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Effect of Probiotics diet on growth and biochemical performance of freshwater fish *Labeo rohita* fingerlings

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Abstract
The nutritional evaluation, survival rate and growth of fresh fish water *Labeo rohita* fingerlings fed with artificial feeds along with supplements like Calcium, Starch and Sardine oil was done using probiotics. The experimental three group's basal diets with probiotics diets include *Bacillus subtilis* + *Lactobacillus rhamnosus* diet-E3, basal with Antibiotics diet-E2 and only basal diet in the control -E1. Feeding trail experiments were conducted for 60 days and growth parameters were monitored through Weight gain, Specific growth rates, Percent survival, Biochemical analysis, Microbial analysis and Antibacterial activity of probiotics were significantly (P<0.05) higher in probiotics diets (Calcium)- E3 incorporated diet fed fish followed by other experimental groups when compared with control deits-E1. The FCR values also recorded to be better with diet-E2 compared to Control diet- E1, demonstrates the efficient utilization of feed by fishes. The present study conclude that probiotics with starch was the best feed concentration food for the freshwater fish *L. rohita*.

Keywords: Probiotics, Antibiotics, Calcium, Starch and Sardine oil, *Labeo rohita*

Introduction
Aquaculture is one of the fastest growing food production activities in the world rapidly during the last decade. Aquaculture culture has been recognized as a growth area of economic importance in countries and has attracted the attention of both public and private sectors [1]. The world's most important aquaculture species Carps group of in terms of production. The freshwater fish Rohu (*L. rohita*) is one of the most popular species especially in Asia and fetches high price. Its total global production is over 1.5 million tons in 2012 [2]. The increased intensity of aquaculture has led to a high number of disease outbreaks with an increasing range of pathogens as a result in serious economic losses [3]. With the increase in the intensification and commercialization of aquaculture production come many challenges, such as pathogens diseases especially bacterial infections remain primary constraints to its continued expansion [4, 5, 6]. In addition to feed quality, bacterial infection causes mass mortality [7, 8, 9]. Feed quality and feeding methods therefore need to be thoroughly considered in order to improve growth performance and feed efficiency of the cultured animals. Several previous reports have suggested that probiotic supplementation can reduce disease outbreaks by enhancing the immune system of fish and shrimp and can decrease culture costs by improving the growth and feed efficiency of fish [10, 11, 12, 13]. The problems outlined above and recent restrictions on the use of antibiotics have resulted in natural immunostimulants, probiotics and prebiotics being considered as an alternative strategy to disease management and Controlling and preventing [14, 15, 16, 17].

Due to the abuse of antibiotics in animal growth promoters, antibiotic resistance has become a common characteristic in microorganisms [18, 19] thus caused serious problems in microbial infectious treatments [20]. The use of "probiotics", which are beneficial microorganisms or their products with the benefit effects to the hosts, have been used in aquaculture in order to control disease, as supplements for improving growth and in some cases as a mean of replacing antimicrobial compounds [21]. It was probably Vergio [22] who first introduced the term "probiotic", when he compared in his manuscript "Anti-undprobiotika" the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population with factors ("Probiotika") favorable to the gut micro flora. Lilly and Stillwell [23] referred to probiotics as

"Microorganisms promoting the growth of other microorganisms". Probiotics as feed supplements benefit the host by improving the feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, growth promoting factors and increasing immune response [24, 25]. Therefore, the aim of this study was to evaluate the effects of dietary supplementation of commercially available multispecies (*B. subtilis + L. rhamnosus*) (probiotics) singly or combined on growth, immune parameters and protection against *Aeromonas hydrophila.*

2. Materials and Methods

The fresh water fish *L .rohita* fingerlings were collected from Bhavani sagar Government Fish Development Corporation, Erode district, Tamil Nadu. They were transported safely and brought to the laboratory in well-oxygenated plastic bags. They were stocked in large cement tank (6' × 4' × 3') and acclimatized to the laboratory condition for 2 weeks before the commencement of experiments.

2.1 Experimental set up.

An indoor experiment was conducted in 6 plastic tubs. The tubs had an effective bottom area of 379.94cm^2 and 60-litre volume. All tubs were given proper aeration and water was changed daily. About 300 fingerlings of Rohu (L. *rohita*) having an initial body weight of 0.38 $\pm$ 0.0152 and length of 3.00$\pm$ 0.1.

