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SUPPLEMENTATION OF AN ISOLATED FISH GUT BACTERIUM, *BACILLUS CIRCULANS*, IN FORMULATED DIETS FOR ROHU, *LABEO ROHITA*, FINGERLINGS

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Abstract

An extracellular protease producing bacteria *Bacillus circulans* (Lr 1.1) was isolated from the gut of rohu, *Labeo rohita,* fingerlings, and used as a supplement in five diets for rohu fingerlings. The effect of the supplement on the growth performance and feed utilization efficiency of the rohu was evaluated. Rohu fingerlings (avg wt 0.83±0.02 g) were fed diets supplemented with 1.5 x 10^4 (diet D2), 1.5 x 10^5 (diet D3), 1.5 x 10^6 (diet D4), 1.5 x 10^7 (diet D5) or 1.5 x 10^8 (diet D6) *B. circulans* cells per 100 g feed for 60 days at 3% of the body weight in triplicate treatments. The control diet (diet D1) was not supplemented with the bacteria. Diet D3 resulted in significantly better growth, a lower feed conversion ratio and a higher protein efficiency ratio than the other experimental diets. Intestinal α-amylase activity did not differ significantly beyond the lowest inclusion level (diet D2), whereas protease activity increased significantly with diets D2 and D3. Apparent dry matter and protein digestibility did not significantly correlate. However, lipid digestibility decreased with the increasing level of *B. circulans*.

Introduction

The use of probiotics has a long tradition in animal husbandry (Stavric and Kornegay, 1995) but has rarely been applied in aquaculture. The first trial in which probiotics were incorporated into aquaculture feeds used commercial preparations designed for land animals (Gatesoupe, 1999). The trials with commercial probiotics for land animals were important as they showed interest in bacterial additives to aquaculture feeds, but the survival of the microbes in the gastrointestinal tract of the aquatic animals was uncertain.

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Therefore, most attempts (Gildberg et al., 1997; Gatesoupe, 1999) aimed at finding autochthonous strains with probiotic properties - microorganisms which are able to colonize the epithelial surface of the stomach, small intestine or large intestine of the host animal (Savage, 1989). The beneficial effects of probiotic microorganisms are well-known in veterinary medicine, and target species are cattle, domestic pets, goats, horses, pigs, poultry and sheep (Fuller, 1989; Sissons, 1989). Probiotics might be used in two ways in aquaculture: by introducing a specific bacterial flora to the digestive system of the fish via the diet, or by inoculating the beneficial bacterial flora into the rearing water. Introducing bacterial flora into the rearing water is probably appropriate only when the fish are held in sea water because of the high drinking rate to compensate for gradual dehydration. Therefore, the main strategy in use today is to isolate intestinal bacteria with favorable properties and include high numbers of these bacteria in the feed of immature animals of the same species.

The extracellular protease producer *Bacillus circulans* was isolated from the gut of rohu, *Labeo rohita,* fingerlings. Characterization of the bacterial flora showed its potential for use as a probiotic (Ghosh et al., 2002a). In this experiment, the probiotic effect of the bacterial strain was evaluated by calculating growth performance and feed utilization in rohu fingerlings.

Materials and Methods

Isolation and selection of gut bacterial flora. Ten healthy fingerlings of rohu, *Labeo rohita* (avg wt 3.51±0.31 g), were starved for 24 hours and sacrificed. The ventral surfaces of the fish were scrubbed with a 1% iodine solution (Trust and Sparrow, 1974) before opening the intestinal wall. The intestines from the fish were removed aseptically and homogenized with a 0.89% NaCl solution (10:1; Das and Tripathi, 1991). The homogenate was used as an inoculum. One ml of the homogenate was spread on sterilized soybean-casein digest agar (tryptone soya agar, TSA, HiMedia, India) plates and incubated at 37°C for 24 hours in duplicate. Colonies with a different morphological appearance were isolated and streaked separately on TSA plates to check their purity. Isolated colonies were characterized and identified (Ghosh et al., 2002a). Among them, the Lr 1.1 strain of *Bacillus circulans* was selected for incorporation into the experimental diets because of its excellent protease and moderate cellulase producing capacities.

