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An investigation on the use of *Prosopis juliflora* pods as a carbohydrate source supplemented with probiotics in the diet of *Labeo rohita* fingerlings

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Abstract

The present study investigated the use of fermented *Prosopis juliflora* pods (PJP) as a carbohydrate source alternative to rice bran (RB), and a *P. juliflora* pods based diet formulation supplemented with probiotics *Lactobacillus acidophilus* (LA), *Lactobacillus bulgaricus* 009 (LB) and *Streptococcus thermophilus* (ST) on the growth of *Labeorohita* (rohu) fingerlings. The diets were isonitrogenous (39%) and isoenergetic (15.9 KJg^{-1} gross energy); and were prepared using rice bran (as reference diet RD), crude pods (PJPC), fermented pods (PJPF) and with probiotics *L. acidophilus* (PJPLA), *L. bulgaricus* (PJPLB) and *S. thermophilus* (PJPST). The results showed that fermentation of *P. juliflora* pods with *L. acidophilus* significantly increased (p<0.05) protein, lipid, Ca, Fe, Mg, K and reducing sugar; and completely eliminated phytic acid. The diets PJPF and PJPLA produced significantly higher (p<0.05) percent weight gain, SGR, FCR and PER as compared to PJPC, PJPLB and PJPST diets; and achieved growth performance comparable to RD. An improvement (p<0.05) in the carcass protein has been detected in groups PJPF and PJPLA in comparison to RD, PJPC, PJPLB and PJPST. The *L. acidophilus* was found to be effective in controlling coliform count in gut as compared to *L. bulgaricus* and *S. thermophilus*; and also in controlling total viable count, total coliform count and fecal streptococci count in fecal matter. The results indicate that PJPF and PJPLA deserve further investigations as alternative feed for rohu.

Keywords:*Prosopi sjuliflora* pods, Antinutritional factors, Probiotics, Fermentation, Growth, Microflora.

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Introduction

In India, Indian major carps are fed on supplementary feeds basedon low input technology (Garg *et al*., 2002; Keshavanath *et al*., 2002). The use of locally available cheap and nutritionally acceptable non-conventional feed in the fish diet would certainly have a significant impact on carp culture by the reduction of feed cost. Carbohydrate in fish diet is an inexpensive immediate source of energy with protein sparing effects (Wilson, 1994; Erfanullah and Jafri, 1998). Grains and their by-products are the main carbohydrate sources used in the diet of cultivable fishes (Krogdahl *et al*., 2005). Replacement of yellow maize with a cheap carbohydrate source like barley as well as incorporation of a simple carbohydrate like pitted dates in the feed improved the growth of *O. niloticus* (Belal and Al-Jasser, 1997; Belal, 1999).

Prosopisjuliflora, mesquite, is a leguminous tree (Family: Leguminosae, Sub-family: Mimosoideae). It is widely spread in arid zones of the world, mainly Africa, Pakistan, India, Brazil and Australia (Choge *et al*., 2007). It has been extensively used in animal feed, as a fuel and to control soil erosion (Ravikala *et al*., 1995). The PJ pods are reported to be rich in carbohydrate (30 to 75%) and contains fructose (3.2-12%), glucose (0.8-5%), galactose (0.8 %), sucrose (7.5-75%), maltose (<0.4%), lactose (0.7%), inositol(5%), raffinose(1%) and reducing sugar (2-20%) (Marangoni and Allii, 1988; Sawal *et al*., 2004; Choge *et al*., 2007). The PJP are shown also to be rich in protein and iron (Galera *et al*., 1992; Choge *et al*., 2007). Extensive research is in progress for the utilization of *P.juliflora* pods as animal feed; the *P.juliflora* pods have been used in the diet of feedlot lambs, cattle as well as Omani goats with good nutritional and economic results; and their potentiality as an energy source have been investigated in the diet of Nile tilapia; but they have so far not been investigated as a nutrient source in the diet of carp (Ravikala *et al*., 1995; Batista *et al*., 2002; Mahgoub *et al*., 2005; Mabrouk *et al*., 2008). Moreover, the pods are quite cheaper as compared to rice bran. Looking to the availability, nutritional quality and low cost, *P.juliflora* pods have been selected in the present study as a carbohydrate source.

Lactic fermentation has been suggested to improve the nutritional quality of plant based fish feed; and a clear improvement in the nutritional quality of carbohydrate fractions by lactic acid fermentation of cereal grains and soybean meal has been observed when used in Atlantic salmon (Skrede *et al*., 2002; Refstie *et al*., 2005). Probiotics are increasingly examined as dietary supplements in aquaculture; and their beneficial effects include higher growth and feed efficiency, prevention of intestinal disorders, pre-digestion of antinutritional factors, improvement in health status as well as resistance against infectious agents (Gatesoupe, 1999; Suzer *et al*., 2008). The common probiotics used in aquaculture are *Lactobaccilus, Bacillus, Bifidobacterium, Saccharomyces* and

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Enterococcus (Kumar *et al*., 2008). In rohu, addition of *Bacillus* sp. to leaf meal has been reported to improve their growth rate (Bairagi *et al*., 2004); and in common carp, *Streptococcus faecium* have been shown to eliminate *E. coli* from the alimentary tract and to improve growth rate (Noh *et al*., 1994). Thus, in the present study the effects of supplementation of lactic acid bacteria cultures to *P.juliflora* pods based diets and fermentation of *P.juliflora* pods on growth, feed efficiency, digestibility, digestive enzyme activities as well as gut, fecal matter and tank microflora was investigated.