2.2 Experimental Diets

Three feeds were selected as experimental diets namely, Rice Bran, Groundnut cake, and Starter feed. All the three were taken in the ratio 2:1:1. They were grinded using a mortar and pestle and fed to fishes in all the 6 tubs. The proximate composition of all the three feeds is shown in the table below. 300 fishes collected were segregated into 6 tubes with 50 in each tub. Out of the 6 tubs, 3 served as experimental and 3 as control for Starch, Calcium and Sardine oil respectively. All the 6 tubes were fed twice daily with artificial feeds along with the below mentioned things. Survival rate of fishes were rated daily by counting the live fishes in each tub. Final length and weight were taken after a period of 60 days.

2.3 Probiotic Bacteria for experimental tubs

Probiotic Bacterial consortium containing mainly *B. subtilis + L. rhamnosus* manufactured by J.B. Chemicals and Pharmaceuticals Ltd, Neelam center, 'B' Wing, Hind Cycle Road, Worli, Mumbai was selected for the present study was preparation of probiotic supplemented diet, the bacteria were added to the basal diet (control) at three different inclusion levels i.e 1x10^{7}CFU/g diets mix Starch, Calcium and Sardine oil respectively

2.4 Antibiotics for control tubs

Antibiotic "Lupimox 250" capsules containing mainly Amoxycillin manufactured by Lupina Ltd, 159, C.S.T Road, Kaiina Santacruz (E), Mumbai was used. 0.3g each of antibiotic was introduced into 3 tubs, which served as control along with the feed.

2.5 Addition of Starch

Starch (C_2 H_5 NO) manufactured by Hi-pure fine chem. Industries, Chennai was used. 0.5 gm each of starch was weighed and was given to one experimental and control tub along with the feeds.

2.6 Addition of Oil

Sardine oil manufactured by coastal aquatic protein, post Box No 122, Ist floor, coastal building, Karnataka was used. 0.5 ml each was given to one control and experiment tub along with the feed.

2.7 Addition of Calcium

Calcium tablets, named "Calday" manufactured by Embiotic laboratories (p) ltd, 20C, Kumbalgodu industrial area, 1st phase, Kumbalgodu, Bangalore were used. The calcium tablets were well grinded using mortar and pestle and 0.5gm each of this powder were given to one experimental and control along with feed.

2.8 Biochemical Analysis

The initial and final biochemical analysis such as total content of protein, amino acid, lipid, carbohydrate content were estimated in fish, *L. rohita.* Estimation of Protein [26] Estimation of Amino acid [27], Estimation of Carbohydrate, Estimation of Lipid [28], Estimation of Ash and Moisture content in Fish [29].

2.9 Growth parameters

The growth (net weight gain) and survival percentage were studied following standard methods. The following variables were calculated: [30]

Survival rate = Ntx 100/N0

Specific growth rate (SGR) = (Ln Wt- Ln W0) x 100/t

Increase in biomass = Final weight – Initial weight

$$\text{Condition factor} = \frac{\text{Fish weight in grams}}{\text{Fish length in cm}} \times 100$$

$$\text{Food conversion Ratio (FCR)} = \frac{\text{Total feed fed}}{\text{Total weight gain}}$$

Where: Wt and W0 were final and initial weight of rohu, respectively; Nt and N0 were final and initial number of rohu, respectively; t is duration of experimental days.

2.10 Microbial Analysis [31]

Microbial Preparation of Nutrient Agar, 1.5g of beef extract and 2.5g-peptone powder were weighed and dissolved in 500ml of water in a clean beaker by proper agitation. Adjusted the pH of the medium to 7.2 with 10 N NaOH using a pH meter.10g agar agar was weighed and added to the above solution and sterile in an autoclave.

