FACTA UNIVERSITATIS
Series: **Medicine and Biology** Vol.15, No 3, 2008, pp. 97 - 102 UC 613.2:639.3

PROBIOTIC EFFECT OF *LACTOBACILLUS ISOLATES* AGAINST BACTERIAL PATHOGENS IN *CLARIAS ORIENTALIS*

Dharumaduari Dhanasekaran[1], Subhasish Saha, Noorudin Thajuddin[1], Annamalai Panneerselvam[2]

[1]Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, 620 024
[2]P.G. & Research Department of Botany & Microbiology, A.V.V.M. Sri Pushpam College, (Autonomous), Poondi, 613 503 Thanjavur District, Tamil Nadu, India
E-mail: dhansdd@yahoo.co.in

Summary. *About 59 Lactobacillus isolates were isolated from 5 different fresh water sites such as Cat fish (Clarias orientalis), Hari fish (Anguilla sp), Rohu fish (Labeo rohita), Jillabe fish (Oreochromis sp) and Gende fish (Punitus carnaticus). Among the 59 isolates only 4 Lactobacillus isolates were selected for further study. Based on morphological, biochemical characteristics, the isolates were identified as Lactobacillus sp. The pathogens were isolated, characterized and identified as Vibrio parahaemolyticus, Aeromonas sp and Aeromonas salmonicida. The Lactobacillus isolates were screened for antagonistic activity against Aeromonas, Vibrio sp. by agar diffusion assay. Among the 4 isolates, Lactobacilli RLD2 showed significant antagonistic activity against Aeromonas and Vibrio sp alone. This isolate was further evaluated by standard plate count assay for the viability of pathogen. The isolate was multiplied and the fish feed was supplemented with Lactobacillus isolates. The results reveal that the size and weight of the fish statically increased in comparison to that of control fish. The present study concluded that the Lactobacillus isolates will be used as probiotic bacteria in aquaculture, to manage aeromonasis.*

Key words: *Probiotic bacteria, Lactobacillus, Antagonistic activity, Aeromonas, Vibrio*

Introduction

Fish borne disease is a common problem encountered even in these modern days, which is said to be the period of scientific development and awareness of hygiene. There is an urgent need in aquaculture to develop microbial control strategies, since disease outbreaks are recognized as import constraints to aquaculture production, trade and the development of antibiotic resistance has become a matter of growing concern. Aquaculture of finfish, crustaceans, mollusks and algal plants is one of the fastest growing food producing sectors, having grown at an annual rate of almost 10% from 1984 to 1995 compared with 3% for livestock meat and 1.6% for capture fisheries production (1).

For instance, disease is now considered to be the limiting factor in the shrimp culture sub-sector (2). So far, conventional approaches, such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic disease. Furthermore, there is a growing concern about the use and particularly, the abuse of antimicrobial drugs not only in human medicine and agriculture but also in aquaculture. The massive use of antimicrobials for disease control and growth promotion in animals, increases the production in animals, increases the selective pressure excreted on the microbial world and encourages the natural emergence of bacterial resistance. Not only can resistant bacteria proliferate after an antibiotic has killed off the other bacteria, but they can also transfer their resistance genes to other bacteria that have never been exposed to the antibiotic. The sub-therapeutic (prophylactic) use of antibiotics related to those used in human medicine or the use of any antimicrobial agent for cross-resistance to antimicrobials used in human medicine could pose a particularly significant hazard to human health (3). According to the World Health Organization (WHO), much needs to be done to reduce the overuse and inappropriate use of antimicrobials. The emphasis in disease management should be on prevention, which is likely to be more cost effective then cure. This may lead to less reliance on the use of chemicals.

In recent years, "Probiotics" is defined more precisely as "mono or mixed cultures of live microorganisms which, when applied to animal, beneficially affect the host by improving the properties of the indigenous micro flora". The term "Probiotic" inevitably refers to Gram-positive bacteria associated with the genus *Lactobacillus.* However, nowadays, there has been a renewal of interest in the use of probiotics. In general terms, a group of requirements have been identified as important properties for *Lactobacilli* to be effective probiotic organism (4). These include the ability to adhere to cells, exclude or reduce pathogenic adherence, persist and multiply, produce acid, resist vaginal microflora, be safe and therefore noninvasive, noncarcinogenic and non-pathogenic and, co-aggregate and form a normal.