Materials and methods

Lactic acid bacteria cultures

Three lactic acid bacterial cultures, *L. acidophilus*, *L.bulgaricus* and *S.thermophilus* were procured from the National Dairy Research Institute (NDRI, Chandigarh, India); maintained in sterile reconstituted SAGAR (DudhSagar Dairy, Mahesana, India) skimmed milk (11% wv^{-1}) as stock cultures and stored at 4°C prior to use. The cultures were checked for the purity by Gram's staining and by catalase test (AOAC, 1995). For the propagation of cultures in the feed, 0.2 mL (10^8-10^{10} $CFUmL^{-1}$) of stock cultures were inoculated in 20 mL of sterile pannier whey (pH 5.5 for *L. acidophilus* and *L.bulgaricus*; pH 7.0 for *S.thermophilus*) and incubated at 37°C for 24h. Cells were harvested by centrifugation (REMI CPR-24) at 10,000 × g for 10 min; cell pellets were suspended in sterile normal saline, counted by pour plate method using Lactobacilli deMan, Rogosa and Sharpe agar (MRS agar), lactic agar and lactic agar + sterile skimmed milk (11%wv^{-1}) (Downes and Ito, 2001) for LA, LB and ST respectively; and all the three cultures were added to the diet separately at the rate of 10^7-10^8 $CFUgm^{-1}$. The *L. acidophilus*, *L. bulgaricus* and *S.thermophilus* cultures were checked for their antagonistic action against rohu gut isolates, coliforms and *vibrio sp.* by agar–well diffusion method (Nowroozi *et al*., 2004).

Diet formulation

P.juliflora pods were obtained from the local market, dried at 55°C –60°C, ground into powder to pass through a 0.5mm sieve and used as test feed. The fishmeal of Indian origin was prepared from javla (small shrimps from the cod end of dol net, Jafarabad, Gujarat), oven dried at 50°C–60°C for 24 h and ground to pass through a 0.5mm sieve. Six isonitrogenous (39% crude protein) and isoenergetic(15.9 $KJgm^{-1}$ gross energy) diets were formulated (Table 2). In the control diet (RD), rice bran was used as the main carbohydrate source. Experimental diets were formulated using crude *P.juliflora* pods(PJPC), using *P.juliflora* pods based diets supplemented with *L. acidophilus*(PJPLA), *L. bulgaricus* (PJPLB) and *S. thermophilus* (PJPST) separately as probiotics at the rate of 10^7 to $10^8$$cellsg^{-1}$; and using *P. juliflora* pods fermented with *L. acidophilus* (PJPF) at the rate of 10^7 to 10^8 $cellsg^{-1}$, incubated for 72h at 37°C and dried at 50°C to 60°C. Maize gluten was used

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to adjust total nitrogen content (Bhatt *et al*., 2011). Before diet formulation, the proximate composition of feed ingredients was checked (Table 1). To the autoclaved feed ingredients, required amounts of vitamin pre-mix, mineral pre-mix, oil-mix and binder were added aseptically. Chromic oxide was used as an external marker for the nutrient digestibility study and bentonite was used as a binder. The feed ingredients were mixed and made into moist pellets of 3 mm in diameter with hand pelletizer, oven dried at 45°C for 24h and stored at 4°C. For the probiotic supplemented diets, the total lactic acid bacterium count (n3) has been checked after drying (0 day) and on 7^{th} day of storage under refrigeration; and the survival rate has been calculated as follows:

Survival (%)=(A_o/A_1)×100

Where, A_o=Number of bacteria on 7^{th} day, A_1=Number of bacteria on 0 day

Experimental design

Rohu fingerlings were obtained from Gujarat Govt. Fish Seed Centre, Navali (Dist. Anand), acclimatized to laboratory conditions for 15 days and fed with a 1:1 mixture of finely powdered rice bran and groundnut oil cake. The fingerlings (average mean weight 3.80±0.05g) were stocked in glass aquaria (150L – 0.91×0.38×0.45 m^3) at the rate of 6L$fish^{-1}$ with three replicates for each treatment group. Un-chlorinated tube-well water was used for the experiment. The fish were fed with the formulated diet twice a day at 9.00h and 15.00h at the rate of 5% of the body weight for 60 days. The fish were weighed every week and the feed allowance adjusted accordingly. During experiment, continuous aeration was provided and temperature was maintained at 30°C. The water quality parameters pH, dissolved oxygen (DO) and total organic carbon (TOC) were monitored weekly following the methods of American Public Health Association (APHA, 1998).

For fecal matter collection, first the residual feed particles were siphoned out; and on the next day, faeces were collected before the first feeding by decantation as per the method of Spyridakis *et al*., (1989) during the last two weeks of experiment. The fecal matter was collected as pooled samples from all the replicates of each treatment group, oven dried at 60°C and analyzed separately for the determination of nutrient digestibility; whereas, for the microbiological analysis, from each treatment group, the fecal samples were collected separately from each replicate on the last two days, pooled and analyzed separately. At the termination of the experiment, five fish were sacrificed from each aquarium and analyzed for carcass composition, intestinal digestive enzyme activity and microbiological studies.

Microbiological analysis of fish gut, fecal matter and aquarium water

The microbiological analysis of fish gut and fecal matter for total viable count, total coliforms, fecal streptococci and presumptive *vibrio* sp. were carried out by pour plate method using Trypton Soya Agar (TSA), Violet Red Bile Agar (VRBA), Pfizer Selective Enterococcus

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Agar (PSEA) and Thiosulfate Citrate Bile Sucrose (TCBS)agar, respectively (APHA, 1998). The lactic acid bacterial counts of each culture in the feed, gut contents and fecal matter were carried out by pour plate method. In water samples, total coliforms and total fecal streptococci count were determined by most probable number (MPN) technique; whereas total viable and vibrio count was performed by pour plate method (APHA, 1998).