2.11 Bacteria analysis from Gut

Procedure

Sterilized all equipments, melted the agar in the tubes, cooled it and keep it at 45 ^{0}C. poured about 20ml of the agar medium into each of the petridish. Added the required gut sample from the rearing medium into the sterile water in test tubes, shaken the test tubes and allowed to settle. Using a sterilize 1ml pipette added 1ml sample each into the plates and spreaded the plate by tilting it back and forth or using a sterile 'L' rod, Left a plate as control. Inverted all the plates and incubated them at 30 ^{0}C is an incubator for 2 days. It observed different types of colonies.

2.12 Qualitative Analysis

The qualitative analysis of bacteria was done in Sudharma Meteropolis Medical laboratory, Shornur Road, Thrissur.

2.13 Antibacterial activity of Probiotics

Antimicrobial activity was measured using the standard method of diffusion disc plates on agar. 0.1 ml of each culture of bacteria was spread on agar plate surfaces. For antibacterial assays, all bacterial strains were grown in Muller Hinton Broth medium for 24 hrs at 37 °C. The concentration of bacterial suspensions were adjusted to 10^{-8} colony forming units (10^{-8} cfu) in Muller Hinton Agar. Paper discs (6 mm in diameter) were impregnated on the agar to load 10µl of each sample.

2.14 Statistical Analysis

The statistical analysis was done individually for each sample and the mean value of three individual observations was taken for each parameters. The standard deviation for the sample mean was calculated and is given in appropriate tables. Two-way ANOVA [3] analyzed all measured variables. All the statistical analysis was performed using the SPSS 7.5 programme for windows.

3. Results and Discussions

The growth parameters, such as survival, weight gain, specific growth rate, feed conversion efficiency and protein efficiency rate were significantly (P<0.05) higher in probiotics (Calcium) E3 incorporated diet fed fish followed by other experimental groups when compared with control. Biochemical analysis of fish fed with E-3 experimental diets, i.e., Calcium, Starch, and Sardine oil along with artificial feeds was done. Out of this, protein content was found to be high in all fishes followed by amino acid, carbohydrate and lipid. Probiotics provide beneficial effects and have become an important management tool in aquaculture Balcazar, *et al.*, [33]. In this study, the effects of probiotics, the useful microorganisms on the growth, survival rate and nutritional status of fish *L. rohita* was analyzed. The biochemical composition, mineral content and the gut microflora of *L. rohita* at the initial and final stages was also analyzed [34]. Dietary probiotic supplementation in aquaculture has been reported to improve intestinal balance, growth performance and disease resistance and enhance immune responses [17, 35, 36].

3.1 Biochemical analysis of experimental diets and tissues

Chemical compositions of the experimental tissues were reported in (Table 1). Variation in the values of crude protein content of fed with probiotics diet Experi-E3 significantly higher was (29.20±2.89 to 44.2±1.57%), crude lipid content (10.20±1.67 to 18.30±0.85%) crude carbohydrate content (26.05 ±3.92 to 43.23 ±1.92%), Ash content (2.28±0.17 to 3.92±0.25%) and Moisture content (78.48±1.50 to 80.38±1.25) recorded respectively. Tissue crude lipid content was recorded within the range of (3.27–3.6%). Probiotics as feed supplements benefit the host by improving the feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, growth promoting factors and increasing immune response [24, 25, 37].

3.2 Growth parameters

The growth parameters recorded in this experiment were presented in (Table 2). The experimental (Probiotics) starch performed the best in all the growth-related parameters. The fish fed with diet Probiotics E3 (Starch) showed a high significant (P< 0.05) mean length (3.00±0.1 to 7.58±1.02) and mean weight (0.70±0.15 to 2.13±0.76) and the highest survival rates were recorded as 99.97 % which also corresponds to the highest specific growth rate of fish Probiotics with starch.

3.3 Microbial analysis

Shows the results of biochemical tests conducted for identification of the isolated bacteria from fish organs. Four strains of Gram-positive bacteria and four isolates of Gram–negative bacteria were isolated from the fish skin and internal organs (gonads, stomach, and intestine) Table 4. Show the gut analysis of fresh water fish *L. rohita*. Qualitative bacterial study was done in Sudharma, Metropolis laboratory. The results obtained in the present study about the gut micro biota of *L. rohita* and the works done by the above scientists show similarities, even though the present study does not aim at finding out the dominant species [38, 39].

