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Influence of a fish gut bacterium *Lactobacillus* sp. on the production of swordtail *Xiphophorus helleri* (Heckel 1848)

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Abstract
The influence of a fish gut bacterium *Lactobacillus* sp on the production of swordtail *Xiphophorus helleri* was studied for a period of one year. The *Lactobacillus* sp P21 produced bacteriocin-like inhibitory substance and exhibited wide spectrum of action against *Aeromonas hydrophila*, *Bacillus* spp, *Pseudomonas* spp and *Citrobacter freundi in vitro*. The growth performance of *X. helleri* reared in the presence of *Lactobacillus* P21 at 10^6 /ml rearing water was better than the control. The total plate counts, total MRS agar counts and the counts of motile aeromonads, presumptive pseudomonads, lactose fermenters and lactose non-fermenters in the gut of probiotic group were comparatively low than the control. On day 60 the count of *Lactobacillus* sp P21 was observed to be log 5.28/g in the gut of *X. helleri* indicating colonization of this bacterium in the gastrointestinal tract. The fecundity of *X. helleri* was in the range of 9–134. On average, it produced from 39.42±18.72 fry/female in control group to 53.00±23.57 fry/female in probiotic group. The increase in average fecundity in probiotic group over the control group was about 25%. There existed significant difference between probiotic group and control in respect of average fecundity/female ($p<0.02$), average number of fry survived /female ($p<0.006$) and average number of fry dead/female ($p<0.029$). The results of the present study demonstrated that the rearing of *X. helleri* in probiotic-enriched water have growth inducing ability and favourably influenced the reproductive performance in terms of high fecundity, high fry survival, reduced fry mortality and reduced fry deformity.

Keywords: Probiotic, *Xiphophorus helleri*, *Lactobacillus* sp, Colonization

Introduction

Antibacterial substances are produced by aquatic bacteria isolated from various sources, and seem to play an important role in the antagonism of bacteria in aquatic ecosystems (Dopazo *et al* 1988). In addition, intestinal bacteria from both freshwater and marine fishes are reported to show inhibitory effect in fish pathogenic bacteria (Westerdahl *et al.* 1994, Sugita *et al.* 1996). If these bacteria are members of indigenous microflora, they

could persist for a relatively long-term and might inhibit the establishment of fish pathogens in the fish intestine. These intestinal bacteria are continuously excreted from host fish into culture water and significantly influence the microflora in the culture water. This suggests that antibiotic producing bacteria, which establish in the intestines, might be suitable biocontrol agents or as probiotics in aquaculture systems. The present study reports the influence of a fish gut bacterium, *Lactobacillus* sp on the production of swordtail *Xiphophorus helleri.*

Materials and methods

Experimental fish, probiotic bacterial strain and opportunistic fish pathogens

The swordtail, *Xiphophorus helleri* Heckel, 1848 of weight 0.21–0.58 g and length 24.7 – 38.3 mm were procured from commercial fish breeders of Amtala, 24 Parganas (South) district, West Bengal, India as and when required. The fishes, on receipt, were disinfected by placing in 5 ppm $KMnO_4$ solution up to 15 min and transferred to circular FRP tanks of 500-litre capacity containing filtered bore-well water. The dead and weak fishes were removed immediately and the healthy ones were stocked at 100 numbers/tank, which was aerated continuously. The fishes were fed with commercial fish feed (crude protein (Min 41%), crude fat (Min 6%), crude fiber (Min 3%) and moisture (Max 11%) on demand twice daily. All the fishes were maintained in such condition for at least 10 days prior to experimentation. The wastes and faecal matter were siphoned out on every 3rd day.

The antagonistic bacterium was isolated as described elsewhere (Abraham and Banerjee 2007) from the gut of mrigal, *Cirrhinus mrigala* on de Man Rogosa Sharpe (MRS) agar (pH: 7.0) with 0.2% glucose (Schillinger and Lucke 1989) and used as a probiont. The MRS agar with 0.2% glucose was used for the growth and maintenance of probiont. The probiotic strain maintained on MRS agar slant was revived on agar plate when needed. Prior to experimentation, 10 ml of MRS broth was inoculated with young catalase negative colonies and incubated at 30 ± 2 ^{0}C for 24 h. This 24 h old culture was then transferred to 1000 ml MRS broth and re-incubated at 30 ± 2 ^{0}C for 48 h and used immediately.

