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Antagonistic Fish Gut Bacterium *Lactobacillus* sp. as Biocontrol Agent in Ornamental Fish Culture

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The effect of addition of a fish gut bacterium *Lactobacillus* sp. P21 in to the rearing water on *Pseudomonas fluorescens* induced mortalities in ornamental fishes was examined. The goldfish, *Carassius auratus and* swordtail, *Xiphophorus helleri* were almost 3.7–4.7 times as likely to become infected when exposed to *P. fluorescens* (10^6 cfu/mL) compared to control. The introduction of *Lactobacillus* sp. into the culture system seeded with *P. fluorescens* reduced the relative risk by more than two folds through the production of low molecular weight bacteriocin-like-substance.

Keywords: Ornamental fish culture, *Carassius auratus, Xiphophorus helleri, Pseudomonas fluorescens,* probiotic, *Lactobacillus* sp.

In aquaculture sector, the ornamental fish breeding and trade provide excellent opportunities as a non-food fishery activity for employment and income generation. In India, Kolkata has been emerging as a major ornamental fish-trading center due to the prevailing favourable climatic conditions. Ornamental fish export from Kolkata region leaped from Rs. 163.20 lakhs in 1999-2000 to Rs. 367.00 lakhs in 2002-2003 (Mukherjee, 2004). The role of bacteria as beneficial agents in culture of aquatic organisms has been the subject of many investigations. The use of beneficial bacteria such as *Aeromonas media* (Lategan *et al.*, 2006), *Bacillus* sp. (Hong *et al.*, 2005), *Lactobacillus plantarum* (Uma *et al.*, 1999), *Photorodobacterium* (Inianto & Austin, 2002), *Pseudomonas* spp. (Spanggaard *et al.*, 2001; Vijayan *et al.*, 2006) and *Vibrio* spp. (Austin *et al.*, 1995; Carraturo *et al.*, 2006) in aquaculture has been amply documented. The recent review by Kesarcodi-Watson *et al.* (2008) provided a comprehensive summary of probiotics in aquaculture with special reference to mollusk culture. However, there is dearth of reports regarding the use of bacteria in ornamental fish culture. The present study reports the effect of a gram-positive fish gut antagonistic bacterium, *Lactobacillus* sp. P21 on the *in-vitro* and *in-vivo* inhibition of *Pseudomonas fluorescens* in ornamental fish culture.

Materials and Methods

An antagonistic bacterium, *Lactobacillus* sp. P21 isolated from the gut of mrigal, *Cirrhinus mrigala* on modified de Man Rogosa Sharpe (MRS) agar (pH: 7.0) with 0.2% glucose (Schillinger & Lucke, 1989) was used as a probiont. The tests performed to identify the probiotic strain were Gram reaction, morphology, catalase test, endospore formation, oxidative / fermentative test, gas from glucose, utilization of glucose, lactose, mannitol, arabinose, asculine hydrolysis, arginine dihydrolase, motility, growth in 3%, 6%, 8%, 10% and 12% NaCl, nitrate reduction, methyl red reaction, Voges-Proskauer reaction, iodole, citrate utilization, gelatinase, amylase and lecithinase activity and growth at 5°C (Collins *et al.*, 1989; Austin & Austin, 1993). The

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ornamental fish pathogen, *Pseudomonas fluorescens* 58C was received from the bacterial collections of Department of Fishery Pathology and Microbiology, Faculty of Fishery Sciences, Kolkata. The *Lactobacillus* sp. P21 cells, grown in modified MRS broth, were harvested by centrifugation at 10,000 rpm for 20 min at 25°C. The cell pellets were washed twice by centrifugation with sterile physiological saline and finally resuspended in 50 mL sterile saline. The cells of *P. fluorescens*, grown in tryptic soy broth (TSB), were also harvested as above. A portion of the cell suspension was suitably diluted up to 10^{-8} in sterile saline. The number of cells/ml in the suspension of *Lactobacillus* sp. P21 and *P. fluorescens* 58C was determined by spread plating on modified MRS agar and tryptic soy agar, respectively and incubating at 30 ± 2°C for 24 - 48 h.

