacing probiotic supplement with turmeric (0.5 g).

2.4 Experimental design

The experimental fish molly (*P. sphenops)* was obtained from local fish market located at Rajapalayam, Virudhunagar District, Tamil Nadu, India. The experiment was conducted for a period of 30 days designed into two treatment groups (Test and control) with one set of fish rearing tanks received probiotic feed (T1-Test) and other tanks received pellets without probiotic feed (C-Control). Both groups of tanks were connected with vegetable tray (13cm×6cm×7cm) placed above the tank and filled with small piece of coco coir. Each tank consist of planting set up for fenugreek (*Trigonella foenum-griaecum*) having two replications. Separate recirculation system was established for each group of fish rearing tanks. Temperature in all fish tanks were maintained at range of (29- 32 ºC) and Photoperiod was maintained at 12-12 hours light/dark for all experiments.

2.5 Fish Feeding Experiment

Fishes were divided into two equal groups, in which each 4 L culture tank had 4 fishes and all the tanks were provided with aeration. The untreated diet served as control and the diet mixed with probiotic strains were considered as treatments. The fishes were fed twice daily at the rate of 3% (average body weight) up to 30 days. The fishes in each treatment were counted and weighed on termination of the experiment.

2.6 Plant Experiment

The fenugreek (*Trigonella foenum-graecum*) seeds were first cleaned, freed from broken seeds, dust and other foreign materials and then soaked in tap water for 12 hours at 37 ºC in ratio of 1:5 (w/v). The soaked seeds were rinsed twice in distilled water. Seeds of fenugreek can be planted in the coco coir, a twelve-inch planter, or even an aluminum tray filled with dirt. The tray was placed with full sun, but partial shade. After 30 days fenugreek plant were collected and assessed for growth and total Chlorophyll, phenolics, alkaloids, flavanoid and tannin content (Yaser *et al*., 2013) [9].

3. Results and Discussion

The growth performance of the fish which were fed with probiotics, are compared to control group in terms of length gain, weight gain, specific growth rate and survival percentage. The fish in tank was provided with recycled fish effluent through plant growing container.

3.1 Isolation and Biochemical Characterization of Probiotic strain

This work was initiated with isolation and biochemical characterization the lactic acid bacteria from milk. Among the 20 isolates the selected LABT1showed positive rod for Gram's staining and negative for spore staining thus confirming that it was a non-spore former gram positive rod. The isolate form white, creamy, smooth and mucoid colonies and was found to be D-glucose, lactose and mannose fermenting with production of acid. The LABT1 strain was catalase negative, Indole, Citrate and Voges Proskauer (VP) negative, methyl red positive, H_2S non-producing and urea negative (Table 1.). Hence the selected isolate LABT1 was identified as *Lactobacillus delbrueckii*. Similarity, Sameul *et al*., (2016) [10] performed isolation and identification of *Lactobacillus* spp. in various food products. Vinothini and Radhathirumalaiarasu (2016) [11] isolated and characterized probiotic bacteria from curd sample as *Enterobacter avium* VEA4 and evaluated its effect on improving chick growth. Lactobacilli ferment lactose to lactic acid, thereby reduce the pH or produce inhibitory substance that harms pathogen.

Table 1: Biochemical Characterization of LABT1 isolate

Biochemical test	Isolate LAB1	Carbohydrate fermentation test	Isolate LAB1
Indole test	-ve	Glucose	A^+G^-
Methyl red	+ve	Fructose	A^+G^-
Voges Proskauer	-ve	Lactose	A^+G^-
Citrate	-ve	Mannose	A^+G^-
Catalase	-ve	Maltose	A^-G^-
Urease test	-ve	Xylose	A^-G^-
Nitrate reduction	-ve	Sucrose	A^-G^-
Gelatin hydrolysis	-ve	Mannitol	A^-G^-
Starch hydrolysis	+ve	Rhamnose	A^+G^-
Oxidase	-ve	Arabinose	A^-G^-

A^+ = Acid Positive, A^-= Acid Negative,
G^+ = Gas Positive, G^-= Gas Negative

3.2 In-vitro Antagonistic activity

The antagonistic activity of the identified probiotic bacteria *L. delbrueckii* LABT1 against the bacterial pathogen *Pseudomonas* sp., *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. were analyzed (Table 2.). The results showed that *Lactobacillus* sp. LABT1 exhibited strong inhibition on the growth of *Vibrio* sp. (19±0.04 mm). Similarly, selected Lactic Acid Bacilli from gut of *R. kanagurta* shows effective antibacterial effect against pathogen *Vibrio* sp., *Campylobacter* sp. and *Salmonella* sp. with zone of inhibition of 11-13 mm (Ghosh *et al*. 2014) [12]. Probiotic antagonism of *Sphingomonas* sp. against *Vibrio anguillarum* exposed *Labeo rohita* fingerlings was observed with maximum growth inhibition zone of 15 mm (Chaudhary and Qazi, 2014) [13]. Chen *et al.* (2016) [14] found improvement of disease resistance against *Vibrio* sp. with probiotic *Bacillus* sp. The need for sustainable aquaculture has promoted research into the use of probiotics on aquatic organisms. Hence the probiotic isolate can be evaluated for improving growth of fish in an innovative recirculating system.