All the media used for the present study were purchased from HiMedia® (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Chemical analysis

Feed ingredients, experimental feeds, fecal samples and fish carcass were analyzed for their proximate composition following the methods of Association of Official Analytical Chemists (AOAC, 1995) as follows: Moisture was determined by oven drying at 105°C for 24h, protein (N×6.25) by Micro Kjeldahl digestion and distillation after acid digestion, ash by ignition at 550°C in a muffle furnace to constant weight, total dietary fibers by SIGMA KIT (Catalog no. TDF-100A; Sigma, St. Louis, MO, USA), lipid by Folch *et al*. (1957), total carbohydrate by Anthrone method (Hedge and Hofreiter, 1962) and reducing sugar by 3,5- dinitrosalicylic acid (DNS) method (Miller, 1959). In crude and processed PJPs, spectrophotometer methods used to estimate the relevant parameters are as follows: total phenol (Kakade *et al*., 1969), tannin (Schanderi, 1970), phytic acid (Wheeler and Ferrel, 1971), trypsin inhibitor (TI) (Baccou *et al*., 1977) and saponin (Molick and Singh, 1980). The mineral contents of calcium, iron, magnesium and potassium were analyzed by ICP (ICP–Inductively Coupled Plasma spectrometer at SICART, Vallbh Vidyanagar, Gujarat). The energy content of the diets was calculated using the average caloric conversion factors as 9.45 $Kcalg^{-1}$, 4.10 $Kcalg^{-1}$ and 5.65 $Kcalg^{-1}$ for lipid, carbohydrate and protein, respectively (Henken *et al*., 1986). Nitrogen-free extract (NFE) was computed by taking the sum of values for crude protein, crude lipid, ash, crude fiber, moisture; and subtracting this from 100 (Maynard *et al*., 1979). Chromic oxide in the diets and faeces was estimated by the method of Furukawa and Tsukahara (1966).

Chemicals used for the analysis of above parameters were purchased from Qualigens® (Qualigen Fine Chemicals, Mumbai, India).

Gut crude digestive enzyme extraction and estimation

The gut was rinsed with chilled distilled water, homogenized with phosphate buffer pH 7.2 (1:10 wv^{-1}) at 4°C, centrifuged (10,000 g × 10 min at 4°C) and the supernatant was stored at -20°C for enzyme analysis. The concentration of soluble protein in pooled samples was determined by the method of Lowry *et al*. (1951). In the supernatant, α-amylase, protease and lipase activities were assayed by using soluble starch (Qualigens®, Mumbai, India), casein (Qualigens®, Mumbai, India) and olive oil (Figaro, Madrid, Spain) as substrates

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respectively (Bernfeld, 1955; Kunitz, 1974; Rathelot *et al*., 1975).

Calculations and statistical analysis

Growth parameters of the fish were calculated according to Steffens (1989).

Specific growth rate (SGR; % day^{-1}) = $(\log_e W_2 - \log_e W_1)/t \times 100$.

Feed conversion ratio (FCR) = Dry feed consumed (g) / live weight gain (g).

Protein efficiency ratio (PER) = Weight gain (g) / crude protein consumed (g).

Coefficients of total tract apparent digestibility (CTTAD) of dietary components were calculated as follows:

$$CTTAD = 1 - \frac{DC_F ID}{DC_D IF}$$

Where CTTAD is the coefficient of apparent digestibility of dietary component in the assay diet, DC_F the dietary component concentration in faeces (gkg^{-1}), ID the indicator concentration in the assay diet (gkg^{-1}), DC_D the dietary component concentration in the assay diet (gkg^{-1}) and IF the indicator concentration in faeces (gkg^{-1}).

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Tukeys multiple range test ($p<0.05$) using the SPSS Version 15. Values are expressed as means and S.E.M.

Results

Composition of experimental diets

The proximate composition of feed ingredients and experimental diets are presented in Table 1. Fermentation of *P.juliflora* pods resulted in significant increase ($p<0.05$) in the levels of proteins and lipids along with the improvement in reducing sugar contents. The fiber content of *P.juliflora* pods was observed to be high (22.40%); and not influenced by fermentation (22.24%). In fermented *P.juliflora* pods, phytic acid was completely eliminated; though significant changes in saponins, tannins and total phenol was not detected. The minerals Ca, Fe, Mg and K were found to be significantly higher ($p<0.05$) in fermented *P.juliflora* pods as compared to crude *P.juliflora* pods. In RD, the gross energy and total lipid were comparatively higher, and fiber was lower as compared to test diets; and in PJPF, the gross energy was slightly lower and nitrogen was somewhat higher among the diet groups (Table 2). The survival rates of lactic cultures *L. acidophilus*, *L.bulgaricus* and *S.thermophilus* in *P.juliflora* pods based diet after drying (0 day) and after 7 days of storage (at 4°C) were observed as 4.69×10^7, 3.61×10^7 and 2.23×10^7 as well as 2.33×10^7, 1.71×10^7 and 0.95×10^7, respectively. The percent survival rate of *L. acidophilus*, *L.bulgaricus* and *S.thermophilus* has been calculated as 49%, 47% and 42%, respectively.

Fish growth and feed utilization

During experiment, rohu fingerlings consumed the feed actively and no mortality was observed. The values of growth performance, feed utilization and digestibility are presented in Table 3. The rohu fingerlings fed with PJPF exhibited significantly higher ($p<0.05$)

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growth and higher feed efficiency in terms of weight gain (325.63±5.44), specific growth rate (SGR) (2.36±0.02), feed conversion ratio (FCR) (1.07±0.01) and protein efficiency ratio (PER) (2.37±0.02) as compared to PJPC; and these values are comparable to those in RD (Table 3). When fish were fed with probiotic supplemented diets, significantly higher (p<0.05) weight gain, SGR, FCR and PER were observed with *L. acidophilus* (PJPLA) as compared to the diet without probiotics (PJPC), and also as compared to diets supplemented with *L.bulgaricus* (PJPLB) and *S.thermophilus* (PJPST) (Table 3). The digestibility of protein, lipid and energy were identical in all dietary treatment groups (Table 3).

