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Original Research Article

Studies on probiotics administration and its influence on gut microflora of ornamental fish *Brachydanio rerio* larvae

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Keywords

Probiotics, Enrichment, *Brachydanio rerio*, *Artemia parthenogenetica*.

A B S T R A C T

The present study was conducted to assess the establishment and effect of probiotic bacteria such as *Lactobacillus rhamnosus*, *Bacillus coagulans*, *Bacillus mesentericus*, *Bifidobacterium infantis* and *Bifidobacterium longum* inhabiting in the gut of 60 days old post larvae of the freshwater ornamental fish, *Brachydanio rerio*. Post-larvae of *B. rerio* were divided in six experimental groups with three replicates. Groups T_1, T_2, T_3, T_4, and T_5 were fed with nauplii of *Artemia parthenogenetica* enriched with *Lactobacillus rhamnosus*, *Bacillus coagulans*, *B. mesentericus*, *Bifidobacterium infantis* and *B. longum* respectively. Group T_0 was served as control (without probiotic treated *A. parthenogenetica*). The experiment lasts for 40 days. Total plate count of initial gut analysis of larvae showed significant level of pathogenic bacteria ($p<0.05$) and were enumerated as 1.35×10^4 CFU ml^{-1}. After 40 days of probiotics treatment, final gut microflora of post-larvae showed decreased level of pathogens. The total plate count of T_1, T_2, T_3, T_4, T_5 and T_0 were reported to be as $1.05x10^4$, $1.08x10^4$, $1.02x10^4$, $1.06x10^4$, $1.01x10^4$ and $2.9x10^5$ CFU ml^{-1}, respectively. All the experimental groups were significantly differed from control group ($p<0.05$). At the end of the experiment, establishment of probiotics were examined. One week after probiotics administration, gut analysis of fish larvae showed, high spore formation of *L. rhamnosus* ($2.1x10^3$ CFU ml^{-1}), when compared to *B. longum* ($3.25x10^3$ CFU ml^{-1}) and *B. mesentericus* ($3.15x10^3$ CFU ml^{-1}). The results from the study suggest that the probiotic bacteria significantly established in gut of *B. rerio* and significant effects on the pathogenic gut inhabitants of the fish.

Introduction

Probiotics are live microorganisms that confer a beneficial effect on the host when administered in proper amounts (Kalliomaki et al., 2001; Brown and Valiere, 2004).

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). Probiotic microorganisms are mostly of human or animal origin; however, some studies show that strains recognized as probiotics are also found in non-dairy fermented substrates (Schrezenmeir and de Vrese, 2001). There is little information about the microorganisms challenges for survival, the criteria for fermentation, their use as starters, and their relationship with other microorganisms (Kedia et al., 2007).

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). *Artemia* are indispensable food organisms in the industrial larviculture of fish and crustaceans (Lee and Ostrowski 2001; Liao et al., 2001; Shields 2001; Marte 2003) owing to their small size, slow swimming behaviour, rapid reproduction rate, ability to be cultured at high density (Lubzens 1987; Lubzens et al., 1989; Lubzens et al., 2001), and usefulness for transferring probiotic bacteria to host aquatic animals (Makridis et al., 2000; Ziaei-Nejad et al., 2006).

In the present study *Artemia parthenogenetica* nauplii were used as a vector for fish larvae. The present aimed to investigate the establishment of probiotics (*Lactobacillus rhamnosus*, *Bacillus coagulans*, *Bacillus mesentericus*, *Bifidobacterium infantis* and *Bifidobacterium longum*) in the gut of fish, *Brachydanio rerio* larvae and its possible effect on pathogenic microorganism confined to gut of fish larvae.

Materials and Methods

Isolation of bacteria

The ornamental fish, *Brachydanio rerio* fries were collected from a local hatchery at Alathur, near Palakkad Dist, Kerala, India. The initial length and weight of the fishes were (1.58±0.122 cm) and (0.0718±0.002 g) respectively. Upon reaching the laboratory at Department of Aquaculture and Fishery Microbiology, M.E.S. Ponnani College, Ponnani, Kerala, India, the gut of *B. rerio* fry was dissected out into an aseptic condition (divya et al., 2012). Dissected tissues were ground thoroughly using sterile mortar and pestle with sterile water. Sufficient quantity of tissue (0.1mg) homogenate was streaked into the nutrient agar plates. These plates were incubated at 37^{o}C for 24 hr. After incubation, pure culture was prepared for each bacterial strain.

