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Colonization of probiotic bacteria and its impact on ornamental fish *Puntius conchonius*

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Abstract

The present study was conducted to assess the establishment and effect of probiotic bacteria such as *Bacillus coagulan*s, *Bacillus mesentericus*, and *Bifidobacterium infantis* in the gut of freshwater ornamental fish *Puntius conchonius*. Postlarvae of 60 days old *Puntius conchonius* divided in four experimental groups each with three replicates. T_1, T_2 and T_3 groups were fed with *Bacillus coagulan*s, *Bacillus mesentericus* and *Bifidobacterium infantis* enriched copepod *Thermocyclops decipiens* respectively. T_0 was the control group (without probiotic treated *T.decipiens*). The experiment was conducted for 45 days. The initial gut analysis of fish showed significant level of pathogenic bacteria in the gut of fish *($p<0.05$).* Total plate count of initial gut analysis of fish larvae were enumerated as 1.2×10^4 CFU ml^{-1}. The bacteriological study indicated that final gut microflora of post-larvae have decreased level of pathogens. Total plate count of T_1, T_2, T_3 and T_0 were reported to be as $1.08x10^4$, $1.06x10^4$, $1.27x10^4$ and $2.1x10^5$ CFU ml^{-1}, respectively. Three experimental groups (T_1, T_2 and T_3) were significantly different from control group (T_0) *($p<0.05$)*. At the end of the experiment, establishment of probiotics were examined. One week after probiotics administration, gut analysis of fish larvae showed, poor spore formation of *Bacillus coagulans* ($2.3x10^3$ CFU ml^{-1}), when compared to *B. mesentericus* ($3.2\pm0.03x10^3$ CFU ml^{-1}) and *Bifidobacterium infantis* ($3.1x10^3$ CFU ml^{-1}). The results from the study suggest that the probiotic bacteria significantly established in gut of *P. conchonius* and significant effects on the pathogenic gut inhabitants of the fish.

Key words

Probiotics, Enrichment, *Puntius conchonius, Thermocyclops decipiens*

Introduction

Probiotics have been defined as microbial cell preparations that when administrated in adequate amounts, have beneficial effects on the health and well being of the host (Panigrahi and Azad, 2007). Several mechanisms responsible for the protective action of probiotics have been proposed (Balcazar *et al.,* 2006), competitive exclusion of pathogenic bacteria; source of nutrients and enzymatic contribution to digestion and direct uptake of dissolved organic material mediated by the probiotic cells. The probiotics have been suggested as way to step into a more environment friendly aquaculture by reducing the use of chemicals and antibiotics (Taoka *et al.,* 2006; Wang *et al.,* 2008).

Zooplankton are known to regulate bacterial abundance and productivity, either by direct feeding or via cascading effects of feeding on bacterial predators (Zöllner *et al*., 2003; Grosshart *et al*., 2008). In the sea, copepods are regarded as hotspots for bacterial

growth, as they concentrate organic matter in their guts and faecal pellets (Tang, 2005) which are providing attachment sites for bacterial colonization (Moller *et al.,* 2007). Several studies have reported the massive colonization on faecal pellets and on the body surface of crustacean zooplankton, especially around the oral region (Nagasawa, 1988).

Several bacteria have been used as probiotics in the larval culture of aquatic organisms and they can be either delivered directly into the water, or via live carrier such as *Artemia* nauplii and rotifers, or else added to pelletized dry food (Gomez-Gil *et al.,* 2000). Among zooplankton, copepods, especially their nauplii, are natural prey for fish larvae (Chesney, 2005; Chen *et al.,* 2006). Copepods are important food source for developing larvae, postlarvae, juveniles of many fishes and crustaceans (Pinto *et al.,* 2001). In the present study, freshwater copepod *Thermocyclops decipiens* (Kiefer, 1929) (Crustacea: Cyclopoida) were used as vector to fish larvae. The tropics state of lakes or reservoirs seems to determine the occurrence of these species. The present study aimed to investigate the establishment of probiotics (*Bacillus coagulans, Bacillus mesentericus* and *Bifidobacterium infantis*) in the gut of fish, *Puntius conchonius* and its possible effect on pathogenic microorganisms confined to gut.

