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Cirrhinus mrigala growth and health management: application of Lactobacillus acidophilus probiotic supplementation

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KEYWORDS

ABSTRACT

Labeo calbasu, Cyprinus carpio, Cirrhinus mrigala, Catla catla, and Labeo rohita are the major Indian carps and are widely consumed throughout India. Cirrhinus mrigala is vulnerable to bacterial infection in various ecological niches. They are easily infected by common bacterial pathogens, viz., Pseudomonas fluorescens and Aeromonas hydrophila. The survey revealed its occurrence in most of the waterbodies in Maharashtra, India. Pseudomonas fluorescens and Aeromonas hydrophila are known as opportunistic pathogens and have been associated with mass mortality incidences across the world. Opportunistic infections like Pseudomonas fluorescens and Aeromonas hydrophila are known to cause mass deaths all over the world. Antibiotics are frequently used by fish producers to combat these illness outbreaks. The rise of resistant pathogens and frequent treatment failures are nevertheless the results of preventive abuse. Probiotic lactic acid bacteria are being utilised more frequently in aquaculture to enhance the quality of seed production while reducing the need for antibiotics.

Twenty-seven isolates of lactic acid bacteria were isolated from different sources. The primary screening comprised the in-vitro inhibition of pathogenic Pseudomonas fluorescens and Aeromonas hydrophila isolates. Selected isolates of lactic acid bacteria with antagonistic properties were further subjected to biofilm formation. The strains were characterised morphologically and by biochemical tests. In-vivo experiments were undertaken with Pseudomonas fluorescens and Aeromonas hydrophila isolates on fingerlings of Cirrhinus mrigala. Lactobacillus acidophilus T22 was thus selected based on its attributes as a probiotic. The strain showed antagonistic activity, adhered strongly to polystyrene plates, and secured Cirrhinus mrigala with the best protection against Pseudomonas fluorescens and Aeromonas hydrophila isolates.

The lactic acid bacteria L. acidophillus T22 modified the profile of the bacterial community associated with fish larvae compared to the control group and reduced the bacterial load in fish larvae of Cirrhinus mrigala. The fish larvae's body bulk and length rose as a result of the probiotic treatment. The management of oxidative stress by L. acidophilus T22 appeared to be more effective due to two physiological markers for gene expression.

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Introduction

Cirrhinus mrigala is indigenous to Indo-gangetic river systems, Mrigal (Cirrhinus mrigala) is one of the three main Indian carp species commonly cultivated in Southeast Asia. In polyculture with other indigenous species, mainly in India, this species has long been significant. But only from the early part of the 20th century are archives of its culture available. The initially higher mrigal growth rate combined with its compatibility with others helped make it one of the main component species in pond culture. The species was transplanted and founded in Indian peninsular riverine systems. This extended to the entire of India afterwards.

In both wild and cultivated fish, bacteria are one of the main causative agents of fish diseases and are responsible for significant economic losses. Freshwater and marine fish are affected by very few pathogens. In specific flexibacteria, aeromonads and vibrios, several pathogens may present as only skin infections. Ulcer, hemorrhage, scale reduction, tail and fin rot, dropsy involves many bacterial diseases, are present in fishes. The bacteria of the genus Aeromonas, Pseudomonas, are ubiquitous facultative parasites, where only under unfavorable circumstances, possible pathogenicity becomes threatening for fish. They belong to natural aquarium bacterial flora, hatchery water, fish farms and domestic bodies of water, in which the appearance of the colonization of these bacteria occurs in fish skin fins, gills and intestinal lumens. Most pathogenic fish bacteria can reside in or on/in apparently normal fishes in the environment. Infections are therefore also precipitated by stress that disturbs the normal defenses against the agents (e.g. overcrowding, low DO, high ammonia).

They have received increasing attention and represent a major concern for global aquaculture. To deal with bacterial diseases, fish farmers most often resort to the use of antibiotics (Nikaido, 2009). These massive uses quickly led to the appearance of resistant bacteria that are difficult to treat. They also pose a threat directly for human health and the environment. The risk of selecting strains resistant pathogens therefore encourages limiting the use of antibiotics.