Preparation of probiotic diet and enrichment:

Pure culture of probiotics was centrifuged at 5000 xg for 30 min. After centrifugation, the pellets were washed twice with saline. After washing probiotics were aseptically enriched with 24hrs old *Artemia parthenogenetica* nauplii for 6 hr duration according to (divya et al., 2012). The cysts of *Artemia parthenogenetica* were collected from the saltpans of Kelambakkam, Southeast coast of India and were purified and stored. Purified cysts were hatched out in 35ppt sea water. Freshly hatched Artemia nauplii were harvested and transferred to enrichment containers (2L) at a density of 50 individuals ml^{-1} at room temperature (28 ± 1^{o}C). The nauplii of *A. parthenogenetica* were enriched with different probiotic bacteria, 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 enrichment, the nauplii of *Artemia* were collected from the enrichment medium, and thoroughly washed with sterilized water and fed with the ornamental fish, *Brachydanio rerio*.

Experimental conditions and culture of bacteria

Post larvae of *Brachydanio rerio* (60 days old) were used for the present experiment. The experiments were divided into six experimental groups, with three replicates, and were designated as T_1, T_2, T_3, T_4, T_5 and T_0. In the present study *Lactobacillus rhamnosus, Bacillus coagulans, Bifidobacterium longum, Bifidobacterium infantis* and *Bacillus mesentericus* was a probiotic bacteria, which have obtained from Cochin University, Cochin, Kerala, India. And these were cultured in the nutrient agar slant.

All these experimental groups such as T_1, T_2, T_3, T_4 and T_5 were represented the probiotics *Lactobacillus rhamnosus*, *Bacillus coagulans, B.mesentericus, Bifidobacterium infantis, B. longum* enriched *Artemia* nauplii fed fish larvae respectively. Group T_0 represents the fish larvae fed with unenriched *Artemia* nauplii. This experiment lasts for 40 days.

Total plate count

All samples (fish gut homogenate and Artemia nauplii) 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^{o}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

Initial mircroflora in *Artemia parthenogenetica* nauplii and gut of *Brachydanio rerio* larvae were analysed. Bacterial count in Artemia nauplii were performed in whole individual. The mean bacterial count in before probiotics enriched Artemia nauplii found to be *E.coli* $1.4\pm0.05X10^3$, *Vibrio* Species $1.2\pm0.13X10^3$ and *Pseudomonas* Species $1.6\pm0.05X10^3$ were observed from the Artemia *parthenogenetica* (Table 1). The study also observed the effect of probiotics enrichment in Artemia nauplii. After 6hrs of enrichment, the probiotics such as *Lactobacillus rhamnosus, Bacillus coagulans, B. mesentericus, Bifidobacterium infantis* and *B. longum* showed maximum viability in Artemia nauplii. maximum number of colony formation was obtained with *Lactobacillus rhamnosus* (Table 3).However, the mean bacterial count in before and after probiotics enriched Artemia

nauplii obtained to be significant differences between them($p<0.05$).

The quantitative analysis of initial gut flora of *B. rerio* larvae presumptively identified microbes were *Streptococcus faecalis* (8.5×10^2), *Micrococcus spp* (7.25×10^2), *Enterobacter aerogenes* (8.45×10^2), *Enterococcus spp* (1.8×10^3)., *Pseudomonas aeroginosa* (3.25×10^2), Coagulase negative *Staphylococcus spp* (9.3×10^2), and *Escherichia coli* (1.6×10^3). *E.coli* and *Enterococcus* sp. were high in the gut of zebrafish larvae when compared to other microbes (Table 2).