Materials and Methods

Experimental conditions and culture of bacteria: The experiment was conducted for 45 days. Post larvae of *Puntius conchonius* (60 days old) were used for the experiment. The experiment was divided into four experimental groups each with three replicates following a completely randomized experimental design in 12 uniform size glass tanks (50 l capacity) at ambient temperature (26-28°C). T_{1-} *B.coagulans* treated fish larvae, T_{2-} *B.mesentericus* treated fish larvae, T_{3-} *Bifidobacterium infantis* treated fish larvae. T_0-without probiotics treated larvae. *Bacillus coagulans (Lactobacillus sporogens), Bacillus mesentericus* and *Bifidobacterium infantis* were used as probiotics and were isolated and pure culture maintained at Department of Aquaculture and Fishery Microbiology, M.E.S. Ponnani College, Ponnani, Kerala, India. Probiotics were cultured in a nutrient agar slant. The slant cultures of the selected probiotics were inoculated into specific broth medium under aseptic conditions. *B. coagulans (L. sporogens*) were cultured in lactic acid broth. Whereas, *Bifidobacterium infantis and Bacillus mesentericus* were cultured in nutrient broth. After 48 hr of incubation at 37°C, three probiotics were sub-cultured in the same medium (50 ml of broth culture was prepared).

Preparation of probiotic diet and enrichment: Pure cultures of probiotics were centrifuged at 4000 xg to 6000 xg for 30 min. After centrifugation, the bacteria (i.e. pellet) were washed twice with saline, *i.e.* the pellet was suspended with saline and then centrifuged again. After washing the bacteria with sterilized water, the pellet was collected and it contained bacteria ranging between 0.8-1.3 g of pellet. The probiotics were aseptically enriched with *Thermocyclops decipiens* nauplii for 6 hr duration.

The fresh water copepod *Thermocyclops decipiens* was cultured in 500 l tanks feeding microalgae *Chlorella vulgaris*, adopting standard procedures (Schipp *et al.,* 1999). *T.decipiens* were collected on a sieve from the mass culture tanks and transferred to enrichment containers (2 l capacity) at a density of 50 individual ml $^{-1}$ at room temperature (28 ± 1°C). The nauplii of *T.decipiens* were fed with different types of probiotics approximately at 6×10^{10} CFU ml^{-1}. Strong aeration was provided to the rearing containers to maintain optimum oxygen level.

Completely randomized experimental design was followed with three replicates. The duration of enrichment was 6 hr. After 6 hr enrichment, the nauplii of *T.decipiens* were collected from the enrichment medium, and thoroughly washed with sterilized water. After that probiotics treated *T.decipiens* were transferred to the larval rearing tanks of *P.conchonius*

Isolation of bacteria from the gut of fish, *Puntius conchonius*: The gut of *P. conchonius* fry was dissected into an aseptic chamber using a sterile scalpel and forceps. Dissected tissues were ground thoroughly in a sterile mortar and pestle with sterile water. Sufficient quantity of tissue sample (0.1-1 mg) was taken and pour into the nutrient agar plates. These plates were incubated at 37°C for 24 hr. After incubation, pure culture was prepared for each bacterial strain.

Total plate count: All samples were processed to estimate the total plate count (TPC) using pour plate method, with agar as the medium. The samples were serially diluted and plated on Agar. The plates were incubated at 37°C for 24 hr. Plates with 30-300 colonies were taken to determine the counts. Representative colonies were picked for further identification. The young cultures of bacterial isolates were subjected to Gram's staining and spore staining for morphological characterization. Isolated colonies were purified and pure cultures were used for biochemical tests as described by Buchanan and Gibbons (1979).

Statistical analysis: The results from the enumeration of bacteria given as mean values and standard deviations (SD) and were analysed by one – way analysis of variance (ANOVA). Duncan multiple range tests were used to identify difference for each treatment among the different experiment using SPSS 13.0 statistical software.

Results and Discussion

In the quantitative analysis of gut microflora of fish, before feeding probiotics were found to be *Staphylococcus* sps ($2.032\text{x}10^3$ CFUml^{-1}), *Streptococcus bovis* ($9.32\text{x}10^2$ CFU ml^{-1}), *Klebsiella* ($1.93\text{x}10^3$ CFUml^{-1}), *Enterobacter aerugenes* ($1.83\text{x}10^3$ CFU ml^{-1}), *Enterococcus* sps ($1.79\text{x}10^3$ CFU ml^{-1}), *Pseudomonas* sps ($1.02\text{x}10^3$ CFUml^{-1}) and *Micrococcus* sps ($1.3\text{x}10^3$ CFU ml^{-1}). Predominant bacteria that inhabited in gut of fish larvae were coagulase negative *Staphylococcus* species ($2.03\text{x}10^3$ CFU ml^{-1}). Total plate count of gut microflora of fish larvae, before feeding probiotics were enumerated as $1.2\text{x}10^4$CFU ml^{-1} (Table 1).