Following the limitations use of antibiotics, numerous studies report trials of the use of probiotics in aquaculture (Irianto and Austin, 2002). Indeed, the use of treatments microbial probiotics improve the quality of the fry produced. Probiotics offered in aquaculture mainly belong either to the group of lactic acid bacteria or to the genus Bacillus. These probiotics are found in commercial form and are introduced as additives in the feed of farmed fish, crustaceans and molluscs (Moriarty, 1998; Wang Y et al., 2005; Castex et al., 2009). Currently, lactic acid bacteria which benefit from GRAS (Generally Recognized status As Safe) are the most used probiotic microorganisms in aquaculture (Gatesoupe et al., 2013; Merrifield et al., 2011; Picchietti et al., 2008). In fact, lactic acid bacteria are often used with the aim of stabilizing the intestinal flora by notably reducing the development of pathogenic flora while promoting lactic flora. These bacteria can also improve nutrient digestion, growth and immune system of fish (Martínez et al., 2012; Tapia-Paniagua et al., 2012).

The in vitro selection of lactic acid bacteria with an antagonistic effect against strains of Pseudomonas fluorescens and Aeromonas hydrophila were studied. The most suitable isolates influencing growth and survival of Cirrhinus mrigala was further implicated in the study.

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Material and Methods:

Isolation and identification of bacterial strains

For the bacterial diversity and bacterial load, the bacterial parameters of the widely cultivated and consumed fish Cirrhinus mrigala have been evaluated and compared for both cultivated and natural systems. The fishes were retrieved with the help of fishing net whereas the samples of water were collected in sterile bottles and polythene bags. The isolation of heterotrophic, aerobic and anaerobic bacterial communities in terms of cfu /ml were identified and enumerated by the standard methods described by Cheesbrough (1989) and Bergey's Manual for Systematic Bacteriology (1986). All the bacterial isolates used in the study were isolated from naturally infected fishes. These bacterial isolates were used for further experimental works along with other bacteria isolated through the study period. Cultures were preserved by asceptically transferring the bacterial cultures to freshly prepared sterile nutrient agar slants after every three week. The stock cultures were stored at 4°C. For experimental works, subcultures were made from the stock cultures in suitable media before use. The cultures were also examined at regular intervals to test their pathogenicity.

Cultures of Lactobacillus acidophilus

Lactobacillus acidophilus isolates were obtained from Department of Biotechnology, Yeshwant Mahavidyalya, Nanded and were maintained on MRS medium. The bacterial cells used in the following treatment were in logarithmic phase. Bacterial suspension with 4 X 10^5 CFU mL−1 of Lactobacillus acidophilus in sterile saline solution was prepared.

Screening of lactic acid bacteria with antagonistic effect against Pseudomonas fluorescens and Aeromonas hydrophila

Twenty-seven lactic acid bacteria isolates were screened for their antibacterial potential against the Pseudomonas fluorescens and Aeromonas hydrophila . This study was carried out using the well diffusion plate method (Vaseeharan and Ramasamy, 2003). Wells of 5 mm of diameter in the agar, at the rate of four plates per condition and with two repetitions at different times. The plates were spread with a suspension of the pathogenic strain of Pseudomonas fluorescens and Aeromonas hydrophila, and the wells were filled with 80 µL of a fresh culture of probiotic bacteria. After incubation for 4 hours at 27±3°C, the diameters of the inhibition zones appearing around wells were measured.

In vivo effect of probiotic on growth of C. mrigala

Fingerlings of C. mrigala of average weight (1.59±0.02) g were used for the feeding trial experiment. Fingerlings were kept in plastic tubs (HDPE) of 50 L capacity in laboratory conditions where temperature at (26.5±0.05)°C, was maintained. Fishes were acclimatized for 10 d prior to the start of experiment. Chlorine-free tap water was used throughout the experiment.