Yasuda (5) anticipated that bacteria would be found to be useful both as food and as biological control agents of disease and activators of the rate of nutrient regeneration in aquaculture. *V. alginolyticus* has been employed as a probiotic in many Ecuodoran Shrimp hatcheries since late 1992. The overall antibiotic use was decreased by 94% between 1991 and 1994. Also *Aermonas hydrophila* has been reported as a normal microflora of aquatic and terrestrial organisms as well as etiological agents of disease in numerous cold-blooded and warm-blooded animals including humans (6).

Recently, the cultivation of cat fish in Vellore District, Tamil Nadu, has become a good employment for farmers and unemployed youth to fulfill the food need, but the cat fish cultivates face bacterial diseases problems. Hence the present work was selected to screen the probiotic bacterial from fresh water and their antagonistic evaluation of bacterial diseases control with following objectives. Isolation and identification of probiotic bacteria from different fresh water fishes in Vellore District, Tamil Nadu. Screening of antagonistic activity of *Lactobacillus* isolates against fresh bacterial pathogens. In vitro evaluation of bacterial pathogen control using antagonistic *Lactobacillus sp.*

Materials and Methods

Materials

In the present investigation, a total of five fresh-water ponds were selected randomly in Vellore District, located at Pottuthaku, Walaja, Ranipet, Otteri, Sathuvachari and the bacteria-infected and healthy fish were collected from these ponds during the first week of August 2005 using cast net. All the infected and healthy fish were then examined for pathogenic and probiotic bacteria respectively.

Methods

Using sterile swabs the specimens from oral and gut region of healthy and infected fish were collected for *Lactobacilli* and other pathogens respectively. The collected swabs were inoculated in *Lactobacilli* MRS agar, MRS broth, TCBS agar and SAA. The agar plates and broth were incubated at 37°C for 24-48 hours and the bacterial colonies were examined for further characterization and identification. The colony morphology such as colour, size and margin were recorded. The bacterial colonies were then subjected to Gram staining reaction and motility test. Similarly, the cultures were also biochemically analysed.

The *Lactobacilli* species were inoculated in MRS broth and incubated for 7 days on rotary shaker at 28± 2°C. The cultures were then centrifuged and the supernatant fluid sterilized by syringe filter to collect the bacteriocin. The Muller Hinton agar plates were seeded with 24-hour bacterial pathogen *Aeromonas sp.* and *Vibrio parahaemolyticus*. The culture fluid was loaded in the wells made on the agar surface and the plates were incubated at 37°C for 24 – 48 hours.

The fish feed was prepared as ground nut cake (40%), Soyabean (20%), rice bran (33%), meal (5%), Vitamin and mineral mixer (2%). The *Lactobacillus sp* was grown for 72 hrs at 25°C in MRS agar and harvested by centrifugation (10000 rpm for 15 min). The cells were re-suspended in 100ml of saline. Egg albumin was added and this emulsion was then applied to fish feed by mixing in a drum mixer for 15 min. Control diets were prepared as feed devoid of *Lactobacillus sp*

Juveniles of cat fish (Clarias orientalis) were obtained from pond, Poothuthaku, Vellore District and transported to the Zoology Laboratory, APCAS, Kalavai and were stocked for acclimation in rectangular tanks for 10 days, 6 rectangular tanks were each stacked with 4 juveniles averaging 0.5± 0.06g which were fed at maintenance level for 10 days prior to the experiments. All the tanks were aerated and the experiments were carried out at the water temperature of 28 ± 1°C. Fish were fed twice a day, using feed coated with *Lactobacilli* sp. The control fish was fed with feed free from *Lactobacilli* sp. level of the nutrient below the minimum requirement. The experiment lasted up to 2 month.

Two sets of experiment, one for *Aeromonas* and another for *Vibrio parahaemolyticus,* were conducted for 15 days. For each bacterium there were 10 conical flasks (100 ml) of 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 contained 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 culture method, similar methods were followed for both pathogens at 5 days intervals.