Table 1 : Bacterial count (CFU ml^{-1}) of different microbes in the gut of ornamental fish, *Puntius conchonius* before and after for 45 days of probiotic treatment

Microorganisms	Before probiotic treatment	After probiotic treatment			
		T1	T2	T3	T0
Total plate count	$1.2x10^4$	$1.08x10^4$	$1.06x10^4$	$1.27x10^4$	$2.10X10^4$
Moraxella sps	—	—	—	—	$3.27±0.08x10^3$
Micrococcus sps	$9.32±0.45x10^{2a}$	—	—	$2.63±0.06x10^{2b}$	$2.03±0.11x10^{3c}$
Streptococcus bovis	$2.03±0.04x10^{3a}$	—	—	$1.60±0.09x10^{c}$	$9.31±0.51x10^{2b}$
Staphylococcus sps	$1.79±0.008x10^{3a}$	—	$1.26±0.04x10^{2\ b}$	—	—
Streptococcus faecalis	$1.62±0.031x10^{3b}$	—	—	—	$2.35±0.30x10^{3a}$
Pseudomonas sps	$1.02±0.031x10^{3a}$	$5.05±5.72x10^{2b}$	—	$5.46±6.13x10^{2\ b}$	—
Enterobacter aerugenes	$1.83±0.05x10^{3b}$	$4.85±0.35x10^{2c}$	—	—	$3.59±0.03x10^{3a}$
Klebsiella	$1.93±0.05x10^{3a}$	—	—	$8.25±0.91x10^{2b}$	$1.98±0.17x10^{3a}$
Achromobacter sps	—	$1.85±0.35x10^{2d}$	$3.75±0.21x10^{2c}$	$5.59±0.12x10^{2b}$	$4.47±0.69x10^{3a}$
E.coli	—	—	$5.36±5.99x10^{2c}$	$7.05±0.35x10^{2b}$	$2.36±0.07x10^{3a}$
Bacillus coagulans	—	$9.67±0.007x10^3$	—	—	—
Bacillus mesentericus	—	—	$9.63±0.06x10^3$	—	—
Bifidobacterium infantis	—	—	—	$9.83±0.02x10^3$	—

Values are means of three replicates ± SD. Means with different superscript within the same row are significantly different ($P<0.05$) ; T_1 = *B. coagulans* treated fish larvae, T_2 = *B. mesentericus* treated fish larvae and T_3 = *Bifidobacterium infantis* treated fish larvae.

Table 2 : Bacterial count of copepod, *Thermocyclops decipiens* before probiotics (bacterial) enrichment

Microorganisms	Bacterial count (CFUml^{-1})
Total plate count	$3.8x10^3$
Achromobacter sps	$1.37±0.09x10^{3c}$
Pseudomonas sps	$1.58±0.06x10^{3b}$
E.coli	$2.13±0.04x10^{3a}$

Total count of microflora was made on whole organism. Counts are average of five copepods. Values are means of three replicates ± SD. Means with different superscript within the same column are significantly different ($P<0.05$)

Table 3 : Viable count of probiotics in copepod, *Thermocyclops decipiens* after 6 hr enrichment

Probiotics	Count of probiotics	Bacterial count of *T. decipiens* after probiotic enrichment
Bacillus coagulans	$5.8±0.007x10^{10b}$	$5.49±0.09x10^{6a}$
Bacillus mesentericus	$5.9±0.02x10^{10a}$	$5.36±0.06x10^{6b}$
Bifidobacterium infantis	$5.9±0.04x10^{10a}$	$5.27±0.11x10^{6c}$

Values are means of three replicates ± SD. Means with different superscript within the same column are significantly different ($P<0.05$)

Table 4 : Establishment of probiotics in fish *Puntius conchonius* after 5 days of probiotics administration

Probiotics	Bacterial count in fish gut (CFU ml^{-1})
Bacillus coagulans	$2.3±0.002x10^{3c}$
Baccillus mesentericus	$3.2±0.03x10^{3a}$
Bifidobacteium infantis	$3.1±0.02x10^{3b}$

Values are means of three replicates ± SD. Means with different superscript within the same column are significantly different ($P<0.05$)