The experiment was conducted with two treatments, each in three replicates, and each containing 15 fingerlings/plastic tub. In treatment one, fishes were fed on control diet (ingredient composition in kg g–1, ground nut oil cake, 650; rice bran 42; hydrothermically processed soybean 266; wheat flour 32; mineral mixture 10) without probiotic bacterium while for the second treatment the diet was supplemented with probiotic bacterium Lactobacillus acidophilus was mass cultured

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and added to feed in the proportion of 2×106 cells 100 g–1 of feed (see Table 6 for proximate composition).

The feed was spread in the sterile tray and the absorption was achieved by spraying the probiotic bacterium Lactobacillus acidophilus, air dried and finally stored in vacuum sealed plastic containers at 4°C. In both the treatments, the fish

In vivo effect of probiotic on growth of C. mrigala

Fingerlings of C. mrigala of average weight (1.395±0.02) g were used for the feeding trial experiment. Fingerlings were kept in plastic tubs (HDPE) of 20 L capacity in laboratory conditions where temperature at 27±3 °C, was maintained. Fishes were acclimatized for 10 d prior to the start of experiment. Chlorine-free tap water was used throughout the experiment. The experiment was conducted with two treatments, each in three replicates, and each containing 10 fingerlings/plastic tub. In treatment one, fishes were fed on control diet as given in table without and supplemented with probiotic bacterium The culture was mass cultured and added to feed in the proportion of 4×10^5 cells 100 g–1 of feed. The feed was spread in the sterile tray and the absorption was achieved by spraying the probiotic bacterium Lactobacillus acidophilus, air dried and finally stored in vacuum sealed plastic containers at 4°C. In both the treatments, the fishes were fed with respective diets daily at 4% body weight in two instalments at 8:00 and 16:30 h for 90 d. Growth parameters and other analyses were done using standard methods. At the termination of experiment, from each treatment, five fish were randomly sampled and kept on ice to remove the intestines which were processed for the determination of various content (Sadasivam and Manickam 1996).

Apparent protein digestibility (APD) of the diets was calculated according to the methods of Cho et al. (1982). Live weight gain (g), percent weight gain, specific growth rate, feed consumption per day in percentage of body weight, feed conversion ratio (FCR), gross conversion efficiency (GCE), and protein efficiency ratio (GER) were calculated using standard method (Steffens 1989). Gross energy content of the diet and fish carcasses was calculated using the average caloric conversion factor of 0.3954, 0.1715, and 0.2364 kJ g –1 for lipid, carbohydrate, and protein, respectively (Henken et al. 1986), whereas metabolizable energy in diets and feeds was calculated using caloric conversion factors: 0.335, 0.138, and 0.188 kJ g–1 for lipid, carbohydrate, and protein, respectively.

Statistical analysis

Significant differences among treatment groups were tested by using Students t-test (Snedecor and Cochran 1989). Statistical significance was tested at a probability value of $P<0.05$ (More, and Baig, 2013).

Result:

Bacterial diversity of from skin: The predominant bacterial genera isolated from the skin of Cirrhinus mrigala were Micrococcus, Enterobacter, Chromobacterium, Serratia, Corynebacterium, Alcaligens, Pseudomonas, E. coli, Aeromonas, Bacillus, Staphylococcus, but Klebsiella, Clostridium, Flavobacterium, Citrobacter, Shigella, Proteus, Streptococcus, Salmonella, were found only moderately. In morphological and physiological grouping of intestinal microflora of Cirrhinus mrigala, occurrence of Gram-negative rods was around 75% and that of Gram-positive was 25%.