Experimental diets. Six isocaloric (4.26 kcal/g) and isonitrogenous (35% crude protein) diets (D1-D6) were prepared. The feed ingredients were finely powdered and fortified with an equiproportional mixture of cod liver oil and sunflower oil as lipid sources of essential fatty acids (18_{n-3} and 18_{n-6}) to meet the requirements of the fish (Bromley, 1980). A ready-made vitamin-mineral mixture (Vitaminetes Forte, Roche India Ltd., Mumbai, India) was added to the diets before pelleting. Chromic oxide (1% w/w) was added to each formulated diet as an external marker. The experimental diets (diets D2-D6) were supplemented with the isolated *Bacillus circulans* cells at 1.5×10^4 (diet D2), 1.5×10^5 (diet D3), 1.5×10^6 (diet D4), 1.5×10^7 (diet D5) and 1.5×10^8 (diet D6) per 100 g of feed. The reference diet (D1) was not supplemented with bacteria. Each experimental diet was mixed with the desired quantity of bacteria cells in a tryptone soya broth suspension culture. The mixtures were passed through an electrically operated semiautomatic pelletizer (pellet size 1.5 mm diameter). The pellets were dried at 40°C in a biological oxygen demand (BOD) incubator for 72 hours, packed in airtight plastic bags and stored in a refrigerator until used. The pellets were crumbled before being dispensed. The ingredients and proximate compositions of the diets are presented in Table 1.

Experimental design. Rohu fingerlings were obtained from a local fish seed dealer and acclimatized to the laboratory conditions for 15 days before beginning the experiment. A sample of fingerlings was sacrificed and used to determine the initial carcass composition. The fish were macerated with a mortar and pestle for proximate analysis. The fingerlings (avg wt 0.83±0.02 g) were randomly distributed in a static water system containing 90

Table 1. Ingredients (% dry weight) and proximate composition (on dry matter basis; n = 3) of the experimental diets.

Ingredient	*%*
Fish meal	39
Rice bran	28
Mustard oil cake	26
Sunflower oil	2
Cod liver oil	2
Vitamin premix[1]	2
Chromic oxide[2]	1

	Experimental diets					
	D1	*D2*	*D3*	*D4*	*D5*	*D6*
B. circulans cells (1.5 x)	*no supplement*	*10^4*	*10^5*	*10^6*	*10^7*	*10^8*
Proximate composition(%)						
Moisture	9.21	9.40	8.80	9.15	8.75	9.92
Dry matter	90.79	90.60	91.20	90.85	91.25	90.08
Crude protein	34.99	35.24	35.11	35.57	35.96	35.55
Crude lipid	9.52	9.97	9.28	10.08	10.23	9.88
Ash	12.47	13.17	13.24	12.52	13.19	12.85
Crude fiber	10.28	10.47	9.99	10.50	10.05	10.13
Nitrogen-free extract	23.53	21.75	23.58	22.18	21.82	21.67
Organic matter	78.32	77.43	77.96	78.33	78.06	77.23
Gross energy (kcal/g)	4.26	4.25	4.23	4.30	4.31	4.25

[1] Vitamin and mineral mixture (Vitaminetes Forte, Roche Products Ltd., 2428 Pt. M. M. Malaviya Road, Mumbai 400 034, India).
[2] External digestibility marker

l glass aquaria. The fingerlings were stocked at a stocking density of 15 fish per aquarium with three replicates for each dietary treatment. The feeding trial continued for a period of 60 days under laboratory conditions with continuous aeration by an air compressor. The water from each aquarium was replenished twice daily. Water quality parameters during the experiment were: temperature 18-25°C, pH 6.4-7.5 and dissolved oxygen 5.0-7.8 mg/l.

Fish were fed twice a day at 08:00 and 12:00, the feeding rate being 3% of the total body weight per day. The daily ration was adjusted every ten days on the basis of the weight increment. Uneaten feed was removed and stored separately to calculate the feed conversion ratio. Fecal samples were collected by pipetting (Spyridakis et al., 1989). The fecal samples were oven dried (60°C) and analyzed for digestibility determinations. At the end of the experiment, fish from all treatments were weighed, sacrificed and treated as previously described for subsequent proximate analysis.