3.4 Antibacterial activity of probiotics

Antibacterial activities of probiotics obtained from *S. serrata* against 5 human pathogenic bacteria are presented in (Table-5). Effect of Probiotics on pathogenic bacteria revealed that, the highest activity 12.6 ± 0.42mm Probiotics observed against *E. coli* of control compared with Antibiotic as a positive control 6.00 ± 1.00mm. The lowest activity was observed against *P. aeruginosa* inhibition zones in 8.05± 0.42mm Probiotics inhibition zone when compared with commercial antibiotics all the statistical values were significant at (P<0.05). Probiotics have also been found to enhance the disease resistance, and thus, could be alternatives to antibiotics and other drugs [18, 19]. In the present study, *L. rohita* fishes were fed with probiotics along with artificial feeds supplemented with feeds like calcium, starch and sardine oil. Csengeri and Petitijean [40] reported a survival rate of 80% in cyprinid larvae using a liver based diet whereas more than 80% survival rates were achieved by Koushik Ghosh *et al*., [41] in their study where they used fish meal, mustard oil cake, rice bran, cod liver oil and vitamin premix to feed *L. rohita*. Koushik Ghosh *et al*., [41] also reported that the differences in growth and survival in growth and survival of rohu spawn between treatments could be attributed to the quality of diets. Supplementation of intestinal microflora increased the nutritional efficiency of the formulated diet and may be due to the probiotic effect of bacteria used [42, 43, 44]. In their studies found out that in Rainbow trout (*Onchorhynchus mykiss*) survival rate increased in probiotics supplement. Higher mean weight and total length were recorded in fish fed with probiotic supplement. In this study also gut analysis of *L. rohita* shows the presence of probiotic bacteria like *Lactobacillus, Enterococcus, Clostridium* and *Bacillus* spp. (Table 4). Like the above findings, in the present study too fishes fed with probiotics shows high survival rate, high mean weight and length. The highest attainment in fish body weight, length and SGR was recorded in groups of fishes fed with 40% carbohydrate diet. Moisture and ash content decreased while protein and fat levels increased. The protein deposition in fish muscle corresponds to the fish growth and signifies that the growth of fish was due to mainly increase in muscle protein [45]. In fish amino acid composition of the diet is also one factor determining protein requirement [46]. In this study, protein is found to the high in starch fed fishes. All the parameters like Length, weight moisture, ash, lipid, carbohydrate amino acid was found to the high in experiment compared to control. Seenappa and Devaraj [47] reported that there was significant increase in the survival rate of fishes fed with proteins and carbohydrates. The studies by Ali *et al*., [48]

showed that among the 5 fish species studied water content and protein content was high in *L. rohita.* Any balanced formula for fish diets must include an energy source plus sufficient indispensable amino acid, essential fatty acids and specific vitamins and minerals to support life and promote growth [49]. Amzad Hossain and Masayuki Furuichi [50] in their study reported a weight gain in fish *Sebastiscus marmoratus* fed with calcium lactate. All the studies show that readily available calcium supplement to the diet appears to the essential for optimum growth and for maintaining normal growth, feed utilization and bone mineralization. In the present study there was an increase in the composition of calcium in fishes fed with calcium and the weight increase is found to be slightly low compared to fishes fed with starch and sardine oil (Table:2). This all shows that probiotics have a very positive effect in aquaculture that is it will give immunity to fishes and enables digestion which will helps in the fish culture and will also help to improve nutritional quality of fishes. In the present investigation, it was found out that the *L. rohita* fed with probiotics had shows an increase in growth, nutritional status and 100% survival rate compared to control. The information generated from present investigation might contribute to the incorporation of the bacteria in commercial aquaculture as supplement in formulated fish feed to achieve colonization in fish gut in higher degree.