After 45 days of experimental period administration of probiotics such as *B. coagulans, B. mesentericus and B. infantis* made strong influence on the gut inhabitants of fish larvae. General trend observed in the final gut analysis was increased proportion of aerobic, anaerobic spore forming bacteria and decreased or completely fleshed out anaerobic cocci, coliforms and bacteriods such as *E.coli*, *Klebsiella*, *Enterobacter aerugenes*, *Moraxella*, *Streptococcus bovis* and *Pseudomonas* sps (Table 1). Total plate count of T_1, T_2, T_3 and T_0 were reported to be as $1.08x10^4$, $1.06x10^4$, $1.27\ x10^4$ and $2.1x10^5$ CFU ml^{-1}, respectively. T_0 ($2.1x10^4$ CFU ml^{-1}) (without probiotic treated larvae) showed significant difference over T_1, T_2 and T_3 ($P<0.05$). The results are in agreement with several authors, who have demonstrated that probiotics microorganisms reduce the proliferation of pathogens by competing for attachment sites (Rinkinen *et al.,* 2003; Chabrillon *et al.,* 2005). Panchayuthapani *et al*. (1995), Venkat *et al.* (2004) reported the inhibitory effects of probiotics strains *Lactobacillus acidophilus* and *Lactobacillus sporogenes*. Probiotics were found to inhibit the growth of Gram negative gut microflora, which were dominant in the gut of the postlarvae *Macrobrachium rosenbergii.* The inhibitory effect may be due to lowering of pH. Due to the production of organic acids normally produced by the lactic acid bacteria or due to the competition for the nutrients or by bacteriocin production, some probiotics strains including *Lactobacillus* and *Lactococcus* have been reported to inhibit the adhesion of pathogenic bacteria to intestinal cells (Mukai *et al.,* 2002; Gueimonde *et al.,* 2006).

It has been observed that feeding probiotics to *T.decipiens*, followed by feeding these enriched *T. decipiens* to the fish larvae, was an effective means through which, delivery the probiotics to the fish larvae will be ensured at a high rate. Final gut analysis of *P. conchonius* revealed that ingested food and probiotics used for the trials strongly influenced the colonization of pathogenic bacteria in fish gut. Number of pathogenic bacteria was decreased when

compared to initial results. Bacteria isolated from the copepods were reflected in the gut of fish and these results confirm, observations made by Hansen *et al.* (1992) and Gatesoupe (1999). They have reported that the intestinal microflora of fish reflects the bacterial content of ingested food. However the microflora varies with salinity, use of antibiotics, diet and dietary components (Ringo *et al.,* 1995). The microbial community of feeding larvae has been found commonly to reflect the microbial composition of the live prey (Korsnes *et al.,* 2006; Verner-Jeffreys *et al.,* 2003).

The mean bacterial count in, before probiotics enriched *T.decipiens* found to be $3.8x10^3$CFUml^{-1}(Table 2). *Achromobacter* sps ($1.37x10^3$CFU ml^{-1}), *Pseudomonas* ($1.58x10^3$ CFU ml^{-1}) and *E. coli* ($2.13x10^3$CFU ml^{-1}) were enumerated from the *T. decipiens.* The investigation also examined the effectiveness of *T.decipiens* enrichment with probiotics such as *B. coagulans*, *B. mesentericus* and *B. infantis*. After, six hr of probiotics enrichment in *T.decipiens,* maximum enumeration of bacteria conferred with *B. coagulans* (5.49 $x10^3$ CFU ml^{-1}) (Table 3). However, the mean bacterial count in before and after probiotics enriched *T.decipiens* found to be siginificant differences between them ($p<0.05$).

Colonization can be defined as the potency for a probiotic to persist in the body for longer period than the inert marker (Marteau and Vesa, 1998). Adhesion properties are considered as an important issue, and particularly, ability to adhere to intestinal mucosa is one of the essential selection criteria for probiotics, since adhesion to intestinal mucosa represents the first step in colonization process (Tuomola *et al.,* 2001). In the present study, one week after probiotics administration the gut analysis of fish showed *Bacillus coagulans* ($2.3x10^3$ CFU ml^{-1}) conferred poor colonization when compared to *Bacillus mesentericus* ($3.2x10^3$ CFU ml^{-1}) and *Bifidobacterium infantis* ($3.1x10^3$ CFU ml^{-1})(Table 4). *B. coagulans* seems to be characterized by the inability to adhere to intestinal epithelium in piglets, where it is considered as a transient colonizer lost one week after administration (Adami and Cavazzoni, 1999).

To exert their beneficial effects probiotics must resist to the acidity of the stomach, lysozyme and bile acids (Tuomola *et al.,* 2001).Few data on acid and bile stability of *B.coagulans* are available (Hyronimus *et al.,* 2000). Thus, spores of *B. coagulans* could likely survive at gastric pH and reach the intestine, where sporulation could occur. After 45 days of experiment, the gut analysis of fish showed significant level of spore formation reported with *B.coagulans.* This study found that feeding probiotics to copepod *T.decipiens*, followed by feeding this enriched *T.decipiens* to fish *P.conchonius* was an effective means through which the probiotics delivery could be boosted. The administration of probiotics significantly changed the proportion of gut microflora of *P.conchonius*.

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