Carcass composition and gut digestive enzyme activity

Fish fed with PJPLA (15.90±0.11) and PJPF (16.03±0.11) had significantly higher (p<0.05) carcass crude protein as compared to RD (14.92±0.11) and other experimental diets (Table 4). No clear changes in carcass lipid, moisture and ash as well as in gut α-amylase, protease and lipase activities were detected in different diet groups.

Microbiological analysis of fish gut, fecal matter and aquarium water

In gut homogenate (Fig. 1), LA supplementation (PJPLA) resulted in significant decrease (p<0.05) in coliform counts (3.90±0.12 $CFUg^{-1}$) as compared to groups without probiotic supplementation (PJPC=4.84±0.09; PJPFLA=4.80±0.22; RD=5.14±0.09 $CFUg^{-1}$); and in all the probiotic supplemented groups, complete elimination of vibrios, significant decrease (p<0.05) in fecal streptococci and increase in the total viable count were detected. When the fecal matter was subjected to microbiological analysis, total viable count, total coliform count, fecal streptococci count and presumptive vibrios count were found to be significantly lower (p<0.05) in the *L. acidophilus* supplemented group as compared to those in the group without probiotic supplementation (PJPF, RD) (Fig. 2). The water quality parameters, pH, DO and TOC, during the experiment were found to be 6.9-7.4, 6.5-7.6 mgL^{-1} and 110-115 mgL^{-1}, respectively. In the tank water of probiotic supplemented groups, vibrios were not detected; and total coliforms (PJPLA=110 MPN $index100^{-1}$ mL^{-1}; PJPLB=540 MPN $index100^{-1}$ mL^{-1}; PJPST=540 MPN $index100^{-1}$ mL^{-1}) as well as fecal streptococci count (PJPLA=22 MPN $index100^{-1}$ mL^{-1}; PJPLB=11 MPN $index100^{-1}$ mL^{-1}; PJPST=17 MPN $index100^{-1}$ mL^{-1}) were decreased (Table 5).

The L. acidophilus, L.bulgaricus and S.thermophilusin gut, fecal matter and tank water

With diets PJPLA, PJPLB and PJPST, a colonization of lactic cultures in gut was detected at the end of the 60 days feeding experiment as compared to non-detectable level at the start of the experiment. The count of *L. acidophilus*, *L.bulgaricus* and *S.thermophilus* were measured as 0.76×10^5 $CFUg^{-1}$, 0.53×10^5 $CFUg^{-1}$ and

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0.39×10^5 $CFUg^{-1}$, respectively in gut; 0.23×10^4 $CFUg^{-1}$, 0.35×10^4 $CFUg^{-1}$ and 0.42×10^4 $CFUg^{-1}$, respectively in fecal matter; and 0.21×10^3 $CFUmL^{-1}$, 0.31×10^3 $CFUmL^{-1}$ and 0.18×10^3 $CFUmL^{-1}$, respectively in tank water.

Table 1: Proximate composition of diet ingredients and antinutritional factors of treated and untreated *Prosopis juliflora* pod meal (as % dry matter unless otherwise stated).

Components	Fish meal	Maize gluten	Rice bran	Unfermented *Prosopisjuliflora**	Fermented *Prosopisjuliflora**	S.E.M.	*P* value
Dry matter	96.47	91.39	91.1	90.68	90.77	6.38	0.997
Crude protein	60.37	63.18	13.98	12.05	15.08	0.18	<0.05
Crude lipid	10.08	7.86	10.32	3.20	4.15	0.16	<0.05
Ash	15.58	3.88	8.5	4.76	4.82	0.17	0.988
Total dietary fiber	2.95	2.15	7.01	22.40	22.24	0.26	0.906
Reducing sugar			5.20	5.60	6.25	0.03	<0.05
NFE[a]	7.49	11.16	56.44	50.32	46.69	-	-
Gross energy ($KJgm^{-1}$)	19.07	19.79	14.22	14.46	15.59	-	-
Antinutritional factors							
Total phenols				0.582	0.598	0.02	0.850
Tannins				0.973	0.958	0.01	0.797
Phytic acids				0.188	ND	0.05	<0.05
Saponin				0.393	0.385	0.02	0.978
Trypsin inhibitor ($TIUgm^{-1}$)				ND	ND	-	-
Minerals ($mgkg^{-1}$)							
Calcium(Ca)				2718.4	3265.3	1.16	<0.05
Iron(Fe)				289.98	1007.5	1.57	<0.05
Magnesium(Mg)				1189.4	1261.2	0.98	<0.05
Potassium(K)				16936	18893	2.04	<0.05

[a] Nitrogen free extract calculated as dried matter bases100 – (moisture+crudeprotein+crude lipid+ ash+crude fiber) %
ND=not detected.
*S.E.M. and P values are shown for unfermented and fermented PJPs. (n=3)

Table 2: Ingredient and proximate composition of reference diet and *Prosopis juliflora* pods based experimental diets.