Table 1: Biochemical analysis of *L. rohita* (mg/gm) fed with antibiotics and probiotics

Parameters	Food supplements	Initial	Final		
			Control-E1	Experi-E2 (Antibiotics)	Experi-E3 (Probiotics)
Protein	Starch	29.2 ± 2.89	30.60 ± 0.61^{b}	34.29 ± 1.96^{ab}	41.1 ±1.93^{a}
	Sardine Oil	29.2 ± 2.89	32.54 ± 1.17^{c}	35.34 ± 2.99^{b}	42.33 ± 1.70^{a}
	Calcium	29.2 ± 2.89	33.66 ± 0.62^{b}	35.89 ±1.27^{ab}	44.26±1.57^{a}
Lipids	Starch	10.2 ± 1.67	12.77 ± 0.52^{c}	17.10 ±0.85^{b}	17.06 ±0.62^{a}
	Sardine Oil	10.2 ± 1.67	14.82 ± 1.83^{c}	18.47 ±3.96^{b}	16.00 ± 2.79^{a}
	Calcium	10.2 ± 1.67	16.36 ±1.69^{b}	17.98 ±1.62^{ab}	18.36 ± 0.50^{a}
Carbohydrate	Starch	26 ± 3.92	27.92 ± 0.62^{c}	31.03 ±1.56^{b}	34.79 ± 1.49^{a}
	Sardine Oil	26 ± 3.92	28.92 ± 1.93^{c}	32.97 ±3.29^{b}	38.58 ± 1.09^{a}
	Calcium	26 ± 3.92	30.52 ± 2.63^{b}	33.63 ±5.77^{ab}	43.24 ± 1.92^{a}
Amino acid	Starch	22.4 ± 3.04	25.80 ± 1.72^{c}	32.87 ±3.34^{b}	35.82 ± 0.64^{a}
	Sardine Oil	23.4 ± 3.04	26.96 ± 1.52^{b}	33.02 ±3.46^{ab}	36.43 ± 1.73^{a}
	Calcium	24.4 ± 3.04	28.63 ± 0.41^{c}	33.78 ± 1.49^{b}	36.96 ± 1.30^{a}
Moisture	Starch	78.4 ± 1.50	76.71 ± 1.72^{b}	78.42 ± 0.99^{ab}	79.41 ± 1.18^{a}
	Sardine Oil	78.4 ± 1.50	77.81 ± 1.83^{c}	79.57 ± 0.60^{b}	80.73 ± 1.25^{a}
	Calcium	78.4 ± 1.50	78.54 ± 1.39^{c}	79.93 ± 1.34^{b}	80.58 ± 3.16^{a}
Ash Content	Starch	2.28 ± 0.177	2.92 ± 1.72^{c}	4.10 ± 0.22^{b}	4.29 ± 0.10^{a}
	Sardine Oil	2.28 ± 0.177	3.38 ± 0.61^{b}	3.88 ± 0.18^{ab}	4.06 ± 0.37^{a}
	Calcium	2.28 ± 0.177	3.96 ± 0.16^{b}	4.06 ± 0.96^{ab}	3.92 ± 0.20^{a}

Mean values within the same row sharing the same superscript are significantly different (P<0.05); mean ± SD

Table 2: Growth parameters of *L. rohita* fed with antibiotics and probiotics

Parameters (%)	Food supplements	Initial	Final		
			Control-E1	Experi-E2 (Antibiotics)	Experi-E3 (Probiotics)
Length (cm %)	Calcium	3.00 ± 0.1	2.83 ± 1.93^{b}	5.5 ± 0.60^{ab}	5.9 ± 0.55^{a}
	Sardine Oil	3.00 ± 0.1	3.94 ± 0.62^{c}	6.1 ± 0.41^{b}	6.5 ± 0.81^{a}
	Starch	3.00 ± 0.1	4.48 ± 2.35^{b}	7.1 ± 0.85^{ab}	7.5 ± 1.02^{a}
Weight (gm %)	Calcium	0.70 ± 0.15	0.94 ± 0.06^{c}	1.66 ± 0.07^{b}	1.75 ± 0.05^{a}
	Sardine Oil	0.70 ± 0.15	1.11 ± 0.76^{c}	1.71 ± 0.03^{b}	1.89 ± 0.04^{a}
	Starch	0.70 ± 0.15	1.43 ± 0.10^{b}	1.83 ± 0.16^{ab}	2.13 ± 0.76^{a}
Survival rate (%)	Calcium	100. ±019	92.05±0.12^{c}	97.09±0.41^{b}	99.05 ± 0.83^{a}
	Sardine Oil	100. ±019	94.62±0.07^{b}	98.08±0.03^{c}	99.06 ± 0.37^{a}
	Starch	100. ±019	96.68±0.8^{c}	98.18±0.08b	99.97 ± 0.18^{a}
Food conversion Ratio (FCR)	Calcium	1.41±0.057	1.99±0.49^{c}	1.29±0.12^{b}	1.11±0.07^{a}
	Sardine Oil	1.41±0.057	2.27±0.83^{b}	1.67±0.87^{ab}	1.02±1.93^{a}
	Starch	1.41±0.057	3.04±1.22^{c}	2.04±1.52^{c}	0.9±1.73^{a}