The opportunistic fish bacterial pathogens such as *Aeromonas hydrophila* (n=9) *Bacillus* spp (n=2), *Pseudomonas* spp (n=4), isolated from diseased ornamental fishes (Abraham *et al.* 2004), were used in *in-vitro* antagonistic assay with probiotic strain. Besides these, one each of *Aeromonas hydrophila, Citrobacter freundii* and *Pseudomonas aeruginosa,* strains received as gift from Dr. I. Karunasagar, Department of Fishery Microbiology, College of Fisheries, Mangalore were also used for the antagonistic assay.

In-vitro antagonistic assay

In vitro antagonistic activity of probiotic strain was tested against 18 opportunistic fish bacterial pathogens by cross streak technique on MRS agar with 0.2% glucose as

described in Lemos *et al.* (1985). Overnight grown slant culture of probiont was inoculated as a straight line on MRS agar plates and incubated for 48 h at 30±2 °C. Young cultures of fish bacterial pathogens (n=18) from TSA were then carefully inoculated perpendicular to the growth of probiont on MRS agar. The plates were re-incubated for 48 h and the zone of inhibition measured using a caliper.

Effect of fish gut antagonistic bacterium on the survival of Xiphophorus helleri

The effect of fish gut antagonistic bacterium on the survival of *X. helleri* was tested by immersion assay (Austin *et al.* 1995). Twenty *Xiphophorus helleri* of average weight 0.56 g and average length 36.11mm were introduced in to each glass aquaria, in duplicate, which contained 20-litre water. The bacterial cells, grown in MRS broth, were harvested by centrifugation at 7500 rpm for 20 min at 25 ^{0}C in a centrifuge. The cell pellets were washed twice by centrifugation with sterile physiological saline and finally resuspended in 50 ml sterile saline. A portion of the cell suspension was suitably diluted up to 10^{-8} in sterile saline and the number of cells/ml of suspension was determined by spread plating on MRS agar with 0.2% glucose after incubation at 30±2 ^{0}C for 48 h. The cell suspension was added in to the aquaria in such a way to get a level of 10^7 cells/ml of rearing water. The control tanks received no bacterial inoculum. The fishes were maintained in their respective aquaria for 15 days at about 22 ^{0}C water temperature and fed daily with commercial fish feed on demand. The accumulated faecal matter and other wastes were siphoned-out on every 3rd day. Mortality, external signs of infections and behavioural abnormalities were recorded daily. The relative percentage survival (RPS) was calculated as RPS = (1 - Mortality in treatment/Mortality in control) x 100.

Influence of fish gut antagonistic bacterium on Xiphophorus helleri production

Before being used, the rearing water, drawn from a bore well, was passed through a sand and gravel filter provided with a charcoal bed of 20 cm thick. The required numbers of uniform sized healthy fishes (groups A and B) were transferred to respective rectangular FRP tanks containing 100-litre water and acclimatized for 3 days. The fishes (0.215–0.238 g) were stocked at the rate of 50 each/tank, in triplicate. The probiont cells were inoculated in to the rearing waters of group A at a level 10^6 cells/ml on every 3rd day for a period of one year between June 2002 and May 2003. The tanks of group B received no bacterium and served as control. The fishes were fed with commercial feed on demand. The faecal matter and other wastes were siphoned out and 60% of water was exchanged on every 3rd day followed by the addition of fresh probiont cells. In control tanks also water exchange was done as in test tanks. The water temperature was in the range of 16 ^{0}C during winter and 30 ^{0}C during summer. During winter, the water temperature was maintained at about 16 ^{0}C using heaters. The fishes were observed for mortality daily. The length and weight of fishes of group A and group B were noted at intervals for first 60 days (June - July 2002) and determined the survival

percentage, feed conversion ratio (FCR), specific growth rate (SGR) from the first 60 days observations as described below:

Food conversion ratio (FCR) = Total feed consumed on dry weight basis (g) / Growth in terms of wet weight gain (g)

Specific growth rate (SGR) = $(\ln W_t - \ln W_0 / \text{Days of culture}) \times 100$

Where, W_t = Final average wet weight (g), W_0 = Initial average wet weight (g)

The gut bacterial flora of *X. helleri* fry on the 1st and 60th day was enumerated by the method of Huys *et al.* (2001) with modification. On each sampling day, five fries were scooped out randomly from each category and aseptically transferred to test tubes containing 10 ml 0.1% (w/v) benzal konium chloride (BKC) solution for 15 sec to remove the surface bacteria. The BKC solution was decanted after 15 sec and the fries were rinsed thrice, each rinse for 15 sec, in sterile physiological saline. The fries were aseptically transferred to pre-weighed sterile homogenizer containing 10 ml saline. The fries along with saline were weighed again and homogenized. The homogenized sample was vortexed and diluted by 10 fold serial dilution to appropriate levels and used for the enumeration of different groups of bacteria immediately. The probiont counts and total MRS counts on MRS agar with 0.2% glucose (Schillinger and Lucke 1989), total plate counts on TSA, presumptive pseudomonads on *Pseudomonas* isolation agar, lactose fermenters and lactose non-fermenters on MacConkey agar and motile aeromonads on starch ampicillin agar (Palumbo *et al.*1985) were enumerated by spread plating.