	Reference diet	Crude *Prosopisjuliflora* pods	Live cells supplemented diets			Fermented
	RD	PJPC	PJPLA	PJPLB	PJPST	PJPF
Ingredient composition (% dry weight)						
Fish meal	50.0	50.0	50.0	50.0	50.0	50.0
Prosopisjuliflora	-	33.4	33.4	33.4	33.4	33.4
Maize gluten [a]	08.0	08.0	08.0	08.0	08.0	08.0
Rice bran	33.4	-	-	-	-	-
Mineral Premix[b]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin Premix[c]	1.5	1.5	1.5	1.5	1.5	1.5
Oil pre mix[d]	5.0	5.0	5.0	5.0	5.0	5.0
Bentonite[e]	0.1	0.1	0.1	0.1	0.1	0.1
Cr_2O_3[f]	1.0	1.0	1.0	1.0	1.0	1.0
Proximate Composition (% dry matter)						
Crude protein	39.85	39.25	39.25	39.25	39.25	40.26
Lipid	9.10	6.72	6.72	6.72	6.72	7.04
Ash	10.93	9.68	9.68	9.68	9.68	9.70
Total dietary fiber	3.98	9.12	9.12	9.12	9.12	9.06
NFE[g]	25.0	23.8	23.2	23.5	23.7	22.6
Gross energy ($KJgm^{-1}$)	16.09	15.84	15.84	15.84	15.84	15.30

[a]Maize gluten and Rice bran were purchased form Charotar Animal Feeds Pvt. Ltd., GIDC, V.V.Nagar, Gujarat, India.
[b] Mineral premix (mg or g kg^{-1} diet): KI (1%), 75 mg; $CoCl_2{\cdot}6H_2O$ (1%), 30 mg; $CuSO_4{\cdot}5H_2O$, 75 mg; $FeSO_4{\cdot}H_2O$, 750 mg; $ZnSO_4{\cdot}H_2O$, 1.57 g; $MnSO_4{\cdot}H_2O$, 80 mg; $Na_2SeO_3{\cdot}5\ H_2O$,45 mg; $MgSO_4{\cdot}7H_2O$, 3000 mg; Molybdenum, 0.15 mg.
[c] Vitamin premix (mg or IU kg^{-1} diet): thiamin, 6 mg; riboflavin, 16 mg; pyridoxine HCL, 9 mg; vitamin B12, 0.44 mg; vitamin K, 0.8 mg; pantothenic acid, 40 mg; niacin 100 mg; folic acid, 0.4 mg, biotin, 0.02 mg; retinol acetate, 24000 IU; Vitamin E, 80 mg; Vitamin D3, 5000 IU.
[d] Oil Pre mix [2 Corn oil (Tirupati active, N.K.Proteins Co., Mehsana, Gujarat, India) : 1 Cod liver oil (Seacod, Universal Medicare Pvt. Ltd., Mumbai, India)].
[e]Bentonite purchased from Gujarat Minechem, Bhavnagar, Gujarat, India.
[f] Chromic Oxide (Cr_2O_3): Qualigens®, Mumbai, India.
[g] Nitrogen-free extract
RD – Reference diet, PJPC – Prosopis control diet, PJPLA– Prosopis pod meal with *Lactobacillus acidophilus*, PJPLB – Prosopis pod meal with *Lactobacillus bulgaricus009*, PJPST – Prosopis pod meal with *Streptococcus thermophilus*, PJPF – Prosopis pod meal fermented with *Lactobacillus acidophilus*

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Table 3: Growth performance, feed utilization efficiencies and apparent digestibility in *Labeo rohita* fingerlings fed experimental diets for 60-days[a].

Parameters	RD	PJPC	PJPLA	PJPLB	PJPST	PJPF	S.E.M	*p* value
Initial body weight(g)	3.97	3.87	3.51	3.94	3.94	3.59	0.05	0.693
Final body weight(g)	14.54^{w}	12.07^{x}	14.16^{zw}	13.23^{yz}	13.05^{y}	14.50^{w}	0.11	<0.05
Weight gain (%)	279.93^{yz}	225.72^{x}	313.44^{z}	244.55^{xy}	241.47^{xy}	325.63^{z}	5.44	<0.05
SGR	2.19^{yz}	1.93^{x}	2.33^{z}	2.04^{xy}	2.02^{xy}	2.36^{z}	0.02	<0.05
FCR	1.15^{xy}	1.31^{z}	1.10^{x}	1.21^{yz}	1.23^{z}	1.07^{x}	0.01	<0.05
PER	2.21^{yz}	1.96^{x}	2.35^{z}	2.16^{y}	2.12^{xy}	2.37^{z}	0.02	<0.05
Feed intake	191.35	173.66	181.43	179.65	178.75	185.23	0.05	0.173
Coefficients of total tract apparent digestibility (CTTD)[b]								
Dry matter	0.80	0.76	0.77	0.76	0.76	0.78		
Protein	0.90	0.89	0.90	0.90	0.90	0.90		
Lipid	0.88	0.86	0.87	0.87	0.87	0.87		
Energy	0.88	0.86	0.87	0.87	0.87	0.87		
Survival (%)	100	100	100	100	100	100		

[a]Means value with the same superscript letters in the same row is not significantly different (p>0.05) from each other.
[b]Statistical analysis was not possible as determinations were performed on pooled samples.
RD – Reference diet, PJPC – Prosopis control diet, PJPLA– Prosopis pod meal with *Lactobacillus acidophilus*, PJPLB – Prosopis pod meal with *Lactobacillus bulgaricus009*, PJPST – Prosopis pod meal with *Streptococcus thermophilus*, PJPF – Prosopis pod meal fermented with *Lactobacillus acidophilus*

Table 4: Proximate carcass composition (% wet weight) and digestive enzyme activity of the experimental fishes at the end of the 60-day feeding experiment[a].

