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Short Communication

EFFECT OF PROBIOTIC BACTERIA CULTURE ON PATHOGENIC BACTERIA FROM FRESH WATER FISH *OREOCHROMIS MOSSAMBICUS*

Johndevadoss Gobinath and Ravichandran Ramanibai*

Department of Zoology, University of Madras, Guindy campus, Chennai 600 025, Tamil Nadu, India

*Corresponding Author e-mail: rramani8@hotmail.com

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Abstract

Fish diseases are one of the major problems in fresh water biota. There is an urgent need in aquaculture to develop microbial control strategies, since diseases epidemics are recognized as important constraints to aquaculture production, trade and the development of antibiotic resistance has become a matter of growing concern. In this study, we evaluated the tilapia fish (*Oreochromis mossambicus*) microbial population and their bio efficacy on aquaculture. Totally 2 rectangular tanks was each stacked with 15 infected fish averaging 22.80 ± 1.0 g and those were fed at maintenance level for 15 days prior to the experiments. About 29 *Lactobacillus* isolates were isolated from *O. mossambicus*. Among the 29 *Lactobacillus* isolates, the isolate RR17 was selected for further study. Based on morphological, biochemical characteristics, the isolates were identified as *Lactobacillus* sp. The pathogens were isolated, characterized and identified as *Aeromonas* sp., *Vibrio* sp., *Escherichia coli*, *Pseudomonas* sp. and *Salmonella shigella*. The isolate *Lactobacillus* sp. RR17 was screened for antagonistic activity against all the pathogens by agar diffusion assay. The isolate *Lactobacillus* sp. RR17 showed significant antagonistic activity against only three pathogens. The isolate was multiplied and the fish feed was supplemented with the isolate *Lactobacillus* sp. RR17. The results revealed that the size and weight of the fish drastically increased when compare to control fish. Thus, the present study clearly demonstrated that the *Lactobacillus* isolates could be used successfully as probiotic bacteria in aquaculture.

***Keywords*:** Probiotic, *Lactobacillus*, *Oreochromis mossambicus*, Antagonistic activity, Tilapia fish

INTRODUCTION

The term “probiotic” was early 1970s to introduce the microbial feed supplements to both human and animals (Berg, 1998). The first study on the screening of probiotic bacteria from the aquaculture environment was reported in 1980s (Dopazo *et al.*, 1988; Kamei *et al.*, 1988). In aquacultural practices, the interaction between the microbiota and the host is not limited only to the intestinal tract alone. Probiotic bacteria can also be active on the gills or the skin of the host or in its ambient environment. Fish exposed to various diseases are one of the major concerns the field of fresh water biota. There is an urgent need in aquaculture discipline to develop microbial control strategies, since diseases epidemics are recognized as important constraints to aquaculture production, trade and the development of antibiotic resistance has become a matter of growing concern. Thus, the use of probiotics for aquatic animals is increasing with the demand for ecofriendly sustainable aquaculture practices (Gatesoupe, 1997). The benefits of probiotic dietary supplements include improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, growth promoting factors and increased immune response (Verschuere *et al.*, 2000). The effect of probiotics for aquaculture has been reported earlier (Mohanty *et al.*, 1993; 1996; Sharma and Bhukhar, 2000; Wang *et al.*, 2005; Wang and Xu, 2006).

Therefore, this study aimed to examine the inhibitory activity of *Lactobacillus* sp. isolated from the fish intestinal tract, identify and to evaluate the bacteria for disease prevention in tilapia fish (*O. mossambicus*) also to test the beneficial bacterial isolates for antagonistic activity against putative fresh water fish pathogenic bacteria.

MATERIAL AND METHODS

Sample collection

The bacteria infected and healthy *Oreochromis mossambicus* fishes were collected from Krishnagiri Reservoir Project (KRP) dam, Krishnagiri, Dharmapuri district, Tamil Nadu, India during July 2010 to March 2011 using cast net. All the infected and healthy fishes were examined for pathogenic and probiotic bacteria separately.

Isolation and cultivation of beneficial and pathogenic bacteria from *O. mossambicus*

From the swabs of the specimens' gills, muscle and intestine regions of healthy and infected fish were collected and inoculated onto culture media such as MRS agar, MRS broth, TCBS agar and SSA. The agar plates and broth were incubated at 37°C for 24 to 48 hours and the bacterial colonies were observed and sub-cultured for further characterization and identification. The colony morphology such as color, size and margin were recorded then subjected to Gram staining reaction and motility test.

Biochemical characterization

The biochemical characterization such as Gram staining motility, starch hydrolysis, nitrate reduction, oxidase catalase, indole, H_2S production and carbohydrate fermentation were carried out according to the guidelines outlined by Griffin, (1992).

