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Original Paper

PROBIOTIC EFFECT OF *Lactobacillus* isolates AGAINST BACTERIAL PATHOGENS IN FRESH WATER FISH

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ABSTRACT

A total of 59 Lactobacillus isolates were isolated from 5 different fresh water fish 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 and biochemical characteristics, the isolates were identified as Lactobacillus sp. The pathogen were isolated from infected cat fishes, 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 RLD_2 showed significant antagonistic activity against Aeromonas and Vibrio sp alone. and was further evaluated by standard plate count assay for the viability of pathogen. The isolate was multiplied and the fish feed was supplement with Lactobacillus isolates. The results reveal that the size, weight of the fish was statically increased in comparison to that of control fish. The present study concluded that the Lactobacillus isolates could be used as probiotic bacteria in aquaculture, to manage aeromonasis.

Keywords: Probiotic bacteria**,** *Lactobacillus,* Antagonistic activity, *Aeromonas*, *Vibrio*

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INTRODUCTION

Fish borne disease is a common problems 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

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1.6% for capture fisheries production (Rana, 1997).

For instance, disease is now considered to be the limiting factor in the shrimp culture sub sector (Lin, 1995). 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 increase the production in animals, increase 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 also they can 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 known to select for cross-resistance to antimicrobials used in human medicine could pose a particularly significant hazard to human health (Witte *et al.*, 1999). According to the Word 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” defined as more precisely “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, now a day, there has been a renewal of interest in the use of probiotics (Irianto and Austin, 2002). In general terms a group of requirements have been identified as important properties for *Lactobacilli* to be effective probiotic organism (Reid *et al.*, 1999). These include the ability to, adhere to cells, exclude or reduce pathogenic adherence, persist and multiply, produce acid, resist vaginal micro flora, be safe and therefore noninvasive, noncarcinogenic and nonpathogenic and, co aggregate and form a normal.

Yasuda and Taga, 1980, anticipated in 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. *Vibrio alginolyticus* has been employed as a probiotic in many Ecuodoran Shrimp hatcheries since late 1992 (Garriques and Arevalo, 1995). The overall antibiotic use was decreased by 94% between 1991 and 1994 (Rico-Mora *et al.*, 1998). 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 (Cahill, 1990).

Recently the cultivation of cat fish in Vellore District, Tamil Nadu become a good employment for farmers and unemployed youth to fulfill the food need, but the cat fish cultivation facing 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.

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MATERIALS AND METHODS

Sampling of Fish

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 bacterial infected and healthy fish were collected from these ponds during the first week of August 2005 using cast net. All the infected and healthy fishes were then examined for pathogenic and probiotic bacteria respectively.

Bacterial Isolation

Using sterile swabs the specimen from oral and gut region of healthy and infected fishes for *Lactobacilli* and other pathogens respectively were collected. 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.

Probiotic Test

The *Lactobacilli* species were inoculated in MRS broth and incubated for 7 days on rotary shaker at 28± 2°C. The culture were then centrifuged and the supernatant fluid sterilized by syringe filter to collect the bacteriocin. The Muller Hinton agar plates were seed with 24hours 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.

Fish Feed Preparation

The fish feed were 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 hr at 25°C in MRS agar and harvested by centrifugation (10000 rpm for 15 min). The cells were resuspended in 100ml of saline. To this egg albumin was mixed this emulsion was then applied to fish feed by mixing in a drum mixer for 15 min. Control diets was prepared as feed devoid of *Lactobacillus* sp.

Feeding Fishes with Probiotic Feed

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 were fed at maintenance level for 10 days prior to the experiments. All the tanks were aerated and the experiments were carried out with a water temperature of 28 ± 1°C. Fish were fed twice daily, using fed with *Lactobacilli* sp. coated feed The control fish was fed with feed free from *Lactobacilli* sp. level of the nutrient below the minimum requirement. The experiment was carried out up to 2 month.

Antibacterial Assay of Probiotic Bacterial Culture

Two sets of experiment, one for *Aeromonas* and another for *Vibrio parahaemolyticus* 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

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bacteria and without the addition of probiotic bacteria. The entire flasks were incubated at 37^{o}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 in ever 5 days intervals.

RESULT AND DISCUSSION

RESULTS

From the 5 gut samples collected, totally 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 various according to the generic variation of fresh water fish (**Table 1**).

Among the 59 *Lactobacillus* isolates, cultural characteristically distinct four isolates were selected for further study. These four *Lactobacillus* isolates morphologically characterized, all four isolates namely RLD_1, RLD_2, RLD_3 and RLD_4 were gram positive, non-motile, the shape of the isolates were varies rod, stout rod, bacilli, short bacilli of RLD_1,RLD_2,RLD_3 and RLD_4 isolates respectively.