Parameters	RD	PJPC	PJPLA	PJPLB	PJPST	PJPF	S.E.M.	*p* value
Carcass composition								
Moisture	73.32	73.75	73.13	73.35	73.47	73.10	1.90	1.000
Protein	14.92^{x}	14.87^{x}	15.90^{y}	14.98^{x}	14.95^{x}	16.03^{y}	0.11	<0.05
Lipid	4.45	4.40	4.75	4.50	4.45	4.81	0.05	0.958
Ash	3.08	3.15	2.93	2.98	3.03	2.90	0.07	1.000
Digestive enzymes activity								
α-Amylase[b]	2.10	2.04	2.23	2.15	2.12	2.26	0.05	1.000
Protease[b]	0.48	0.45	0.50	0.48	0.46	0.49	0.008	0.052
Lipase[b]	5.15	5.10	5.22	5.18	5.15	5.26	0.04	0.292

[a] Means value with the same superscript letters in the same row is not significantly different (p>0.05) from each other.
[b]α-Amylase = mg maltose liberated/h/mg protein; Protease = mg tyrosine liberated/h/mg protein; Lipase = U/mg protein
RD – Reference diet, PJPC – Prosopis control diet, PJPLA– Prosopis pod meal with *Lactobacillus acidophilus*, PJPLB – Prosopis pod meal with *Lactobacillus bulgaricus009*, PJPST – Prosopis pod meal with *Streptococcus thermophilus*, PJPF – Prosopis pod meal fermented with *Lactobacillus acidophilus*

Table 5: Microbiological analysis of aquarium water sample.

Aquarium	No. of coliforms (MPN index100mL^{-1})[b]	No. of fecal streptococci (MPN index 100mL^{-1})[b]	Total viable count (CFUmL^{-1})[a]	Presumptive *Vibrio* sp. (CFUmL^{-1})[a]
RD	>1600	49	$0.23^{x} \times 10^{5}$	165^{x}
PJPC	>1600	26	$0.34^{y} \times 10^{5}$	148^{y}
PJPLA	110	22	$0.49^{z} \times 10^{5}$	ND
PJPLB	540	11	$0.54^{z} \times 10^{5}$	ND
PJPST	540	17	$0.45^{yz} \times 10^{5}$	ND
PJPF	>1600	27	$0.37^{y} \times 10^{5}$	138^{z}

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Table 5 continued:		
S.E.M.	0.02	1.82
p value	<0.05	<0.05

ND=not detected

[a] Means value with the same superscript letters in the same column is not significantly different ($p>0.05$) from each other.

[b] The statistics is not required as the data suggest most probable numbers (MPN method).

RD – Reference diet, PJPC – Prosopis control diet, PJPLA– Prosopis pod meal with *Lactobacillus acidophilus,* PJPLB – Prosopis pod meal with *Lactobacillus bulgaricus009,* PJPST – Prosopis pod meal with *Streptococcus thermophilus,* PJPF – Prosopis pod meal fermented with *Lactobacillus acidophilus*

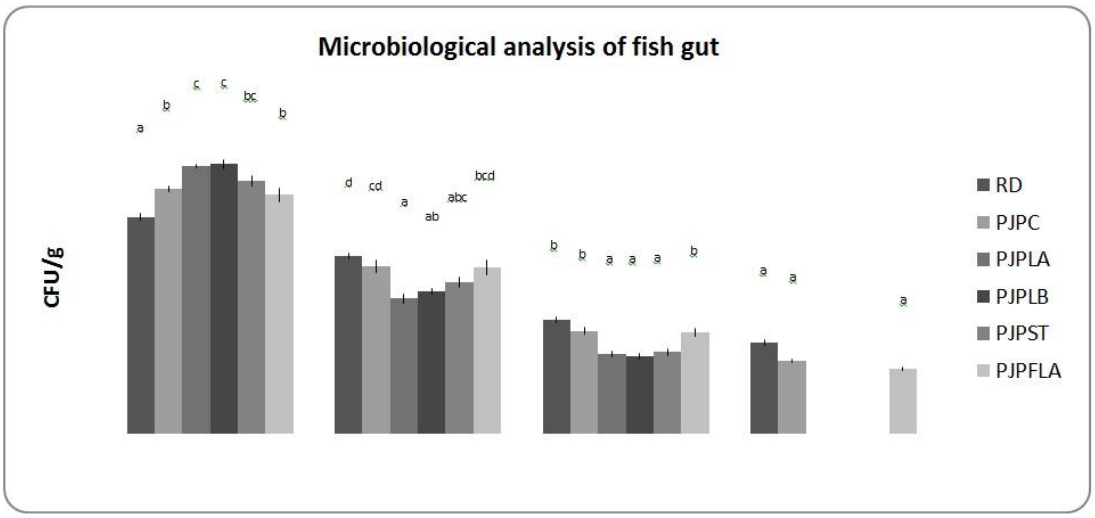

Figure 1: Microbiological analysis of fish gut. [Means within a column with different superscripts are significantly different ($p<0.05$)].