Table 1: Bacteria genera associated with Cirrhinus mrigala

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Bacterial genera	% incidence of colonies isolated Skin
Gram Positive bacteria	
Bacillus	6.46
Clostridium	3.25
Corynebacterium	7.37
Micrococcus	9.06
Staphylococcus	5.07
Streptococcus	1.64
Gram negative bacteria	
Aeromonas	6.81
Alcaligens	7.28
Chromobacterium	8.06
Citrobacter	2.54
E. coli	6.88
Enterobacter	8.89
Flavobacterium	2.77
Klebsiella	3.56
Proteus	1.75
Pseudomonas	7.08
Salmonella	1.47
Serratia	7.58
Shigella	2.50

Antibacterial activity of Lactobacillus acidophilus against Aeromonas hydrophila and Psuedomonas fluorescens: Seven morphologically different bacterial colonies were found as predominant organisms. Of the twenty seven isolates, seven isolates were assayed for their ability to inhibit the growth of target strains Aeromonas hydrophila and Psuedomonas fluorescens. It was found that the inhibitory effects of Lactobacillus isolates were varied with different target strains (from 5.6 to 26 mm diameter). The T22 isolate showed maximum zone of inhibition of 26 mm diameter for both the test pathogens (Figure 1).

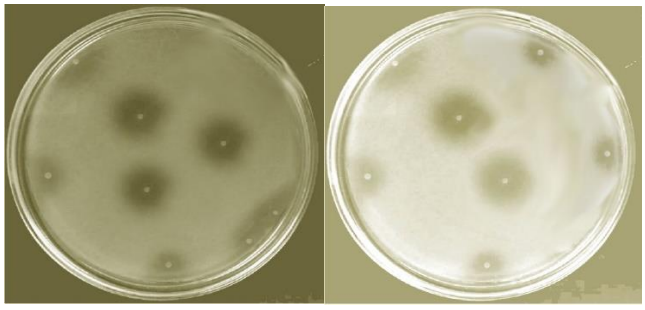

Fig. 1 Inhibition of growth of Aeromonas hydrophila and Psuedomonas fluorescens by L.acidophilus as expressed by Zone of inhibition on nutrient agar plate.

Biochemical composition of feeds: The biochemical compositions of experimental diet is presented in table 2. Crude Protein content of the diets was 32.12±0.05 percent whereas the lipid content was 55.43 ± 0.35. The carbohydrate content was 49. 59±0.42 percent whereas the overall energy of the diet was 383.04±0.53 percent. The probiotic L.acidophilus count at the time of preparation in the diet while preparation was 4 X 10^5. The

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assessment of the diet after 30 day pf preparation revealed that L.acidophilus count after 30 days decreased to 2 X 10^5 indicating nearly fifty percent decrease in the count . The diets prepared were statistically similar to their protein, lipid and carbohydrate content.

Table 2. Composition of diet ingredients of feed.

Ingredients	Composition
Ground nut oil cake	40 gm
Wheat flour	15 gm
Soybean oil cake	15 gm
Rice bran	10 gm
Fish meal	13 gm
Fish oil	5.0 gm
Vitamin mineral mixture	1.0 gm
Carboxymethylcellulose (binder)	1.0 gm
Crude protein	32.12±0.05 %
Crude lipid	5.43 ± 0.35 %
Carbohydrate	49. 59±0.42 %
Ash	14.37±0.21 %
Energy (kcal/100g)	383.04±0.53 %
L.acidophilus count at the time of preparation	4 X 10^5
L.acidophilus count after 30 days	2 X 10^5

Growth, survival and digestibility

The growth of fish in terms of weight gain (g), growth per day in percentage body weight, specific growth rate (SGR) were significantly ($P<0.05$) higher in diet containing probiotics Lactobacillus acidophilus (Table 3). Digestibility parameters, e.g., apparent protein digestibility (APD) and gross conversion efficiency (GCE) were higher in diet containing probiotics Lactobacillus acidophilus containing probiotics at the rate of 2×10^5 cells 100 g–1 of feed whereas significantly ($P<0.05$) lower values (2.89±0.01) of feed conversion ratio (FCR) were observed when compared with values of diet without probiotic (3.13±0.05).