Microbial analysis of gut from Brachydanio rerio larvae at 40 days revealed that probiotics (such as *Lactobacillus rhamnosus*, *Bacillus coagulans*, *B. mesentericus*, *Bifidobacterium infantis* and *B. longum*) 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*, *Streptococcus* sp. and *Pseudomonas* sp. (Table 2).

Total plate count of T_1, T_2, T_3, T_4 and T_5 were reported to be $1.05x10^4$, $1.08x10^4$, $1.02x10^4$, $1.06x10^4$, and $1.01x10^4$ CFU ml-1 and respectively. T_0 ($2.9x10^5$ CFU ml^{-1}) (without probiotic treated larvae) showed significant difference over T_1, T_2, T_3, T_4, and T_5 ($P<0.05$).

The present study also examined the establishment of probiotics in the gut of zebrafish after one week of probiotics administration. 40 days of probiotics treatment significantly affected the viability of probiotics in the gut of fish larvae. *Lactobacillus rhamnosus* showed high spore formation in the gut of fish when compared to other probiotics (Table 4). It ensures the effective delivery probiotic through the enriched *Artemia* nauplii feeding.

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 of the probiotic bacteria are considered as an important issue, and particularly, ability to adhere to intestinal mucosa is one of the essential selection criteria, 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 *Lactobacillus rhamnosus* ($2.1x10^3$ CFU ml^{-1}) conferred poor colonization when compared to *Bifidobacterium longum* ($3.2x10^3$ CFU ml^{-1}) and *Bacillus mesentericus* ($3.15x10^3$ CFU ml^{-1}). *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).

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.

Table.1 Bacterial Count of *Artemia parthenogenetica* before Probiotics (Bacteria) enrichment

Microorganisms Total plate count	Bacterial count (CFU ml^{-1})
Total plate count	$4X10^{3}$
E.coli	$1.4\pm0.05X10^{3b}$
Vibrio species	$1.2\pm0.13X10^{3c}$
Pseudomonas species	$1.6\pm0.05X10^{3a}$

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

Table.2 Bacterial count of (CFU ml-1) of different microbes in the gut of ornamental fish, *Brachydanio rerio* before probiotic treatment

S.No	Bacteria	Colony count
	Total plate count	8.195×10^{3}
1.	*Streptococcus faecalis*	$8.50\pm0.071 \times 10^{2cd}$
2.	*Micrococcus* species	$7.25\pm0.035x\ 10^{2bc}$
3.	*Enterobacter* species	$8.45\pm0.0636 \times 10^{2cd}$
4.	*Streptococcus bovis*	$3.30\pm0.0424 \times 10^{2a}$
5.	*Enterococcus species*	$1.875\pm0.1061x\ 10^{3f}$
6.	*Pseudomonas* species	$3.25\pm0.0354 \times 10^{2a}$
7.	*Staphylococcus* species	$9.30\pm0.0424 \times 10^{2d}$
8.	*Escherichia coli*	$1.675\pm0.106x\ 10^{3e}$
9.	*Klebsiella*	$6.40\pm0.0566 \times 10^{2b}$

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

Table.3 Bacterial Count of Artemia nauplii, before and after Probiotic enrichment

Probiotics	Before enrichment CFU/ml^{-1}	After enrichment (*Artemia* gut) CFU/ml^{-1}	After 5days of administration (Fish gut) $_1$ CFU/ml^{-}
Lactobacilus rhamnosus	$5.9\ \pm0.707x10^{10}$	$6\ \pm0.141x10^{6}$	$2.1\ \pm0.141x10^{3a}$
Bacillus coagulans	$5.8\ \pm0.566x10^{10}$	$5.8\ \pm0.424x10^{6}$	$3.05\ \pm0.212x10^{3b}$
Bifidobacterium infantis	$6\ \pm0.566x10^{10}$	$5.9\ \pm0.565x10^{6}$	$3\ \pm0.282x10^{3b}$
Bifidobacterium longum	$5.7\ \pm0.424x10^{10}$	$5.87\ \pm0.282x10^{6}$	$3.25\ \pm0.212x10^{3b}$
Bacillus mesentericus	$5.7\ \pm0.849x10^{10}$	$5.85\ \pm0.707x10^{6}$	$3.15\pm0.070x10^{3b}$