The supernatant was decanted thoroughly into a sterile conical flask aseptically and stored at 4°C for the extraction of antibacterial substance by ammonium sulphate precipitation. The supernatant fraction was filtered through a membrane filter (pore size 0.22 μ) to remove cellular debris. About 100 mL cell free supernatant was made up to 65% saturation by slow addition of ammonium sulphate and held overnight at 4°C. The sample was then centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant fraction decanted. The precipitate was collected and resuspended in 5 mL of 10 mM phosphate buffer containing 0.1 mM EDTA, pH 7.0. To collect the crude extract of the antibacterial substance, the concentrated precipitate was exhaustively dialyzed against the same buffer for 24 h at 4°C in a dialysis membrane with molecular weight cut-off 3500 Da. The cell pellet of *Lactobacillus* sp. was also used immediately for the extraction of cell bound antibacterial compound. The extraction procedure described by Wratten *et al.* (1977) was modified and adopted. The cell pellet in sterile polypropylene container was suspended in 5 mL ethyl acetate for 2 h. The pellet was removed by filtration and the extract was collected in a sterile container. The ethyl acetate extract was vacuum evaporated at 40-50°C to get a concentrated crude extract.

The antibacterial activity of the cell free supernatant, dialyzed ammonium sulphate precipitate and crude ethyl acetate extract was done by well diffusion assay (Tagg & McGiven, 1971). Wells of 5.0 mm diameter were made on hardened modified MRS agar plates. The bottom of each well was sealed with MRS soft agar (MRSB + 0.8 % agar). The cell-free supernatant and the crude extracts of antibacterial substance were tyndallized at 100°C for 30 min prior to use. The wells were filled with 50 μl each of test samples and allowed to diffuse for 2 h. The plates were then overlaid with 10 mL of tryptic soy soft agar (TSB + 0.8% agar) seeded separately with 10 μL of overnight grown *P. fluorescens* 58C. The overlaid plates were incubated at 30 ± 2°C for 24 h and examined for a definite zone of clearance around the wells.

The experimental goldfish, *Carassius auratus* (0.52 ± 0.03 g) and swordtail, *Xiphophorus helleri* (0.165 ± 0.006 g) were procured from the ornamental fish breeders in and around Kolkata, West Bengal, India. The fishes, on receipt, were disinfected by placing in 5-ppm potassium permanganate solution for up to 15 min and transferred to circular fiberglass reinforced plastic (FRP) tanks of 500-litre capacity containing aerated bore-well water. The dead and morbid fishes were removed immediately and the healthy ones were stocked at 100 numbers each in FRP tanks. Twenty-five *C. auratus* were introduced into each of the 12 glass aquaria containing 35 liter aerated bore-well water. Likewise, twenty-five *X. helleri* were introduced into each of the 12 glass aquaria. The fishes were acclimatized in glass aquaria for 3 days. The cell suspension of *Lactobacillus* sp. P21 and *P. fluorescens* 58C were added in to the aquaria in such a way to get a level of 10^7 and 10^6 cells/mL of rearing water, respectively as described below:

Treatment 1 *Lactobacillus* sp. P21 at 10^7 cfu/mL rearing water, in triplicate for both *C. auratus* and *X. helleri*

Treatment 2 *Pseudomonas fluorescens* 58C at 10^6 cfu/mL rearing water, in triplicate for both *C. auratus* and *X. helleri*

Treatment 3 *Lactobacillus* sp. P21 at 10^7 cfu/mL and *P. fluorescens* 58C at 10^6 cfu/mL rearing water, in triplicate for both *C. auratus* and *X. helleri*

Treatment 4 No bacterial inoculum — served as control

The fishes were fed with commercial pelleted diet on demand daily for a period of 30 days. The bacterial cell suspensions were added into the respective tanks on every 3rd day following the removal of wastes and faecal matter, and replenishment of 50% of the rearing water. The fishes were observed for mortality daily and dead ones removed immediately. Relative risk (RR), i.e., the ratio of the incidence rate in the exposed group to the incidence rate in the unexposed group, was calculated using Win Episcope version 2.0. Chi-square test was used to test the significance of difference in the mortality between treatments.