Mean values within the same row sharing the same superscript are significantly different (P<0.05); mean ± SD

Table 3: Table showing Nutritional indices of *L. rohita* fed with antibiotics and probiotics

Type of Nutritional indices (%)	Food supplements	Initial	Final		
			Control-E1	Experi-E2 (Antibiotics)	Experi-E3 (Probiotics)
Increase in Biomass	Calcium	0.162±1.98	1.34±0.28^{bc}	0.28±2.87^{b}	0.47±2.76^{a}
	Sardine Oil	0.171±0.53	0.456±3.74^{c}	0.755±3.65^{ab}	1.05±1.36^{a}
	Starch	0.235±1.22	0.357±0.13^{c}	0.633±2.47^{b}	1.01±2.15^{a}
Specific Growth Rate (SGR)	Calcium	0.236±2.76	0.345±0.27^{cd}	0.519±1.67^{b}	0.629±1.36^{a}
	Sardine Oil	0.297±1.26	0.656±2.11^{cd}	0.753±3.19^{ab}	0.855±1.45^{a}
	Starch	0.457±2.78	0.565±2.65^{bc}	0.704±1.23^{c}	0.843±1.29^{a}
Condition factor	Calcium	3.56±1.84	4.76±1.99^{c}	12.86±1.62^{b}	14.4±2.11^{a}
	Sardine Oil	4.67±2.65	6.75±2.54^{d}	15.91±2.76^{ab}	19.06±1.57^{a}
	Starch	7.95±2.18	9.54±0.53^{cb}	16.55±2.55^{b}	21.3±0.98^{a}

Mean values within the same row sharing the same superscript are significantly different (P<0.05); mean ± SD

Table 4: Bacteria found in the gut of *L. rohita* fed with antibiotics and probiotics

Control-E1	Experi-E2 (Antibiotics)	Experi-E3 (Probiotics)
Pseudomonas spp. *Aeromonas spp.* *Streptococcus spp.* *E. coli* *Acinetobacter spp.* *Micrococcus spp.*	*E. Coli* *Streptococcus spp.* *Pseudomonas spp.*	*E. Coli* *Streptococcus spp.* *Pseudomonas spp.* *Bacillus mesentericus* *Clostridium butyricum* *Enterococcus faecalis* *Lactobacillus sporogenes* *Micrococcus spp.*

Table 5: Antibacterial activity of Probiotics against bacterial pathogens

S. No	Organism Dilution mg/ml	Diameter of clear zone (mm) pathogens				
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumonia***	***Aeromonas hydrophila***
1.	Control	7.0±1.59	4.0±0.63	6.0± 1.00	5.0±0.21	4.0±0.12
2.	Antibiotics	7.35±0.51	6.5±0.62	8.7±0.86	6.85±0.74	5.2±0.72
3.	Probiotics	9.5±.06	8.05± 0.42	12.6 ± 0.42	10.5±0.71	8.6±0.98

4. Conclusion

The nutritional evaluation, survival rate and growth of fishes were done using probiotics. The fishes were fed with artificial feeds along with supplements like Calcium, Starch and Sardine oil. Upon the biochemical evaluation protein shows highest value followed by other biochemical constituents like amino acids, carbohydrates and lipids. The analysis of gut microbiota was also done. The analysis of gut of fishes fed with probiotics showed the presence of useful bacteria and the exclusion of pathogenic bacteria. The information generated from present investigation might contribute to the incorporation of the bacteria in commercial aquaculture as supplement in formulated fish feed to achieve colonization in fish gut in higher degree.

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