Results

From the 5 gut samples collected, a total of 59 *Lactobacillus* isolates were isolated. The maximum *Lactobacilllus* isolates was observed in cat fish (*Clarias orientalis*) (23 isolates) and minimum in Gende fish (*Punitus carnaticus*) (3 isolates). Hari fish (*Anguilla sp*), Rohu fish (Labeo *rohita*) and Jilabe fish (*Oreochromis sp*) contain 16, 10 and 7 isolates respectively. This finding reveals the Probiotic (*Lactobacillus)* bacterial distribution varies according to the generic variation of fresh water fish (Table 1).

Among the 59 *Lactobacillus* isolates, 4 distinct isolates were selected for further study. These four Lactobacillus isolates were morphologically characterized, all four isolates, namely RLD_1, RLD_2, RLD_3 and RLD_4, were Gram positive, non-motile, the shapes of the isolates were various rod, stout rod, bacilli, short bacilli of RLD_1, RLD_2, RLD_3 and RLD_4 isolates respectively.

In MRS agar, these four isolates showed distinct variation i.e., creamy smooth edges, convex, dry rough,

irregular, white irregular, opaque, shiny smooth irregular colonies of isolates RLD_1, RLD_2, RLD_3 and RLD_4 respectively. This finding indicates that Lactobacillus isolates vary in taxonomic characteristics (Table 2).

Table 1. *Lactobacillus* isolates from freshwater fish

S.No.	Name of the	Total count CFU /g
1	Cat fish (*Clarias orientalis*)	23
2	Hari fish (*Anguilla sp*)	16
3	Rohu fish (*Labeo rohita*)	10
4	Jilebi fish (*Oreochromis sp*)	7
5	Gende fish (*Punitus carnaticus*)	3
Total		59

Among the various biochemicals studied, positive results were observed in all four isolates such as catalase, glucose, lactose and maltose and negative results were observed in Indole, Methyl red, Voges Proskauer, Citrate, Nitrate reduction. The following test such as urease, fructose were positive for isolates RLD_1, RLD_2 whereas isolates RLD_3, RLD_4 were negative for the above test.

The fructose, mannitol and rhamnose were fermented by isolates RLD_1, Fructose, Mannitol were fermented by isolate RLD_2, RLD_3 and RLD_4 respectively the other sugar were not utilized by RLD_2, RLD_3 and RLD_4 isolates. (Table 3). Based on the morphological, biochemical properties, the isolate RLD_1, RLD_2, RLD_3, RLD_4 were identified as *Lactobacillus sp.*

The fish pathogens were isolated from the gut based on cultural characteristics and 3 distinct isolates were selected for further study. These three pathogens were morphologically characterized; all three isolates were A_1, A_2 and V_1. They were gram negative, non-motile (except V_1), the shape of the isolates were various rods, long bacilli of A_1, A_2 and V_1 isolated respectively.

In SAA agar, these two isolates showed distinct variations i.e. regular, creamy, shiny, smooth edges, brown colour colonies of isolates A_1 and A_2 and on TCBS agar, V_3 isolates were showed green in colour, round, regular colony respectively. This finding indicates that pathogen *Vibrio* sp and *Aeromonas* sp isolates have taxonomic characteristics (Table 4).

Table 3. Biochemical characteristics of *Lactobacillus* isolates

Characters	*Lactobacillus* isolates			
	RLD_1	RLD_2	RLD_3	RLD_4
Biochemical				
Indole	–	–	–	–
Methyl Red	–	–	–	–
Voges Proskauer	–	–	–	–
Citrate utilization	–	–	–	–
Nitrate reduction	–	–	–	–
Urease	+	+	–	–
TS1	K/A	K/A	K/K	K/K
Catalase	+	+	+	+
Oxidase	–	–	–	–
Sugars Assimilation				
Arabinose	–	–	–	–
Fructose	+	+	–	–
Glucose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	–	+	+
Rhamnose	+	–	–	–

\+ : Positive – : Negative
K/A : Alkaline/Acid K/K : Alkaline/Alkaline

Among the various biochemicals studied, positive results were observed in all three isolates such as catalase, oxidase, glucose, lactose, mannitol, maltose, rhamnose, sucrose and negative results were observed in indole, voges proskauer, nitrate reduction. The following test such as gelatin, methyl red were showed positive for isolates A_1, A_2 and V_1 for the above test.