Chemical analysis and data collection. Experimental diets and fecal samples were analyzed for proximate composition (AOAC, 1990) as follows: moisture content by oven drying at 105°C for 24 hours; protein (N x 6.25) by the micro-kjeldhal technique using the Kjeltec System (Tecator, Sweden); lipid by extracting the residue with 40-60°C petroleum ether for 8 hours in a Soxhlet apparatus; crude fiber using the Fibertec System (Tecator, Sweden). Nitrogen free extract (NFE) was computed by subtracting the values for crude protein, lipid, ash, fiber and moisture from 100 (Maynard et al., 1979). Chromic oxide in the diets and in the feces was estimated spectrophotometrically (Bolin et al., 1952). The proximate analyses of the carcasses were done before initiation and after termination of the experiment following the procedures used for the diets. Water quality parameters were monitored following methods outlined by APHA (1995).

Fish from each experimental set were dissected on an ice tray at the beginning and at the end of the experiment. The intestine was removed to determine the digestive enzyme activities. The intestinal tissues were removed, thoroughly washed with chilled distilled water to remove blood and mesenteries, collected in ice-cooled petri dishes, weighed and cut into small pieces. A 50% homogenate was prepared with an ice-cooled 0.1 M phosphate buffer (pH 7.0) and centrifuged in a refrigerated centrifuge (Beckman-GS-15R, USA) at 2,500 rpm for 15 minutes. The supernatant was used for enzyme assay. α-amylase was quantitatively determined following the method described by Bernfeld (1955). Protease activity was measured according to the method of Moore and Stein (1948) using bovine serum albumin as the substrate.

Average live weight gain (%), specific growth rate (SGR; %/day), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using standard formulae outlined by Steffens (1989).

The apparent digestibility (AD) of nutrients was calculated according to De Silva and Anderson (1995), using the following formula: AD(%) = 100 – 100 x (% Cr_2O_3 in diet/% Cr_2O_3 in feces) x (% nutrient in feces/% nutrient in diet).

Statistical analysis. Analysis of variance (ANOVA) was performed using Microsoft Excel software. Duncan's multiple range test (Duncan, 1955) was employed to test differences among means. The compared parameters were final weight, % weight gain, SGR, FCR, carcass composition, nutrient digestibility, and intestinal amylase and protease activities.

Results

Fish growth and performance. Performance and survival rates of the fish are presented in Table 2. The highest weight gain was obtained in the group fed diet D3, followed by diets D2 and D4. Group D3 had a significantly higher final weight, % weight gain, SGR and PER and a significantly lower FCR than the other experimental diets ($p<0.05$). Diets D2 and D3 resulted in better performance than the reference diet (D1) but diets with a higher inclusion level (D5 and D6) resulted in poorer performance. The FCR was inversely

Table 2. Growth and apparent nutrient digestibility of fish fed experimental diets for 60 days. Results are means ± SD of three determinations.

	Experimental diets					
	D1	*D2*	*D3*	*D4*	*D5*	*D6*
Bacillus circulans cells (1.5 x)	*no supplement*	*10^4*	*10^5*	*10^6*	*10^7*	*10^8*
Initial weight (g)	0.83±0.02	0.83±0.02	0.83±0.02	0.83±0.02	0.83±0.02	0.83±0.02
Final weight (g)	1.69±0.02^c	1.91±0.02^b	2.01±0.03^a	1.76±0.02^c	1.62±0.03^d	1.58±0.01^d
Average weight gain (%)	103.61±2.18^c	130.12±3.50^b	142.17±2.11^a	112.05±3.60^c	95.18±1.75^d	90.36±2.52^d
Specific growth rate (%/d)	1.19±0.01^c	1.39±0.01^b	1.47±0.02^a	1.25±0.02^c	1.11±0.01^d	1.07±0.01^d
Protein efficiency rate	1.34±0.02^c	1.51±0.02^b	1.65±0.03^a	1.40±0.02^c	1.25±0.01^d	1.20±0.02^d
Food conversion ratio	1.74±0.04^b	1.38±0.05^c	1.27±0.04^d	1.61±0.03^b	1.89±0.07^a	1.99±0.04^a
Survival rate (%)	100	100	100	100	100	100
Apparent digestibility (%)						
Dry matter	50.32±1.15^b	51.05±0.55^{ab}	54.83±1.04^a	51.17±0.55^{ab}	49.53±1.44^b	48.85±1.27^b
Protein	88.79±1.13^{ab}	90.67±1.15^a	90.95±1.45^a	90.16±1.05^a	88.24±1.14^{ab}	84.74±2.01^b
Lipid	89.45±2.47^a	88.79±2.03^a	86.04±2.30^{ab}	85.61±1.18^{ab}	81.42±2.07^b	77.12±3.12^c
Ash	46.15±1.30^a	47.06±1.35^a	46.14±1.30^a	48.25±4.41^a	47.62±1.38^a	45.91±1.29^a

Values with same superscript in the same row are not significantly different ($p < 0.05$).

related to the SGR and PER in all experimental groups. No fish were lost during the trial.