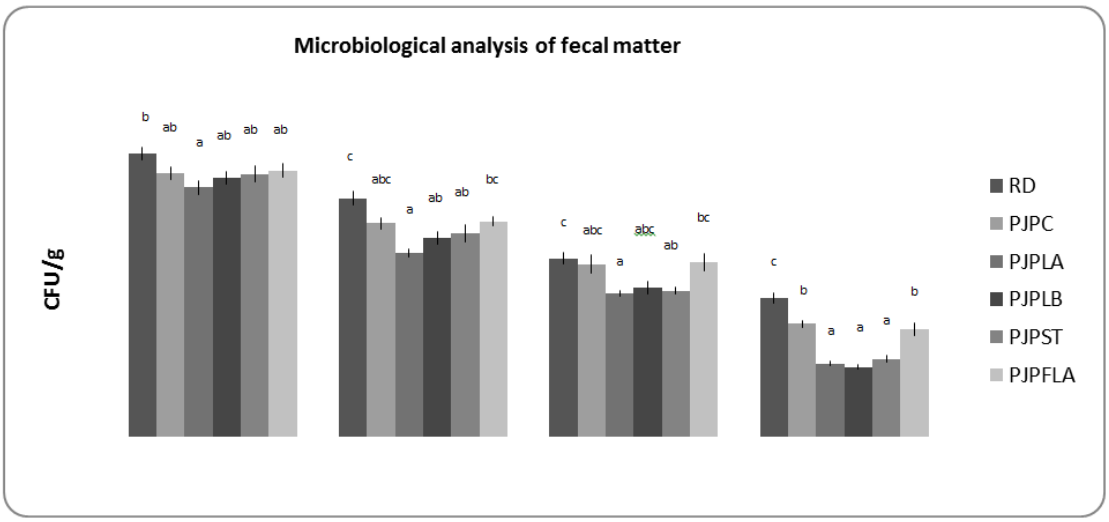

Figure 2: Microbiological analysis of fecal matter. [Means within a column with different superscripts are significantly different ($p<0.05$)].

RD – Reference diet, PJPC – Prosopis control diet, PJPLA– Prosopis pod meal with *Lactobacillus acidophilus,* PJPLB – Prosopis pod meal with *Lactobacillus bulgaricus009,* PJPST – Prosopis pod meal with *Streptococcus thermophilus,* PJPFLA – Prosopis pod meal fermented with *Lactobacillus acidophilus*

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Discussion

The fermentation of PJPs with *L. acidophilus* improved its protein, lipid and mineral contents; and totally eliminated the phytic acid. Moreover, the *P. juliflora* pods based diet, supplemented with *L. acidophilus* as live cells, improved the microflora in the gut, fecal matter and tank. Both of these resulted in higher growth and better carcass quality of fish.

The major carbohydrate sources reported to be used in carp feed as dietary sources of energy are maize, sorghum, wheat, rice and barley (Belal, 1999). The *P. juliflora* pod is a cheaper agro-based unutilized source available in plenty in many parts of the world. The presently used *P. juliflora* pods are found to be rich in protein (12.05%), gross energy (14.46 KJg^{-1}), reducing sugar (5.60%), Ca (2718.4 $mgkg^{-1}$), Mg (1189.4 $mgkg^{-1}$) and K (16936 $mgkg^{-1}$); pods are free from trypsine inhibitor; and they are also found tocontain antinutritional factors like total phenol (0.582%), tannins (0.973%), saponins (0.393%) and fibers (22.40%) (Table 1). These values are comparable to the pod values reported by several investigators as 7 to 22 % protein, 30 to 75 % carbohydrate, 11 to 35 % crude fibers, 1 to 6 % fat and 3 to 6 % ash (Choge *et al*., 2007). The nutritive quality of pods is found to be comparable to several other carbohydrate sources being investigated in the fish diets (Belal, 1999; Skrede *et al*., 2002). Moreover, the carbohydrate contents, mainly sucrose and glucose, are reported to be higher in *P. juliflora* pods as compared to barley and wheat (Skrede *et al*., 2002; Choge *et al*., 2007).

In the present experiment, fermentation of *P. juliflora* pods with *L. acidophilus* resulted in significant increase ($p<0.05$) in protein, lipid content and reducing sugar; it resulted in complete elimination ($p<0.05$) of phytic acid and increase ($p<0.05$) in the levels of minerals Ca, Fe, Mg and K; however, the fermentation did not reduce the level of total dietary fibers, tannin and saponin in pods (Table 1). The nutritional quality of several plant based feeds like sesame seed meal, soybean white flakes, grains, leaf meals and lathyrus seed meal are known to have improved after fermentation with beneficial bacterial strains for the diet of cultivable fish species (Mukhopadhyay and Ray, 1999; Bairagi *et al*., 2002, 2004; Ramachandran *et al*., 2005). Presently observed increase ($p<0.05$) in protein reflect the active protein metabolism by the lactobacilli; and the simultaneous increase ($p<0.05$) in lipid in fermented pods is in agreement with increase in the nutrient level through microbial synthesis during fermentation of plant feed as reported by Wee (1991). Lactic acid fermentation is known to improve the protein and lipid contents in sesame seed meal (Mukhopadhyay and Ray, 1999); and it has altered the peptide composition in soybean white flakes (Refstie *et al*., 2005). In the fermented *P. juliflora* pods, with complete elimination of phytic acid, increase ($p<0.05$) in the levels of minerals indicates effectiveness of lactic culture in the elimination of phytic acid,

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possibly by bacterial phytase production. In monogastric animals, phytic acid was reported to act as chelater and it reduces the availability of minerals and proteins (Skrede *et al*., 2002; Ramachandran *et al*., 2005). Lactic acid fermentation has been reported to increase the levels of glucose, fructose and maltose as well as to lower the levels of sucrose and raffinose in wheat flour; and it is found to reduce non starch carbohydrates in barley whole meal (Skrede *et al*., 2002). The change in the sugar composition of fermented *P. juliflora* pods suggests that lactic culture has metabolized carbohydrates, possibly sucrose. This is possibly because sucrose is known to be the main soluble sugar content of pods (Choge *et al*., 2007); and lactic cultures are known to ferment sucrose actively (Refstie *et al*., 2005). A considerable decrease in fiber and tannin has been reported in leaf meal and grass pea seed meal when fermented with Bacillus (Bairagi *et al*., 2002; 2004). Though presently used lactic acid fermentation has not reduced the level of total dietary fibers, tannin and saponin in pods, the 3.2 gkg^{-1} of tannin and 1.2 gkg^{-1} of saponin in the presently formulated test diets may not have affected the fish growth because test diets with 2.3 to 4.0 gkg^{-1} of tannin and 0.7 to 1.2 gkg^{-1} of saponin were reported to not cause any negative effect on growth and feed utilization in carp (Hossain *et al*., 2001).