Reproductive performance

The fully ripe and ready to spawn female (brooder) was separated on the basis of bulged abdomen, protruded anus and slow swimming. The abdomen of such fishes was smooth to touch. The spawning was done in a special spawning tank, which had two layers to avoid parental cannibalism of the new born. The top layer was a dismantable one, which had closely arranged perforations (16/sq.inch) to allow the fry to pass through. The length and weight of ready to spawn female was determined prior to introduction into the top layer of the spawning tank, covered with a lid and kept undisturbed. Once spawned, the fries settled at the bottom layer of the spawning tank. The mother fish was removed, maintained in a separate tank and reintroduced in to the respective tank after 3 days of spawning. Immediately after spawning, the fries were separated from the bottom layer of the spawning tank using a hand net and counted the total numbers including the dead, immature and deformed ones. The observations on each group were recorded separately. Randomly, five fries were taken and measured the length and combined weight. The average weight of the individual fry was calculated dividing the combined weight by the number of fries taken. The healthy fries, after recording all observations, were transferred to 300-litre capacity rectangular FRP tanks

containing green water separately. Reproductive performance and other parameters were compared by student t-test using Microsoft Excel Package.

Results and discussion

A perusal of literature revealed that studies on the use of biocontrol agents or application of probiotics in ornamental fish culture are scanty. The present study revealed that the growth performance of *X. helleri* reared in the presence of fish gut bacterium, designated as *Lactobacillus* P21 (Table 1), at level of 10^6/ml was better than the control. The mean survival was the same in both probiotic and control groups (91%) in first 60 days. There existed a significant difference ($p<0.03$) in FCR between *Lactobacillus* P21 inoculated (2.803 ± 0.139) and control (3.122 ± 0.104) groups. The difference in SGR between probiotic (1.655 ± 0.633) and control (1.243 ± 0.507) groups was, however, insignificant ($p>0.05$). The use of probiotics in different sectors of aquaculture is well studied with varying reports of their effects on growth, survival and disease resistance of different commercially important aquatic organisms (Verschuere *et al.* 2000, Irianto and Austin 2002).

Table 1. Biochemical characterization and antibiogram of the fish gut antagonist bacterium, *Lactobacillus* sp P21

Parameter	Reaction	Parameter	Reaction
Gram reaction	+	Citrate Utilization	-
Morphology	Rod	Gelatinase	+
Catalase test	-	Amylase (Starch hydrolysis)	-
Endospore formation	-	Lecithinase	-
Oxidative/Fermentative test	+/+	Growth at 5°C	+
Gas from glucose	-	Growth at 10°C	+
Utilization of Glucose	+	Growth at 45°C	-
Lactose	-	Antibiogram (in mm)	
Mannitol	+	Chloramphenicol, 30 μg	27* (S)
Arabinose	-	Ciprofloxacin, 5 μg	22* (S)
Asculine hydrolysis	-	Gentamycin, 10 μg	20* (S)
Arginine dihydrolase	+	Nitrofurantoin, 300 μg	24* (S)
Motility	+	Co-trimoxazole, 25 μg	8* (R)
Growth in 3% NaCl	+	Trimethoprim, 5 μg	9* (R)
6% NaCl	+	Oxytetracycline, 30 μg	26* (S)
8% NaCl	-	Growth on MRS agar	+
10 % NaCl	-	Production of BLIS	+
12% NaCl	-	Hemolysis on 5% goat blood	-
Nitrate reduction	-	agar	
Methyl red reaction	+	Spectrum of action on	
Vogus Prosgauer reaction	-	Gram positive bacteria	Variable
Indole	-	Gram negative bacteria	Variable

(S): Sensitive; (R): Resistant; R: Rod; * Zone size in mm; BLIS – Bacteriocin-like inhibitory substance