Digestibility. Apparent digestibility of dry matter, protein and ash significantly differed among groups. The digestibility of dry matter and protein was higher in fish fed D3 than in those receiving D6; from the inclusion level of D3, digestibility gradually decreased as the inclusion rate of bacteria increased. Lipid digestibility decreased sharply and significantly with the increasing level of *B. circulans* from D2 to D6. Ash digestibility was unaffected by the *B. circulans* supplements.

Proximate composition of carcass. The carcass protein content in fish fed diets D2 and D3 was higher than in those fed the reference diet whereas in those fed diets D4, D5 or D6, carcass protein content did not differ from the reference diet (Fig. 1). Carcass lipid content decreased significantly with the increase of *B. circulans* but there was no specific trend regarding carcass ash content.

Activities of digestive enzymes. Both α-amylase and protease activity increased in all experimental groups beyond the initial value (Fig. 2), but the activity in groups D2 to D6 did not significantly differ from each other. Intestinal protease activity in all experimental groups was significantly higher than in the reference group; it was highest in the D3 group, followed by D2 and D4.

Discussion

The strains of *Bacillus* sp. used as probiotics for terrestrial livestock have telluric origins. They are not autochthonous in the gastrointestinal tract but may be active during intestinal transit (Gournier-Chateau et al., 1994). There are many reports of the isolation of *Bacillus* strains from fish (Hamid et al., 1978; Strom and Olafsen, 1990; Nedoluha and Westhoff, 1995; Sadhukhan et al., 1997; Kennedy et al., 1998; Sugita et al., 1998), crustaceans (Austin and Allen, 1982; Sharmila et al., 1996; Sugita et al., 1996) and bivalves (Sugita et al., 1981).

In the present study, an extracellular enzyme producing strain of *B. circulans* was isolated from the gastrointestinal tract of *L.*

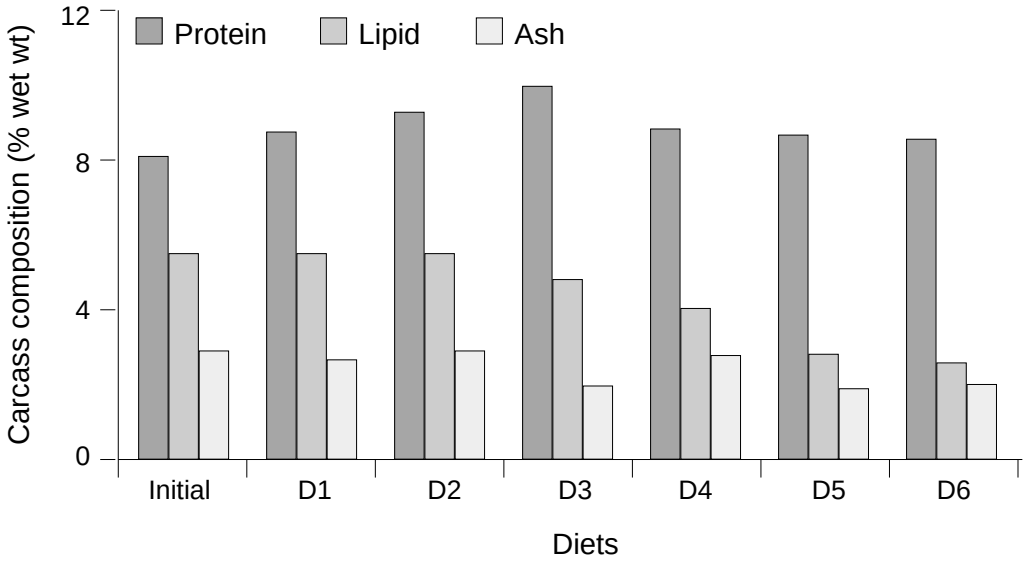

Fig. 1. Proximate carcass composition (% wet weight) of rohu fingerlings fed bacilli incorporated diets for 60 days.