Glucose, fructose, mannitol, maltose, rhamnose, sucrose were fermented by isolated A_1, A_2 and V_1 produce Acid and gas (Table 5).

Based on the morphological, biochemical properties, the isolated V_1, A_1 and A_2 were identified as *Vibrio parahaemolyticus, Aeromonas salmonicida* and *Aero-*

Table 2. Characterization of *Lactobacillus* isolates

Characters	Lactobacillus *isolates*			
	RLD_1	RLD_2	RLD_3	RLD_4
Morphology				
Gram's Staining	G + ve rod	G + ve rod	G + ve rod	G + ve rod
Motility	–	–	–	–
Cultural				
MRS broth	Less turbidity	More turbidity	Turbidity	Turbidity
MRS Agar	Creamy, smooth edges, convex colony.	Dry, rough, irregular colony.	White, irregular, Opaque colony.	Shiny, smooth irregular colony.

\+ : Positive – : Negative

manas hydrophila respectively. The results reveal that the size and weight of the fish increased for about 5 g, in comparison to that of control fish when fed with *Lactobacillus spp*.

Table 4. Characteristics of *Aeromonas* and *Vibrio sp* from cat fish

Characters	*Aeromonas sp*		*Vibrio sp*
	A_1	A_2	V_1
Morphology			
Grams Staining	Gram negative rod	Gram negative rod	Gram negative rod
Motility	+	+	+
Cultural			
Starch Ampicillin agar	Regular, brown colony.	Shiny, smooth opaque cream colour	–
TCBS	NA	NA	Green colour, round, regular colony.
Alkaline peptone water	Less turbid	No turbid	More turbid

NA – Not Applicable

Table 5. Biochemical and characteristics of *Aeromonas* and *Vibrio sp*

Characters	Pathogen isolates		*Vibrio sp*
	Aeromonas A1	*Aeromonas* A2	V_1
Biochemical			
Indole	–	–	–
MR	+	+	+
VP	–	–	–
Citrate	–	–	–
Nitrate Reduction	+	–	–
Gelatin Hydrolysis	+	+	–
Catalase	+	+	+
Oxidase	+	+	+
Sugars Assimilation			
Glucose	–	A/G	A/G
Lactose	+	A/G	A/G
Mannitol	+	+	+
Maltose	+	A/G	+
Rhamnose	+	A/G	+
Sucrose	+	+	+

+ : Positive – : Negative
A/G : Acid/Gas

The present study concluded that the *Lactobacillus* isolates will be used as probiotic bacteria in aquaculture growth improvement and *Aeromonasis* control (Table 6).

Table 6. *In vitro* antibacterial activity of *Lactobacillus* isolates against the *Aeromonas sp*

S. No.	Treament	CFU/ml			
		$1^{st}\times10^5$	$5^{th}\times10^5$	$10^{th}\times10^5$	$15^{th}\times10^5$
1.	Control	3.02	3.48	3.62	3.83
2.	Test 1 – + Pathogen + *Lactobacillus*	3.33	4.04	4.90	5.09
3.	Test 2 – + Pathogen.	5.68	4.23	3.90	3.06

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 bacteria. Probiotics have been defined by the World Health Organization – Food and Agriculture Organization, as "live microorganisms" which when administered in adequate amounts, confer a health benefit on the host. "In the past decade, 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 (7).