The fish gut bacterium was a gram positive, catalase negative, non-hemolytic, asporogenous rod which produced off white to milky white colonies of 2 mm diameter after incubation for 48 h at 30 ^{0}C on MRS agar with 0.2% glucose (Table 1). Likewise, isolation of lactic acid bacteria in fish gut was reported earlier (Ringo *et al.* 1995, Ringo and Gatesoupe 1998). No fish mortality was observed up to day 10 when challenged at above log 7.0/ml water by immersion assay and the relative percentage survival (RPS) was 75 on day 15 (Table 2). The results of the *in-vitro* antagonistic assay by cross streak technique against 18 opportunistic fish pathogenic strains isolated from ornamental fish and other sources revealed that the *Lactobacillus* sp P21 was quite effective against *Aeromonas hydrophila*, *Bacillus* spp, *Pseudomonas* spp and *Citrobacter freundi* (Table 3). Many of these strains were inhibited at varying levels by *Lactobacillus* sp P21, probably due to the production bacteriocin-like inhibitory substance (Table 1). Lactic acid bacteria have been known to inhibit many gram-positive and gram-negative bacteria (Tag *et al.* 1976) mainly through production of organic acids, hydrogen peroxide and bacteriocin-like inhibitory substances (BLIS).

Table 2. Effect of *Lactobacillus* sp P21 on the survival of *Xiphophorus helleri*

Days of immersion	Survival % at temperature 22 °C		RPS
	Lactobacillus sp P21 @ 2.55x10^7/ml	Control	
1	100	100	100
5	100	95	100
10	100	85	100
15	95	80	75

Number of fish tested = 20/tank; TPC of control tank = 1.00x10^4/ml. Relative Percentage Survival (RPS) = {1 - Mortality in treatment / Mortality in control} x 100.

Table 3. *In-vitro* antagonistic activity of *Lactobacillus* sp P21 against fish bacterial pathogens by cross-streak technique

Bacterial strains (N=18)	Zone of inhibition in mm
Aeromonas hydrophila (n=10)	0 – 12.5
Bacillus spp (n=2)	3.0
Citrobacter freundii (n=1)	1.0
Pseudomonas spp (n=4)	1.0 – 12.5
P. aeruginosa (n=1)	4.0

As seen in Table 4, the total plate counts, total MRS agar counts and the counts of motile aeromonads, presumptive pseudomonads, lactose fermenters and lactose non-fermenters in the gut of probiotic group were comparatively low than the control on day 60. The level of *Lactobacillus* sp P21 was maintained always above ≥log 5.80 / ml rearing water throughout the experiment. The *Lactobacillus* sp P21 counts increased to log 5.28/g on day 60 in the gut of *X. helleri*, indicating a slow colonization of this bacterium in the gastrointestinal tract. By colonizing the intestinal mucous layer the probiont may

serve as first defense barrier against invading pathogenic bacteria (Ringo and Gatesoupe 1998). Once colonized, the probiont alter the microbial metabolism by the increase or decrease of relevant enzyme levels, competitively exclude the potential pathogens by the production of inhibitory compounds or competition for nutrients, space or oxygen (Salminen *et al.* 1996, Irianto and Austin 2002). The results, however, indicated insignificant inhibition of gut bacterial flora probably be due the marked differences in the counts of *Lactobacillus* sp P21 and other bacterial groups. It is important to mention here that the total plate counts in the gut of all fish groups were always above log 8.20/g and in the range of log 8.23/g – log 9.64/g, The magnitude of difference between the *Lactobacillus* sp P21 (log 5.28/g) and total plate counts of the fish gut (log 8.36/g) was to the order of log 3.00. The results indicated that the population of *Lactobacillus* sp P21 was not sufficient enough in the gastrointestinal tract of *X. helleri* that could significantly affect the growth of non-beneficial bacteria.

Table 4. Log counts of bacteria in the gut of *Xiphophorus helleri* fry reared in the presence of *Lactobacillus* sp P21

Bacteria	Probiotic group		Control	
	Day 1	Day 60	Day 1	Day 60
Total plate count /g fry	8.23	8.36	8.25	9.64
Total MRS agar count /g fry	7.79	7.82	7.81	8.43
Lactobacillus P21 count /g fry	<4.00	5.28	<4.00	<4.00
Motile aeromonads /g fry	7.71	6.34	7.67	7.73
Presumptive pseudomonads /g fry	5.53	5.02	5.45	6.58
MPN total coliforms /g fry	5.67	4.94	5.67	4.64
Lactose non-fermenters /g fry	7.06	6.15	7.01	7.34
Lactose fermenters /g fry	4.63	4.28	4.66	6.04

Several reports (Verschuere *et al.* 2000, Irianto and Austin 2002) assumed that the lactic acid bacteria (probionts) are important part of the intestinal defense against invading bacterial pathogen. Although the intestinal tract may serve as one portal of entry for fish pathogenic bacteria (Olssen *et al.* 1996), the observations of Spanggaard *et al.* (2000) revealed that fish skin and gills are important site of proliferation and invasion, and the skin area would not be reached by the probiotic bacteria when incorporated in to the fish feed. The results of the present study demonstrated that *Lactobacillus* sp P21 not only colonized the gut, and also on the skin and gills of *X. helleri* (*Lactobacillus* sp P21 count on MRS agar: log 5.72/g fish on day 60) to deter the colonization of undesirable bacteria, which ultimately resulted in better growth performance.