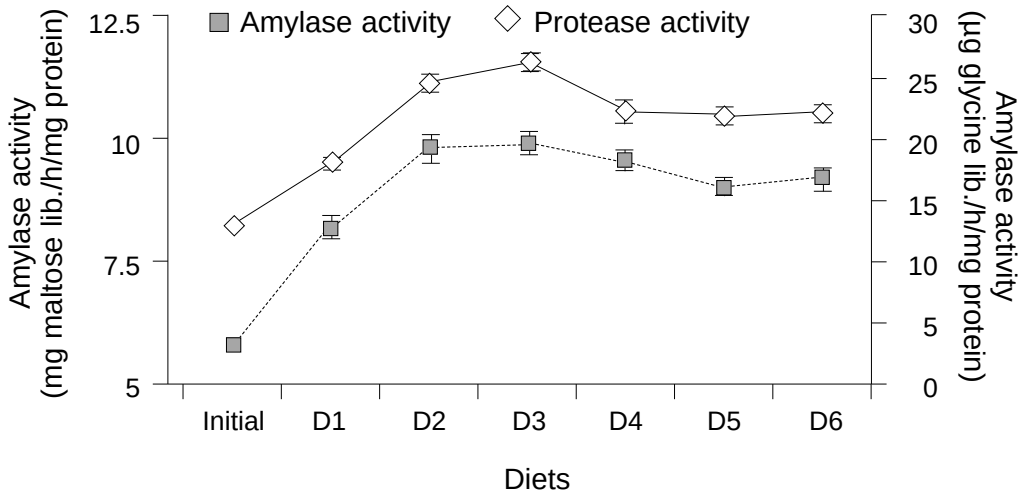

Fig. 2. Intestinal α-amylase and protcase activities of rohu fingerlings fed experimental diets for 60 days.

rohita and used to supplement five isonitrogenous diets. Of the three species of *Bacillus* isolated from the rohu gut, the strain *B. circulans* (Lr 1.1) was selected for supplementing the diets because of its better protease producing ability. In addition, it can produce cellulase moderately. The isolate, however, did not show any amylase producing capacity (Ghosh et al., 2002a). The beneficial influence of *B. circulans* on the growth performance of the rohu fingerlings could be seen. The diet supplemented with 1.5 x 10^4 bacteria cells resulted in the best performance in terms of percent weight gain, SGR, FCR and PER. The fish did not perform better with a higher level of bacteria inclusion.

The beneficial roles of bacterial enzyme on fish growth have been discussed by Ghosh et al. (2001). The strain used in the present study is capable of producing a good amount of proteolytic enzyme and a moderate amount of cellulase. The experimental evidence, therefore, suggests that the strain *B. circulans* (Lr 1.1) might have induced growth in rohu fingerlings by producing essential nutrients not present in the formulated diets or by improving digestion by supplying digestive enzymes to the fish. The same strain was found effective in improving the growth and survival rate of rohu spawns (Ghosh et al., 2002b). However, the poor performance of rohu fingerlings fed diets supplemented with a higher number of bacteria cells is difficult to explain.

Apparent digestibility of ash did not significantly change due to the bacteria supplementation. However, protein digestibility tended to be higher than the reference in fish fed the probiotic up to the D4 diet and, thereafter, it was lower. Apparent protein digestibility was positively correlated to the growth performance of the fish. Lipid digestibility significantly decreased at the highest inclusion rates (D5 and D6 diets).

The activities of the digestive enzymes (protease and α-amylase) in the intestines of the rohu fingerlings were higher than the initial value in all the dietary treatments. The results clearly indicated the diet-related changes in digestive enzyme activities. It is assumed that the supplementation of the enzyme-producing *B. circulans* Lr 1.1 augmented the enzyme activity in the fish (Jobling, 1994).

The supplementation of bacteria cells induced a reduction of lipid deposition, probably by reducing lipid digestibility, and an increase in body protein in the rohu finger-

lings. The protein accretion was highest in the fish fed diet D3. The results clearly indicate that the bacterial enzyme (protease, in particular) helped to better protein utilization, resulting in more protein accretion in the muscle.

It is concluded from the present study that *B. circulans* Lr 1.1 may be used as a supplement in formulated diets for rohu fingerlings for better utilization of nutrients by increasing the endogenous level of enzymes. A search for more autochthonous bacteria strains with probiotic properties for application in practical diet formulation is recommended.

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