In the present study 5 different fresh water fish, such as cat fish (*Clarias orientalis*), Hari fish (*Anguilla sp*), Rohu fish *(Labeo rohita)*, Jilabe fish (*Oreochromis sp*) and Gende fish (*Punitus carnaticus*) were collected and screened for *Lactobacillus* isolates from the above fishes. The maximum *Lactobacillus* population was recorded in cat fish, minimum in Gende fish (*Punitus carnaticus*). The similar study was carried out by Itoh (8). *Lactobacilli* have been found to produce metabolic products that play an important role in controlling undesirable microflora in the gut. Most LAB isolated in our study were assigned to *Carnobacteium* strains belonging to this genus, or to the former species. *L.divergens* and *L.Carnis* have been isolated from fish and sea food (9)

The isolated *Lactobacillus* were culturally, morphologically and biochemically characterized and identified as *Lactobacillus sp.* This finding is similar to the findings of *Lactobacillus* fermentum (ATCC 9328), *L.Saki subsp. Sakei* (DSM 20017), *L. Plantarum* (ATCC 8014), *L.Curvatus sub sp.curvatus* (DSM 20019) and *L.lacits subsp. Lactis* (ATCC 1107). (Wilkinson and Jones 1977). Identification of the 237 rods at the species level was done according to several authors (10).

To evaluate the antagonistic effect of *Lactobacillus* isolates against the fresh water fish pathogen, the *Vibrio* and *Aeromonas* isolates were isolated from the cat fish

(*Clarias orientalis*). The isolates *Vibrio* and *Aeromonas* were culturally, morphologically, biochemically characterized and identified as *Vibrio parahaemolyticus, Aeromonas salmonicida*

These findings have already been reported by Lambert and Nicolas (11) who confirmed that different species and different species and different isolates of the same species of *Vibrio* vary in their pathogenicity for bivalves. Burke Rodgers (12) worked on RSD of Mugicephales of lower Noosa river estuary and Lake Cootharaba of South – eastern Queensland and found that *V. anguillarum* was the sole organism associated with very early lesion. *A. hydrophila* was isolated from advanced lesions of fish taken from fresh water reaches of Noosa River and Cootharaba Lake.

The antagonistic activity of 5 *Lactobacillus* isolates was screened against fish pathogen by agar cup assay method. Among the 5 isolates 3 isolates such as RLD1, RLD2, RLD3 showed anti-*Aeromonas* activity. Only one isolate RLD2 showed anti – *vibiro* activity. This finding coincides with findings of Joborn (13), who reported inhibitory activity against *A. salamonicida* and *V. anguillarum* in intestinal mucus, arising from growth of this strain.

The isolate RLD3 showed broad spectral activity against *Aeromonas* and *Vibrio* was selected for further *in vitro* analysis. The results of *in vivo* studies reveal that treatment showed increased *Aeromonas* isolates in comparison with control. Treatment 2 shared inhibition of *Aeromonas* population in comparison with treatment 1. The antagonistic Lactobacillus is responsible for inhibition of *Aeromonas* populations in cat fish (*Clarias orientalis*). This finding is already reported by Burke Rodgers (12) who worked on RSD of Mugi cephalus of lower Noosa river estuary and lake Cootharaba of South – eastern Queensland and found that *V. anguillarum*, was the sole organism associated with very early lesion. A *hydrophila* was isolated from advanced lesions of fish taken from fresh water reaches of Noosa River and Cootharaba Lake.

The study concluded that the *Lactobacillus* isolates will be helpful in the management of Bacterial disease *Aeromonosis* in cat fish (*Clarias orientalis*). The species identification, optimization of Lactobacillus growth and their *in vivo* effect on pathogen in fish under pathology status will be a further course of work.