Preparation of probiotic feed

The fish feed was prepared out of ground nut cake (40%), Soyabean (20%), rice bran (33%), meal (5%), vitamin and mixer (2%). The *Lactobacillus* sp. RR17 was grown for 72 h at 25°C in MRS broth and harvested by centrifugation (12000 rpm for 15 min). The cells were re-suspended in 100 ml of 0.9% saline. Emulsion of egg albumin was applied to fish feed by mixing in a drum mixer for 15 min. Control diets were prepared as feed lacking of *Lactobacillus* sp. RR17

Maintenance and feeding schedule

Collected tilapia fish *O. mossambicus* were stocked for acclimation in hundred litre rectangular tanks for 15 days, totally 2 rectangular tanks were maintained each stacked with 15 infected fish averaging 22.80 ± 1.0 g. Which were fed at maintenance level for 15 days prior to experiments. All the tanks were aerated and experiments were carried out at the temperature of 29 ± 1°C. Fishes were fed twice a day, using feed coated with *Lactobacillus* sp. RR17. The control fish was fed with feed free from *Lactobacillus* sp. RR17. The experiments lasted up to 3 months.

Antagonistic activity

Five sets of experiment, *Aeromonas* sp., *Vibrio* sp., *E. coli, Pseudomonas* sp. and *salmonella shigella* were conducted for 15 days. For each bacterium 10 conical flasks (100 ml) have culture medium (50 ml) containing pure strain of bacterial fish pathogens. To which 2 ml of probiotic bacterial culture was added to the flask. The control flask was maintained only pathogenic bacteria and without the addition of probiotic bacteria. The entire flasks were incubated at 37°C for 15 days. Starting from first day, the number and growth of organism was monitored using standard dilution technique i.e. 10^{-4} to 10^{-5} in sterilized test tube and finally colony forming units (CFU/ml) was enumerated by pour plate method, similar methods were followed for both pathogens in every 5 days intervals.

RESULTS

Identification and characterization of microorganisms

Twenty nine *Lactobacillus* isolates were obtained from tilapia fish *O. mossambicus* and designated as RR1–RR29. Among the 29 *Lactobacillus* isolates, only one distinct isolate RR17 was selected for further study. Selected isolate *Lactobacillus* sp. RR17 was morphologically characterized as Gram positive, rod shape and non motile. In MRS agar, beneficial bacteria showed district variation i.e., white smooth, irregular colony (Table 1).

Table 1: Characterization of *Lactobacillus* and pathogenic bacteria

Characteristic	***Lactobacillus* sp.**	***Aeromonas* sp.**	***Vibrio* sp.**	***E. coli***	***Pseudomonas* sp.**	***Salmonella shigella***
Morphology	rod	rod	rod	rod	rod	rod
Gram staining	+	–	–		–	–
Motility	–	+	+	+	+	+
Selective Media	MRS Agar White, smooth irregular colony	-	TCBS medium Green color	EMB Violet color	CAB White color irregular colony	SSA Agar Brown color regular colony

+ Positive; – negative

Table 2: Biochemical characteristics of *Lactobacillus* and pathogenic bacteria

Characteristic	***Lactobacillus* sp.**	***Aeromonas* sp.**	***Vibrio* sp.**	***E. coli***	***Pseudomonas* sp.**	***Salmonella shigella***
Indole	–	–	–	+	–	+
Methyl red	–	–	+	+	+	+
Voges proskur	–	–	–	–	+	+
Citrate utilization	+	–	–	–	–	–
Nitrate reduction	–	–	+	+	+	+
Urease	+	+	–	–	–	–
Catalase	–	+	+	+	+	–
Oxidase	–	–	+	+	+	+
Arabinose	–	–	–	A/G	–	–
Fructose	+	+	A/G	A/G	–	A/G
Glucose	+	–	A/G	+	A/G	+
Lactose	+	–	A/G	A/G	A/G	A/G
Maltose	+	+	–	+	A/G	+
Mannitol	+	+	–	+	+	+
Rhamnose	+	+	–	–	A/G	–
Arabinose	–	–	–	A/G	–	A/G

+ Positive; – Negative
A/G: Acid/Gas

Among the various biochemical assays studied the positive results were observed in *Lactobacillus* isolates such as urease, catalase, fructose, glucose, lactose, maltose, mannitol and rhamnose whereas the negative results were indole, methyl red, vogus proskur, citrate utilization, nitrate reduction, oxidase and arabinose. Fish pathogens, such as *Aeromonas* sp., *Vibrio* sp., *E. coli*, *Pseudomonas* sp., *Salmonella shigella* were isolated from the gills, intestine, muscles region of infected fishes. These five pathogens were morphologically characterized as *Aeromonas* sp., *Vibrio* sp., *E. coli, Pseudomonas* sp. They were physically Gram negative, motile and rod shape.