Table 2: *In vitro* antagonistic activity of culture filtrate of *L. delbrueckii* LABT1

Test Pathogens	Inhibition zone (mm)
Pseudomonas sp.	9±0.02
Salmonella sp.	13±0.04
Vibrio sp.	19±0.04
Shigella sp.	11±0.03

3.3 Feed analysis

The protein content of fish feed of test (with probiotic supplement) prepared with agro waste was found as 37 % and control 35% for control (without probiotic supplement) respectively. The feed ingredients were identified moisture content of 78% and 72% was with the percentage of carbohydrate quantified as 52.2% and 53.6% for control and test (Fig. 1.). For commercial culture of fish, utilization of locally available agro-industry byproducts is prerequisite for the formulation of low-cost balanced diet. Recently fish meal has become the most expensive protein ingredient in aquaculture feeds. In this work aqua feed was prepared using agro by product and probiotic supplement includes Soybean meal, milk powder, Wheat bran and rice bran. Growth performance of Nile Tilapia fed with diets which contain different levels of *Spirulina platensis* were improved for the feeding experiment of 75 days (Sahan *et al*., 2015) [15]. Similarly, Ali *et al.*, (2016) [16] observed that fish *Pterophyllum scalare* fed with mixed protein diet showed higher specific growth rate (0.43%) better growth performance in terms of length and weight (19.3 ± 0.72 mm and 0.13 ± 0.01 mg) respectively and better survival rate (92%).

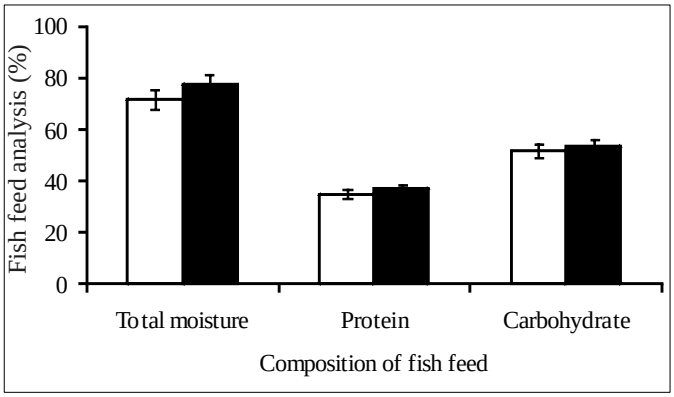


Fig 1: Proximate composition of fish feed with probiotic *Lactobacillus* sp. LABT1 (■) and without probiotic (□).

3.4 Fish growth studies

Results related to length and weight gain, specific growth rate (SGR) and survival of feeding trials of *P. sphenops* with experimental diets are presented in Table 3. The initial and final length of fishes fed with Probiotic diet were 0.8±0.03 and 4.2±0.04 cm, showing a length gain of 3.4±0.01cm whereas fishes grown without probiotic diet had initial and final length of 0.8±0.02 and 2.85±0.04 with length gain of 2.05±0.02. Similarly, the initial and final weights of fishes fed with *L. delbrueckii* LABT1 were 0.9±0.02 and 3.02±0.02 g with a weight gain of 2.12±0.0 g was found to be high compared with fishes reared without probiotic diet with initial and final weights of 0.9±0.02 and 2.07±0.03 with weight gain of 1.17±0.01. The specific growth rate of control fishes and *L. delbrueckii* LABT1 fed fishes were 5.5±0.64 and 7.9±0.52% with their survival of 100%. Abareethan *et al*. (2013) [17] confirmed that the bacterial strain mixed in water enhanced the water qualities and also significantly increased the length and weight of freshwater fish, *Labeo rohita.* However, Pour *et al.* (2014) [1] observed enhancement of growth performance and body composition in molly fish (*P. sphenops*) associated with dietary intake of garlic (*Allium sativum*). On the other hand the growth and survival rate of angel fish *Pterophyllum scalare* varied as when fed with animal based protein diet showed better growth performance in terms of length and weight (19.3 ± 0.72 mm and 0.13 ± 0.01 mg) respectively and better survival rate (92%) than those fed with other protein diets (Ali *et al*., 2016) [16]. Kumar *et al*. (2019) [18] found improved growth performance and carcass characteristic of chicken with probiotic feed.