Results and Discussion

The antagonistic fish gut bacterium was a gram-positive, motile, fermentative, catalase negative, non-hemolytic, asporogenous rod that produced off white to milky white colonies of 2 mm diameter after incubation for 48 h at 30°C on modified MRS agar. It showed positive reaction to mannitol, asculine hydrolysis, arginine dihydrolase, methyl red and gelatinase, and negative reaction to gas from glucose, nitrate reduction, Voges-Proskauer, indole, lactose, arabinose and citrate utilization, amylase and lecithinase. It was capable to growth at 6% NaCl and 5°C but not at 45°C. This strain was identified and designated as *Lactobacillus* sp. P21.

Table 1 and Figs. 1 and 2 present the *in-vivo* effect of *Lactobacillus* sp. P21 to protect *C. auratus* and *X. helleri* against a pathogenic bacterium, *P. fluorescens* 58C. The mortality was observed to be high in *P. fluorescens* 58C inoculated aquaria (86% in *C. auratus* and 82% in *X. helleri*) compared to other treatments. The *Lactobacillus* sp. P21 produced antibacterial substance that excreted into the growth medium. A clear zone of inhibition was observed when well diffusion assay was performed using dialyzed ammonium sulphate precipitate. The cell free supernatant of growth medium and ethyl acetate extract of *Lactobacillus* cells yielded no zone of inhibition.

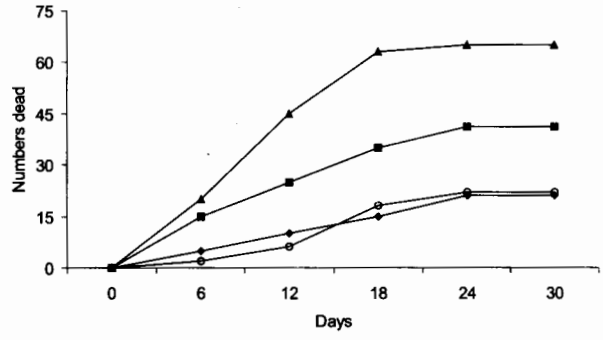

Fig. 1. Cumulative mortality in Carassius auratus. Treatments: (▲) Pseudomonas fluorescens 58C 106 cfu/mL, (■) Pseudomonas fluorescens 58C 106 cfu/mL + Lactobacillus sp. P21 107 cfu/mL, (◆) Lactobacillus sp. P21 107 cfu/mL, (○) Control

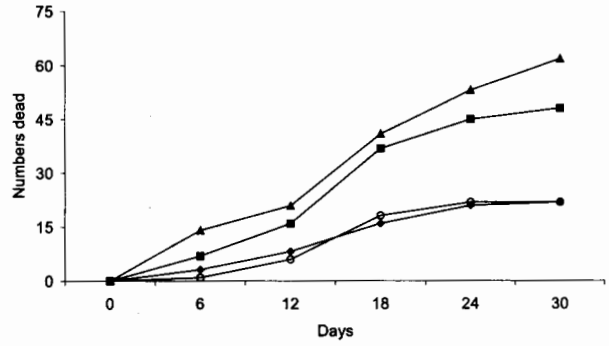

Fig. 2. Cumulative mortality in Xiphophorus helleri. Treatments: (▲) Pseudomonas fluorescens 58C 106 cfu/mL, (■) Pseudomonas fluorescens 58C 106 cfu/mL + Lactobacillus sp. P21 107 cfu/mL, (◆) Lactobacillus sp. P21 107 cfu/mL, (○) Control

Table 1. *In–vivo* inhibition of *Pseudomonas fluorescens* 58C by *Lactobacillus* sp. P21: Mortality and relative risks in *Carassius auratus* and *Xiphophorus helleri*.