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In SSA agar, *Salmonella shigella* showed distinct variations i.e. brown in color, regular colonies of isolates and *Vibrio* sp. showed green color, round regular colony in TCBS agar, and in case of CAB agar, *Pseudomonas* showed white color, irregular colonies of isolates, and *E. coli* showed violet color, regular colonies in EMB agar. These five pathogens isolate

showed distinct taxonomic characteristics described in Table 1.

Among the various biochemical assays studied the positive results were observed in all five pathogens isolates, such as methyl red, nitrate reduction, catalase, oxidase and maltose, mannitol, rhamnose for sugar assimilation. Whereas the negative results were obtained in indole, vogus proskur, citrate utilization, urease and arabinose for sugar assimilation. Arabinose, fructose, glucose, lactose were fermented by isolated *Vibrio* sp., *E. coli*, *Pseudomonas* sp. and *salmonella shigella* produce acid and gas (Table 2).

Based on the morphological characteristics and biochemical properties, the isolated fish pathogens are identified as *Aeromonas* sp., *Vibrio* sp., *E. coli*, *Pseudomonas* sp., *Salmonella shigella*.

Antagonistic effect

To evaluate the antagonistic effect of *Lactobacillus* sp. RR17 isolate against the fresh water fish pathogens, *Vibrio* sp., *Aeromonas* sp., *E. coli*, *Pseudomonas* sp. and *Salmonella shigella* isolates were isolated from the *O. mossambicus*. The antagonistic activity of *Lactobacillus* sp. RR17 isolate was screened against fish pathogens by agar plate assay method. The zone of inhibition showed against only *Aeromonas* sp., *Vibrio* sp. and *E. coli* (Table 3).

The size and weight of the *O. mossambicus* increased nearly 26 g when compared to that of control fish not fed with probiotic feed as 22.8 g. We concluded that the *Lactobacillus* isolates could effectively used as probiotic bacteria in aquaculture farms which showed greater growth improvement.

Table 3: Screening of antagonistic activity in *Lactobacillus* against the five fish pathogens

Pathogens	Zone of inhibition (*Lactobacillus*)
***Aeromonas* sp**	+
***Vibrio* sp**	+
E. coli	+
***Pseudomonas* sp**	–
salmonella shigella	–

+ : Presence; – : Absence

DISCUSSION

The use of probiotics for disease control in aquaculture is an area of increasing interest, as the use of antibiotics is causing concern over the possible development of antibiotic resistant in bacteria, probiotics have been defined by the World Health Organization, Food and Agriculture Organization as “live microorganism” which when administered in adequate amounts, confer a health benefit on the host “In the past decade (Fuller, 1989). Several Gram negative and Gram positive bacteria have been evaluated in the *in vitro* or *in vivo* for their potential to inhibit pathogenic organisms and overcome infections in fish and larvae in aquaculture (Itoh *et al.*, 1995).

In the present study, fresh water fish tilapia *O. mossambicus* were collected and 29 *Lactobacillus* isolates was obtained. The similar passion was also attempted by Hiu *et al.*, (1984). *Lactobacillus* has been found to produce metabolic products that play important role in controlling undesirable microflora in the gut. Collins et al. (1987) reported that most *Lactobacillus* isolated in their study were assigned to *Carnobacteium* strains belonging to this genus, or to the former species *L. divergens* and *L. carnis* have been isolated from a fish and sea food.

The isolated *Lactobacillus* was culturally, morphologically and biochemically characterized and identified. Based on the morphological features such as colony color, shape and size are characterized. In biochemical identification, isolate examined to indole, vogus proskur, citrate utilization, urease, arabinose, methyl red, nitrate reduction, catalase, oxidase and maltose, mannitol, rhamnose for sugars assimilation. Holt (1994) outlined in Bergey’s manual of systematic bacteriology for the characterization of *Lactobacillus* on the basis of morphological, physiological tests. Gibello *et al.*, (2005) also reported the biochemical characterization is commonly used technique to differentiate one bacterial strain to others.

In the present investigation, the antagonistic effects of isolate *Lactobacillus* sp. RR17 against the fresh water fish pathogens. Similarly, this finding is co-inside with the findings of Joborn *et al.* (1997), who reported inhibitory activity against *Aeromonas salamonicida* and *Vibrio anguillarum* in intestinal mucus, arising from growth of this strain. The study concluded that the *Lactobacillus* sp. RR17 isolate will

be helpful in the management of bacterial disease in tilapia *O. mossambicus*. The species identification, optimization of *Lactobacillus* growth, *in vivo* effect on pathogen in fish under pathology status will be a further course of work.

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