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

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**).

Among the various biochemical 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 showed positive for isolates RLD_1, RLD_2 where as isolates RLD_3, RLD_4 were showed 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.*

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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 **-** **:** Negati

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

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	-	-	-	-

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Sugars Assimilation				
Arabinose	-	-	-	-
Fructose	+	+	-	-
Glucose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	-	+	+
Rhamnose	+	-	-	-

+ : Positive - : Negative
K/A : Alkaline/Acid K/K : Alkaline/Alkaline

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

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

Where, NA – Not Applicable

Among the various biochemical 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.

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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 *Aeromanas hydrophila* respectively. The results reveal that the size, weight of the fish was about 5 gm increased in comparison to that of control fish when fed with *Lactobacillus spp*.

The present study concluded that the *Lactobacillus* isolates will be used as probiotic bacteria in aquaculture growth improvement and *Aeromonasis* control (**Table. 6 and 7**).

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

Characters	**Pathogen isolates**		
	Aeromonas A1	*Aeromonas* A2	*Vibrio* sp 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

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Table 6. Screening of antagonistic activity in *Lactobacillus* against the fish pathogen by agar diffusion assay

S. No.	*Lactobacillus* isolates	Pathogen	
		Aeromonas sp	*Vibrio* sp
1.	RLD_1	+	-
2.	RLD_2	+	+
3.	RLD_3	+	-
4.	RLD_4	-	-

\+ : Presence of Zone of inhibition reaction
\- : Absence of inhibition reaction

Table 7. *In vitro* antibacterial activity of *Lactobacillus* isolates against the *Aeromonas* sp

S. No.	Treatment	CFU/ml			
		1^{st} X 10^5	5^{th} X 10^5	10^{th} X 10^5	15^{th} X 10^5
1.	Control	3.02	3.48	3.62	3.83
2.	Test (Pathogen + *Lactobacillus)*	3.33	4.04	4.90	5.09

DISCUSSION

The use of probiotics for disease control in aquaculture is a 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 microorganism" 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 top inhibit pathogenic organisms and over come infections in fish and larvae in acquaculture (Itoh *et al.*, 1995).

In the present study, 5 different fresh water fishes 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 Hiu *et al.*, 1984. *Lactobacilli* have been found to produce metabolic products that play

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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 a fish and sea food (Collins *et al.,* 1987).

The isolated *Lactobacillus* was culturally, morphologically and biochemically characterized 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). Identification of the 237 rods at the species level was done according to several authors.

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 are already reported by Lambert and Nicolas, 1998, confirmed that different species and different species and different isolates of the same species of *Vibrio* vary in their pathogenicity for bivalves. Burke Rodgers, 1981, worked on RSD of Mugicephales of lower Noosa river estuary and Lake Cootharaba of South – eastern queensland 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 – *Vibrio* activity. This finding is co –inside with findings of Joborn *et al.,* 1997, 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. In the *in vivo* result reveals that treatment showed increased *Aeromonas* isolates in comparison with control. Second treatment shared inhibition of *Aeromonas* population in comparison with first treatment. The antagonistic *Lactobacillus* is responsible for inhibition of *Aeromonas* populations in cat fish (*Clarias orientalis*). This finding is already reported by Burke Rodgers, 1981, worked on RSD of Mugi cephalus of lower Noosa river estuary and lake cootharaba of South – eastern queensland 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, their *in vivo* effect on pathogen in fish under pathology status will be a further course of work.

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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

Table 2. Characterization of *Lactobacillus* isolates

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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

Table 3. Biochemical characteristics of *Lactobacillus* isolates

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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

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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

Where, NA – Not Applicable

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

Characters	Pathogen isolates		*Vibrio* sp V_1
	Aeromonas A1	*Aeromonas* A2	
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

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Table 6. Screening of antagonistic activity in *Lactobacillus* against the fish pathogen by agar diffusion assay

S. No.	*Lactobacillus* isolates	Pathogen	
		Aeromonas sp	*Vibrio* sp
1.	RLD_1	+	-
2.	RLD_2	+	+
3.	RLD_3	+	-
4.	RLD_4	-	-

\+ : Presence of Zone of inhibition reaction

\- : Absence of inhibition reaction

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Table 7. *In vitro* antibacterial activity of *Lactobacillus* isolates against the *Aeromonas* sp

S. No.	Treatment	CFU/ml			
		1^{st} X 10^5	5^{th} X 10^5	10^{th} X 10^5	15^{th} X 10^5
1.	Control	3.02	3.48	3.62	3.83
2.	Test (Pathogen + *Lactobacillus)*	3.33	4.04	4.90	5.09