The data on weight gain revealed that, initially up to 15 d, significant ($P>0.05$) variations were not observed in the weight gain of group of fishes which were fed varying dietary treatments. However, an increase in growth rate ($P<0.05$) in the fishes fed on diet with probiotics Lactobacillus acidophilus was evidenced after 15 d and it continued till 90 d.

Table 3 Growth performances and intestinal enzyme activities of C. mrigala fed on diets without and with probiotic bacterium Lactobacillus acidophilus after 90 d feeding trial

Growth parameters1)	Diet	
	Without Lactobacillus	With Lactobacillus
Initial weight (g)	1.395±0.06	1.43±0.09
Final weight (g)	6.115±0.07	6.98±0.02
Live weight gain (g)	3.356±0.01	4.138±0.02
Survival rate (%)	95.4±3.5	95±2.4
Growth gain in BW (%)	224.3±2.79	272.4±1.5
Growth per day in BW (%)	1.17±0.05	1.28±0.02

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Specific growth rate (SGR) (% BW d^{-1})	0.56±0.03	0.63±0.03
Feed conversion ratio (FCR)	3.13±0.05	2.89±0.01
Gross conversion efficiency (GCE)	0.31±0.05	0.34±0.02
Protein efficiency ratio (PER)	0.79±0.01	0.86±0.01
Apparent protein digestibility (APD) (%)	75.7±0.13	79.9±0.30

values in the same line are significantly (P<0.05) two different treatments. All values are means±SE.

Effect of Lactobacillus acidophilus on Carcass composition of C. mrigala

Initial and final carcass composition (g 100 g–1) with respect to proximate nutrients of test fish on basis of feeding trials is shown in Table 3. Crude protein (g 100 g–1) was found to be significantly (P<0.05) higher 16.62±0.31g 100 g–1) and ash 2.63 ±0.13g (without Lactobacillus) and 6.46±0.3g (with Lactobacillus)100 g–1) and crude fat 7.36±0.18g (g 100 g–1) was found to be significantly (P<0.05) lower in fish fed with probiotic treatment. However, no significant variation was found in moisture and gross energy (kJ g–1) between treatments.

Table 4 Proximate carcass composition of fish fed on diet without and with probiotic bacterium Lactobacillus acidophilus after 90 d feeding trial

Proximate composition	Initial value	Without Lactobacillus	With Lactobacillus
Moisture (g 100 g–1)	70.03±0.08	67.6±0.24	66.7±0.43
Crude protein (g 100 g^{-1})	11.32±0.04	13.25±0.21	16.62±0.31
Crude fat (g 100 g–1)	4.12±0.121	7.36±0.18	6.46±0.3
Total ash (g 100 g–1)	2.08±0.221	3.9±0.30	2.63±0.13
Gross energy (kJ g–1) .06	6.72±0.04	8.58±0.10	7.81±0.06

Discussion

The bacterial diversity of the surface of the fish reflects its status before catching. The main bacterial genera in this study have been Alcaligens, Aeromonas, Bacillus, Enterobacter, E. coli, Klebsiella, Pseudomonas, Proteus and Staphylococcus. The above and the Gillespie and MacRae (1975) and Gilmour et al., (1976) studies showed similar types of fish and differed according to the area. Similar and related studies have also been reported on Atlantic salmon's skin in coastal, estuarine and fresh waters by Horsley (1973). Efficient water storage in fish ponds is one of the key factors for fish farming success. However, certain types of bacterial isolates that can function as opportunistic pathogens in the fish body cannot be overlooked. Bacteria such as Aeromonas, Pseudomonas, and Citrobacter are isolated and characterized. E. Coli is known in fish for their pathogenesis. This opinion is backed by many publications (Kumar et al., 1986, Prabhakar et al., 1985, Lesel et al., 1986). This shows the potential role of these isolates as well as other environmental factors in the pathogensis of freshwater fish epizootics. Septicaemical diseases were commonly observed in the Aeromonas, Pseudomonas and Micrococcus fish as secondary bacterial infections; in combination with ulcerative syndrome Aeromonas hydrophila, Acinetobacter and

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Streptococcus were detected in various locations and water bodies in Kerala (Vijayan et al., 2006; Sreedharan et al., 2013).