Total count of microflora was made on whole organism. Counts are average of five Artemia nauplii. Values are means of three replicates ±SD. Means with different superscript with in the same column are significantly different (*P<0.0*

Table.4 Bacterial counts (CFU ml^{-1}) of different microbes in the gut flora of fish *B.rerio* larvae after 40 days of probiotic treatment

Micro organisms	**Treatments**					
	T1	**T2**	**T3**	**T4**	**T5**	**T0**
TPC count	1.05×10^{4d}	1.08×10^{4c}	1.02×10^{4e}	1.06×10^{4b}	1.01×10^{4}	2.9×10^{5a}
Streptococcus faecalis	-	-	-	-	-	$4.250\pm0.071\times10^{3}$
Micrococcus species	-	$3.30\pm0.141\times10^{2d}$	$2.355\pm0.205\times10^{2c}$	$2.15\pm0.353\times10^{2b}$	$1.05\pm0.071\times10^{2a}$	$1.275\pm0.106\times10^{3a}$
Enetrobacter aergenes	-	-	-	-	-	$3.415\pm0.0212\times10^{3}$
Enterococcus species	-	-	-	-	-	$2.050\pm0.071\times10^{3}$
Pseudomonas aeruginosa	$4.64\pm0.205\times10^{2c}$	$4.25\pm0.354\times10^{2c}$	$2.77\pm0.325\times10^{2b}$	$3.02\pm0.374\times10^{2d}$	$1.27\pm0.0989\times10^{2a}$	$1.415\pm0.0212\times10^{3a}$
Staphylococcus species	-	-	-	-	-	$5.30\pm0.424\times10^{2}$
Escherichia coli	-	$2.10\pm0.424\times10^{2b}$	-	-	-	$4.415\pm0.120\times10^{3a}$
Streptococcus salivaris	-	-	-	$1.90\pm0.141\times10^{2}$	-	-
Streptococcus lactis	-	$4.67\pm0.467\times10^{2}$	-	-	-	-
Streptococcus bovis	$9.28\pm0.39\times10^{2}$	-	-	-	-	-
Lactobacillus rhamnosus	-	-	-	-	$6.720\pm0.098\times10^{3}$	-
Bacillus coagulans	-	-	-	$9.907\pm1.52\times10^{3}$	-	-
Bacillus mesentericus	-	-	$8.550\pm0.212\times10^{3}$	-	-	-
Bifidobacterium longum	$9.322\pm0.031\times10^{3}$	-	-	-	-	-
Bifidobacterium infantis	-	$9.412\pm0.581\times10^{3}$	-	-	-	-

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

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).

The results of the present study with the fish gut analysis of *B. rerio*, after 40 days of probiotic treatment, had strong influence over the colonization of probiotic bacteria. Numbers of bacteria was decreased when compared to initial stage of the experiment. Ringo and Birkbeck (1999) reported that in general, *Aeromonas* spp., *Pseudomonas* spp., and bacteria of the *Flavobacterium/ Cytophaga* group were the most common bacteria in the intestines of freshwater fish. There are some reports indicating the presence of lactic acid bacteria in larvae and fry intestines (Kvasnikov et al., 1977; Ringø and Birkbeck, 1999). Hansen et al. (1992) and Gatesoupe, 1999, reported that the intestinal microflora of fish reflects the bacterial content of ingested food and of the environment. The microflora varies with salinity, use of antibiotics, diet and dietary components (Ringø et al., 1995). According to Ringø et al. (1995), the predominant bacterial genera/species isolated from most fish guts were aerobic or total anaerobic microorganisms.

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 40 days of experiment, the gut analysis of fish showed significant level of spore formation reported with *Lactobacillus rhamnosus* followed by *Bacillus coagulans.* This study found that feeding probiotics to *Artemia franciscana* nauplii, followed by feeding this enriched Artemia nauplii to fish *Brachydanio rerio* larvae was an effective means through which the probiotics delivery could be boosted. The administration of probiotics significantly changed the proportion of gut microflora of zebrafish larvae.

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