References

1. Rana KJ. Review of the state of the world Aquaculture. FAO fisheries circular number 886. Rome, Italy: food and Agriculture Organization of the United Nations, 1997:3-16.
2. Lin CK. Progression of intensive marine shrimp culture in Thailand. In Browdy CL, Hopkins JS. (eds.) Swimming through troubled water. Proceedings of the special session on shrimp farming, Aquaculture Society, 1995:13-23.
3. Witte W, Klare I, Werner G. Selective pressure by antibiotics as feed additives. Infection 1999;27:35-8.
4. Reid G, Smeianov V, Bruce AW. Biotherapeutic agents to prevent cystitis in women. In: Elmer GW, McFarland L, Surawicz C (eds), Biotherapeutic agents and infectious diseases. Totowa, N.J, Humana Press Inc, 1999:245-61.
5. Yasuda K, Taga N. A mass-culture method for Artemia salina using bacteria as food. Mer 1980;18:55-62.
6. Cahill MM. Bacterial flora of fishes: a review. Microb Ecol 1990;19:21-41.
7. Irianto A, Austin B. Probiotics in aquaculture. J Dis 2002;25: 633-42.
8. Itoh T, Fujimoto T, Kawai Y, Toba T, Saito T. Inhibition of food borne pathogenic bacteria by bacteriocins from Lactobacillus gasseri. Lett Appl Microbiol 1995;21:137-41.
9. Hiu S, Holt, RA, sriranganathan N, Seidler RJ, Fryer JL. Lactobacillus pisicola, a new species from Salmonid fish. Int J Syst Bacteriol 1984;34:393-400.
10. Collins MD, Farrow JAE, Phillips BA, Ferusu S, Jones D. Classification of Lactobacillus divergens, Lactobacillus piscicola and some catalase negative, asporogenous, rod shaped bacteria from poultry in a new genus, Carnobacterium. Int J Syst Bacteriol 1987; 37:310-6.
11. Lambert C, Nicolas JL. Specific inhibition of chemiluminescent activity by pathogenic Vibrios in hemocytes of two marine bivalves: Pecten maximus. J Invertebr Pathol 1998;71:53-63.
12. Burke J, Rogders L. Identification of pathogenic bacteria associated with the occurrence of 'red spot' in sea mullet, Mugil cephalus L. in south-eastern Queensland. J Fish Dis 1981;4: 153-9.
13. Joborn A, Olsson JC, Westerdahl A, Conway PL, Kjelleberg S. Colonisation in the intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by Carnobcterium sp. Kl J Fish Dis 1997; 20: 383-92.

PROBIOTSKO DEJSTVO IZOLATA *LACTOBACILLUS* NA BAKTERIJSKE PATOGENE *CLARIAS ORIENTALIS*

Dharumaduari Dhanasekaran[1], Subhasish Saha, Noorudin Thajuddin[1], Annamalai Panneerselvam[2]

[1]Departman mikrobiologije, Fakultet prirodnih nauka, Bharathidasan Univerzitet, Tiruchirappalli, 620 024
[2]P.G. & Departman razvoja botanike i mikrobiologije, A.V.V.M. Sri Pushpam koledž, , Poondi, 613 503 Thanjavur Distrikt, Tamil Nadu, Indija
E-mail: dhansdd@yahoo.co.in

Kratak sadržaj: *Na 5 slatkovodnih lokacija, izolovano je oko 59 izolata Lactobacillus-a, kod riba kao što su som (Clarias orientalis), jegulja (Anguilla sp), Rohu šaran (Labeo rohita), tilapija (Oreochomis sp) i vrsta zrakoperki (Puntius carnaticus). Od 59, samo je 4 Lactobacillus izolata odabrano za dalja proučavanja. Na osnovu morfoloških i biohemijskih karakteristika, izolati su identifikovani kao Lactobacillus sp. Izolovani patogeni su karakterizovani i identifikovani kao Vibrio parahaemolyticus, Aeromonas sp i Aeromonas salmonicida. Pmoću agar difuzije testirana je antagonistička aktivnost Lactobacillus izolata na Ae4romonas, Vibrio sp. Od 4 izolata, Lactobacilli RLD2 je pokazao značajnu antagonističku aktivnost samo na Aeromonas i Vibrio sp. Ovaj izolat je dalje testiran klasičnom metodom brojanja (SPC-standard plate count) u cilju odredjivanja sposobnosti opstanka patogena. Izolat je umnožen a riblja hrana je obogaćena izolatima Lactobacillus-a. Rezultati su pokazali da se veličina i težina riba statistički povećala u odnosu na kontrolnu grupu. Ova studija zaključuje da će se Lactobacillus izolati koristiti kao probiotska bakterija u akvakulturi, u cilju sprečavanja aeromonaze.*

Ključne reči: *probiotska bakterija, Lactobacillus, antagonistička aktivnost, Aeromonas, Vibrio*