The *X. helleri* reproduced throughout the year, with the maximum fry production during August and April and the least during winter months - December, January and February. The probiotic addition, in general, increased the fry production and reduced the fry mortality, deformity and immature fry during spawning. As seen in Table 5, the

average fecundity/female and average number of fry survived/female was high in *Lactobacillus* sp P21 inoculated group than in control. The average number of fry dead/ female and average number of deformed fry/female was found to be high in control than in probiotic inoculated group. The average number of immature fry/female was, more or less, the same in both the groups. Significant differences existed in respect of average fecundity/female ($p<0.02$), average number of fry survived/female ($p<0.006$) and average number of fry dead / female ($p<0.029$). The average length ($p<0.02$) and weight ($p<0.0002$) of the female was significantly high in *Lactobacillus* sp P21 inoculated group (53.89±4.46 mm; 1.97±0.44 g) than control (49.06±6.72 mm; 1.48±0.48 g). Likewise, the average length ($p<0.004$) and weight ($p<0.00013$) of the fry of *Lactobacillus* sp P21 inoculated group was also significantly high (7.71±0.31mm; 0.0060±0.0015g) than the control group. The percentage survival of fry was more in *Lactobacillus* sp P21 inoculated group (97.51) than the control (91.16) group thereby revealing its influence on the growth and reproductive performance of *X. helleri.*

Table 5. The growth parameters and reproductive performance of *Xiphophorous helleri* reared in the presence of probiont

Observations	Probiotic group (n = 28) Range and Mean ± SD	Control (n = 31) Range and Mean ± SD
Fecundity*	28 - 134 53.00 ± 23.57[a]	9 - 80 39.42 ± 18.72[a]
Number survived*	26 - 126 51.68 ± 22.87[b]	9 - 80 35.94 ± 18.18[b]
Number dead*	0 - 8 1.32 ± 1.65[c]	0 - 19 3.48 ± 4.92[c]
Deformed fry*	0 - 4 0.29 ± 1.03	0 - 6 0.35 ± 1.15
Immature fry*	0 - 2 0.32 ± 0.66	0 - 6 0.32 ± 1.25
Weight in g	1.20 - 2.80 1.97 ± 0.44[d]	0.70 - 2.20 1.48 ± 0.48[d]
Length in mm	46 - 64 53.9 ± 4.5[e]	36 - 58 49.1 ± 6.7[e]
Fry weight in mg	3.4 - 8.1 6.0 ± 1.4[f]	2.0 - 8.0 4.8 ± 1.7[f]
Fry length in mm	7.6 - 8.0 7.7 ± 0.3[g]	6.8 - 8.1 7.4 ± 0.3[g]
Total fries produced	1484	1222
Percent survived during spawning	97.51	91.16

Values sharing common superscripts within rows are significantly different ($p<0.05$).
*: Average numbers / female. n = number of spawning

The fecundity of *X. helleri* was reported to be as high as 242 fry/female; however, spawns of *X. helleri* average about 30 fry/female, after a gestation period of 26-30 days (Tamaru *et al.* 2001). The results presented in Table 5 revealed that the *X. helleri* produced fry in the range of 9–134. On average, it produced from 39.42±18.72 fry/female in control group to 53.00±23.57 fry/female in probiotic group. The results of the present study (Table 5) demonstrated that the rearing of *X. helleri* in probiotic-enriched water favourably influenced the reproductive performance in terms of high fecundity, high fry survival, reduced fry mortality and reduced fry deformity. More or less, similar results were reported for *X. helleri* when fed with drum-dried flake feed supplemented with various levels of *Daphnia* sp (Kruger *et al.* 2001). It has been reported that lactic acid bacteria produce B group vitamins (Goldin and Gorbach 1992) and probably the production and supply of B group vitamins and certain unknown stimulants could have played a role in the elevated reproductive performance of probiotic fed *X. helleri* compared to control group. The increase in average fecundity in probiotic group over the control group was about 25%, thus revealing the economic benefit of using beneficial fish gut bacterium (probiont) in ornamental fish production.

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