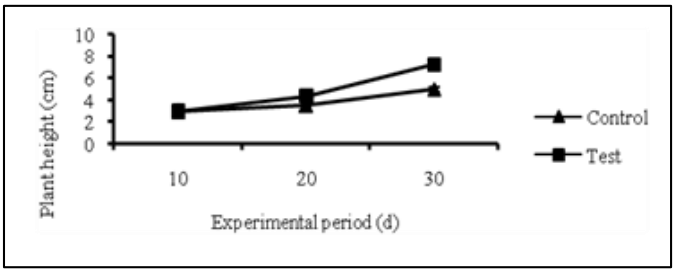
Table 3: Growth performance of *P. sphenops*

Growth performance	Control[a]	Test[b]
Initial length of fishes (cm)	0.8±0.02	0.8±0.03
Final length of fishes (cm)	2.85±0.04	4.2±0.04
Initial weight of fishes (g)	0.9±0.02	0.9±0.02
Final weight of fishes (g)	2.07±0.03	3.02±0.02
Gain in length (cm)	2.05±0.02	3.4±0.01
Gain in weight (g)	1.17±0.01	2.12±0.0
Specific growth rate (%)	5.5±0.64	7.9±0.52
Survival rate (%)	100	100

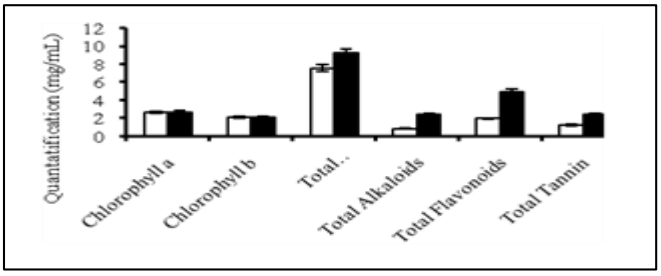
All the Experiments were average of duplicate
[a]Fish fed without *L. delbrueckii* LABT1 supplement
[b]Fish diet with *L. delbrueckii* LABT1 supplement

3.5 Plant study

In the test experiment the fenugreek plant length was significantly higher compared with the control. Among the extract obtain from fenugreek plant leaves of control and test experiment the total Chlorophyll, phenolics, alkaloids, flavanoid and tannin content were found to be highest for the test trial (Fig 2). In the current study Fenugreek could grow without the addition of extra nutrients, and it only comes from molly fish farming waste. Fenugreek growth was well marked with color of fresh green leaves and no signs of nutritional deficiency. Similarly residual water from the intensive tilapia farming provided higher concentrations of macro and micronutrients in the shoots, higher production of fresh matter of shoots (95.48 g plant^{-1}) and a larger number of leaves (14.90 %) for Lettuce cultivation (Geisenhoff *et al.*, 2016) [19]. A quantity of about 1 kg basil leaves were produced in a period of 60 days of growth using aquaponic system with cultured carp Cyprinus carpio (Filep *et al.*, 2016) [20]. Above finding is good concurrence with earlier works where examine the growth of romainelettuce (*Lactuca sativa* L. var. *Longifolia*) in aquaponic system without the addition of artificial nutrient. The nutrient relies solely on wastewater of nile tilapia (*Oreochromis niloticus*) cultivation circulated continuously on the aquaponic system. The results showed that tilapia weight reached 48.49 ± 3.92 g of T3 (tilapia, romaine lettuce, and inoculated bacteria), followed by T2 (tilapia and romaine lettuce) and T1 (tilapia) of 47.80 ± 1.97 and 45.89 ± 1.10 g after 35 days of experiment (Effendi *et al*., 2017) [21].



(a)



(b)

Fig 2: Growth of fenugreek with effluent of Fish cultivation tank in provided with probiotic *Lactobacillus* sp. LABT1 supplemented fish feed (■) and control without probiotic supplemented feed (□). Analysis of plant height (a) and quantification of total Chlorophyll, phenolics, alkaloids, Flavanoid and Tannin content of plant (b).

4. Conclusion

This lactic acid bacteria can be employed for formulation of probiotic fish feed. Further the improvement of fish growth in recycled fish effluent facilitates the easy cultivation of medicinally important plant. From the result it may be concluded that supplementing fishes with probiotic diet works well in Recirculating aquaculture system as molly fish showed increased length and weight gain and measurement of specific growth rate proportional to addition of probiotic *L. delbrueckii* LABT1 in fish feed. Also Fenugreek growth was well recorded in probiotic supplemented fish feed T1 treatment. The plant could utilize nutrient originating from fish culture, it act as filter and provide oxygenated water to be reused in the fish farming. Thus probiotic in RAS worked together in raising economically imperative fishes and pharmaceutically essential plant.

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