The growth performance and feed efficiency in terms of weight gain, SGR, FCR and PER were significantly higher (p<0.05) with fermented *P. juliflora* pods as compared to crude *P. juliflora* pods; the feed intake was found to be similar among experimental groups; and the above values were quite comparable to the RD. Incorporation of pods in animal feed has shown promising results; and has exhibited improvement in dry matter intake, weight gain and feed conversion with decrease in feed cost in livestock (Rao and Reddy, 1983; Habit and Saavedra, 1988; Mahgoub *et al*., 2005). Growth obtained for rohu with fermented *P. juliflora* pods during the present investigation is found to be better than with several other fermented feed like leucaena and duckweed leaf meal (Bairagi *et al*., 2002, 2004) as well as sesame seed meal (Mukhopadhyay and Ray, 1999), grass pea meal (Ramachandran *et al*., 2005) and black gram seed meal (Ramachandran and Ray, 2007). The higher growth and better feed efficiency of rohu fed with fermented PJP appear to be due to improvement in the nutritional quality of fermented pods. At the same time, though the fiber contents of crude and fermented pods are higher as compared to rice bran, the total fiber contents of formulated experimental diets ($90gkg^{-1}$) arein permissible limit for carp as reported by Erfanullah and Jafari (1998). The nutrient digestibility of *P. juliflora* pods based diets indicates that the dry matter digestibility, nutrient digestibility and energy digestibility are in the same range as in the control diets. This indicates that the pods are as digestible as rice bran for rohu fingerlings; and the digestibility of fermented pods is found to be better as

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compared to the digestibility of fermented soybean meal and cereals in the diet of cultivable fish species as reported by Skrede *et al*. (2002), Ramachandran *et al*. (2005) and Refstie *et al*. (2005).

The diet with fermented *P. juliflora* pods produced significantly higher (p<0.05) protein level in carcass. At the same time, it did not cause any significant changes in carcass moisture, lipid and ash as well as in digestive enzyme activities α-amylase, protease and lipase in different groups of fishes (Table 4). This indicates that *P. juliflora* pods as a carbohydrate source did not cause any negative effects on the digestive enzyme activities and carcass composition of fishes. The significant increase in carcass protein in PJPF group is in agreement with Mukhopadhyay and Ray (1999), Bairagi *et al*. (2002) and Ramachandran & Ray (2007). The significant increase in carcass protein is likely to be due to the protein sparing effect of the presently used fermented pods (Wilson, 1994; Erfanullah and Jafri, 1998).

Use of probiotics in feed resulted in complete elimination of vibrios in gut and tank water; and decrease in the count of other pathogenic indicator organisms in gut, fecal matter and tank water (Figs. 1, 2 and Table 5). This indicates the antagonism of pathogenic bacteria and effectiveness of the presently used probiotic cultures in improving fish microbiology, especially with *L. acidophilus*. This improvement in microflora is likely to have been achieved by the production of bacteriocins, organic acids and hydrogen peroxide by probiotic cultures; and/or by competing for essential nutrients as suggested by Fuller (1989). The improvements in growth, feed efficiency and carcass quality with probiotic supplemented feed appear to be related with the improvements in the intestinal microflora of fish as well as the tank microflora. Probiotic supplementation in the diet has been reported to improve the intestinal microflora by enhancing beneficial microorganisms (Suzer *et al*., 2008) and by reducing the pathogenic problems in intestine (Fuller, 1989; Yanbo and Zirong, 2006). In a similar study, Ahilan *et al*., (2004) observed retardation of coliforms by lactobacilli in juvenile gold fish; and probiotic *Halomonas* sp. B_{12} (isolated from the intestine of *Fenneropenaeus chinensis*) was observed to be very effective in reducing the intestinal vibrios count in shrimps (Zhang *et al*., 2009). Recently in aquaculture, use of probiotics to enhance the growth performance of cultivable species and to improve the water quality has received attention (Al-Dohail *et al*., 2009). Moreover, decrease in the antinutritional factors in *L. acidophilus* supplemented feed during the present investigation could also be one of the reasons for the improvement in the growth of the fish because the beneficial microflora have been reported to pre-digest the antinutritonal factors present in the diet (El-Haroun *et al*., 2006; Suzer *et al*., 2008). Incorporation of probiotics in the diet has been reported to improve growth in common carp (Yanbo and Zirong, 2006), nile tilapia (El-Haroun *et*

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al., 2006), shrimps (Venkat *et al*., 2004) and in rohu (Ghosh *et al*., 2003).

In the present study, the viable probiotic count was maintained at about 10^7 cellsg^{-1} of feed during storage at 4°C. Moreover, among the lactic cultures, the survival rate of *L. acidophilus* in *P. juliflora* pods based diet was found to be higher after drying and on the 7th day of storage. Viability of probiotic cultures in the diet before consumption is a very important aspect for its beneficial effects (Havenaar *et al*., 1992). The presently observed level of lactic acid bacteria appears to be sufficient to give probiotic effects as reported by Nousiainen and Setala (1993).

The present investigation suggests that *P. juliflora* pods can be used in the diet of *L.rohita* (rohu) fingerlings as a carbohydrate source after fermentation with *Lacto bacilus acidophilus*. The lactic acid fermentation improves the nutritional value of pods resulting in better growth and carcass composition of rohu. The *L. acidophilus* as live cell supplement to the *P. juliflora* pods based diet also improves the growth and carcass composition of rohu by improving the gut as well as tank microflora.

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