Treatment	Mortality (%) in 30 days		Relative risk	
	Carassius auratus (Mean ± SD)	*Xiphophorus helleri* (Mean ± SD)	*Carassius auratus*	*Xiphophorus helleri*
Lactobacillus sp. P 21	28.00 ± 3.27^{ab}	29.33 ± 1.89^{fg}	0.968	1.000
P. fluorescens 58C	86.67 ± 1.89^{acc}	82.67 ± 1.89^{fhi}	4.707	3.747
Lactobacillus sp. P21 + *P. fluorescens* 58C	54.67 ± 3.77^{bcd}	64.00 ± 8.64^{ghj}	1.665	2.032
Control	29.33 ± 1.89^{de}	29.33 ± 1.89^{ij}	-	-

a-j: values sharing common superscripts within a column differed significantly ($P<0.05$). - : No data. Relative risk is the ratio of the incidence rate in the exposed group to the incidence rate in the unexposed group.

As seen in Table 1, relative risk was the highest in *P. fluorescens* 58C inoculated aquaria, thereby revealing its pathogenic potential on ornamental fishes. The mortalities in the *Lactobacillus* sp. P21 inoculated and control stocks of *C. auratus* and *X. helleri* were almost the same. The RR values of 0.968 and 1.0, respectively for *C. auratus* and *X. helleri* in *Lactobacillus* sp. P21 seeded group indicated no increased risk compared to control. The RR values of 3.747 – 4.707 in *P. fluorescens* 58C seeded groups suggested that the fishes were almost 3.7 – 4.7 times more susceptible to infection when compared to control. The introduction of *Lactobacillus* sp. P21 in to the aquaria seeded with pathogenic *P. fluorescens* 58C, however, reduced the relative risk to 1.7 and 2.0 in *C. auratus* and *X. helleri*, respectively. There existed significant differences ($P<0.05$) in the mortality rates of *C. auratus* between different treatments used in the aquaria namely i) *Lactobacillus* sp. and *P. fluorescens* ii) *Lactobacillus* sp. and mixture of *Lactobacillus* sp. & *P. fluorescens*, III) *P. fluorescens* and mixture of *Lactobacillus* sp. & *P. fluorescens*, IV) *P. fluorescens* and control and V) mixture of *Lactobacillus* sp. & *P. fluorescens* and control. Similar differences in the mortality rates of *X. helleri* ($P<0.05$) were also noticed. In earlier studies also, addition of antagonistic bacteria into the rearing water resulted in *in-vivo* disease reducing effect and or reduction of members of fish pathogenic bacteria in the water (Smith & Davey, 1993; Austin *et al.*, 1995; Moriarty, 1998; Abraham *et al.*, 2001).

From the result of *in-vitro* inhibition of *P. fluorescens* by crude ammonium sulphate precipitate, it can be inferred that the antagonistic principle of *Lactobacillus* sp. P21 was of proteinaceous origin, more particularly of low molecular weight bacteriocin-like-substance. Probably through the production of antibacterial substance, this strain competitively excluded *P. fluorescens* 58C and reduced the mortality rate of *C. auratus* and *X. helleri*. Similarly, numerous strains of lactic acid bacteria are associated with the production of various types of low molecular weight bacteriocins (Davey, 1981; Piard *et al.*, 1992; Bruno & Montville, 1993). Likewise, Carraturo *et al.* (2006) demonstrated inhibition of *V. parahaemolyticus* by a bacteriocin-like inhibitory substance produced by *V. mediterranei* 1. The results of the present study, thus, revealed that the antagonistic bacterium *Lactobacillus* sp. P21 isolated from carp gut inhibited fish pathogen, *P. fluorescens* 58C both *in-vitro* and *in-vivo*. Therefore, colonization of non-pathogenic antagonistic bacterium, not exceeding the threshold level, on ornamental fish seeds would help reduce mortalities by preventing attachment and proliferation of bacterial pathogens on them. It appears to be an effective way of controlling bacterial pathogens in ornamental fish

culture in lieu of the negative impacts of antibiotics.

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