In order to certify the beneficial effect, a probiotic is expected to have a few specific properties and antagonism to pathogens is one such property. A common way to select probiotics is to perform in vitro antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products in a liquid (Vine et al. 2004) or solid (Dopazo et al. 1988; Chythanya et al. 2002) medium. In the present study, selected strain identified as Lactobacillus acidophilus showed the maximum inhibition (26 mm in diameter) against the pathogenic strain A. hydrophila and P. fluorescens , when performed by well diffusion assay (Fig. 1). Earlier reports confirmed that the Bacillus sp. had the ability to inhibit the pathogenic strains (Brett and Groves Powedchagun et al. 2011). Inhibition zone of diameter 23 and 19 mm by probiotic mixture (Lactobacillus sporogenes, Lactobacillus acidophilus and Saccharomyces cerevisiae) against Streptococcus faecalis and A. hydrophila were reported by Sharma et al. (2013).

The results of present studies suggested that Lactobacillus acidophilus with antibacterial properties might inhibit the growth of invading bacteria in the intestine of C. mrigala. There is no evidence that isolate of Lactobacillus acidophilus has any harmful effect on C. mrigala fingerlings since there was no mortality and a complete absence of any pathology or abnormality in the fingerlings. The non-pathogenicity ensures that it is safe for use as a probiotic. Feeding trials in the present study indicated that the probiotic supplemented diet resulted in significantly ($P<0.05$) high growth performance, increase in digestibility and nutrient retention. Similar results were observed by Ghosh et al. (2007) in Indian carps. Lactobacillus has also been studied for its probiotic action in C. mrigala (Prasad & Rangacharulu, 2015), labeo rohita (Ghosh et al., 2004).). Lactobacillus acidophilus, as a feed additive in the formulated plant protein based diet (40% protein) for C. mrigala, exhibited significantly ($P<0.05$) higher growth performance in terms of live weight gain, growth percent gain, body weight, specific growth rate (SGR) and growth per day. The feed conversion ratio (FCR) was low clearly indicating the better utilization of available feed. Protein efficiency ratio (PER), apparent protein digestibility (APD) was also high in the group fed on Lactobacillus acidophilus supplemented diets. Such improvement in growth and FCR established with the dietary supplementation of gut isolated probiotic may be accredited to positive physiological and biological changes in the gastro-intestinal tract of fish. Daniels et al. (2004) have also reported similar response in European lobster (Homarus gammarus L.). Bhatnagar et al. (2012) isolated Bacillus coagulans strain from the gut of C. catla and used as probiotic supplement. They reported significantly ($P<0.05$) higher growth performance of fish with B. coagulans supplemented diet when compared with control. On the contrary, Shelby et al. (2006) reported that no specific effect on growth response was observed in young tilapia, Oreochromis niloticus, treated with the commercial probiotics. The positive growth promoting responses of probiotic supplemented diet in the present study may be attributed to the reason that probiotic selected was isolated from the gut of the same species whereas Shelby et al. (2006) used commercial probiotics revealing the importance of selection of probiotic. According to Lenard (2009) naturally occurring bacteria in the gut, modulate and stimulate immune system activity by enhancing the intestinal barrier function, and suppressing pathogens. Present results demonstrated the beneficial effect of incorporating gut isolated bacteria since they can form a

persistent population in the gut supporting the incorporation of autochthonous bacterial strains with probiotic properties.

It can be concluded that Lactobacillus acidophilus isolates can impart beneficial growth promoting effects as potential probiotic feed additive. This potential probiotic bacterium can improve feed utilization and also enhance accumulation of carcass proteins. Based on the results, use of probiotics as a feed additive is recommended to stimulate productive performances leading to sustainable aquaculture.

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