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Antagonistic Probioticity of Novel Bacterial Isolates from Pakistan against Fish Pathogen *Pseudomonas fluorescens* in *Labeo rohita* Fingerlings

Asma Chaudhary[1], Qurat-ul-Ain Ahmad[1], Afia Muhammad Akram[1], Sadia Roshan[2] and Javed Iqbal Qazi[3]*

[1]*Department of Zoology, Division of Science and Technology, University of Education, Lahore, 54770, Pakistan*
[2]*Department of Zoology, University of Gujrat, Gujrat, Pakistan*
[3]*Department of Zoology, University of the Punjab, Lahore, 54590, Pakistan*

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AC designed and demonstrated the study. QA and AMA analysed experimental data. SR wrote the manuscript. JIQ supervised and guided the trials.

Key words
Probiotics, Virulence, *Pseudomonas fluorescens*, Disease resistance, Antagonistic bacteria to pathogens, *Labeo rohita*

ABSTRACT

The study was endeavoring to investigate *in vitro* as well as *in vivo* antibacterial and probiotic potential of *Sphingomonas* sp. AsCh-P3 (MF543123) and *Bacillus aerius* AsCh-A7 (MF543123) against fish pathogen *Pseudomonas fluorescens*. Antagonism by cross streak was observed in ten isolates out of total twenty. Six isolates showed better inhibition by well as well as disk diffusion methods with maximum growth inhibition zones *i.e.*, 15 and 17 mm by *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 respectively. Six probiotic bacterial isolates of known enumeration (C.F.U.ml^{-1}) showed growth antagonism for the fish pathogen with inocula comprising of less number of C.F.U.ml^{-1}. Highest survival rates *i-e* 63.5 and 70.17% were expressed by *Sphingomonas* sp. AsCh-P3 and *B. aerius* AsCh-A7 respectively by mixing in formulated fish feed, drying and storing in refrigerator. Mortalities of *Labeo rohita* fingerlings challenged to *P. fluorescens* intraperitoneally appeared in a dose dependent manner with symptoms like hemorrhage, swelling of the belly and anus, scale damage etc. The pathogen challenged fish were administered with *Sphingomonas* sp. AsCh-P3 (group-G1) and *B. aerius* AsCh-A7 (group-G2) treated feed in different experimental set ups. Highest Relative Percent Survival (80-90%) was recorded for fish receiving probiotic feed prior and after injection (G1b, G2b). Provision of probiotics was evident to alleviate pathogen virulence in fish. The emerging multi-drug resistance and related side effects of conventional therapies have caused researchers to explore alternate strategies, such as probiotics employment for disease control in aquaculture.

INTRODUCTION

Aquaculture is emerging more speedily compared to other animal food industry. Bacterial diseases are the main hazards to public health and contribute to fish stock fatalities in the industry. To combat diseases, certain measures such as chemotherapy, proper nutrition and immunological manipulation are used.

The indiscriminate consumption of the remedial measures for aquatic animal diseases, including protective chemical additives, veterinary drugs, etc. has supervened the antimicrobial resistance and devastated the bio-environs. There is a dire need to improve microbial control strategies to alleviate the disease incidence and drug resistance (Miranda and Rojas, 2007; Denev *et al*., 2009; Kolndadacha *et al*., 2011; Gobinath and Ramanibai, 2012).

Among pseudomonads, *Pseudomonas fluorescens* and *P. putida* have been known to be very notorious pathogen species of fresh and brackish water fish. According to Lom and Schubert (1983) *P. fluorescens* produced open ulcers among the carp. Its signs were scaly erosions; a swollen body cavity, protrusion and reddening of the anus and hyperemia of the swim bladder. This disease received the designation "Septicemic Pseudomonas infection" of the common carp and silver carp.

The exploitation of probiotics as an alternate strategy reinvigorating the therapeutic channel, has been established as positive promoters to improve aquaculture production and gained momentum in recent years (Verschuere *et al*., 2000; Dahiya *et al*., 2010, 2012; Sihag and Sharma, 2012). Probiotics are live non-carcinogenic microbes having the potential to reduce pathogen adhesion, boost host's immune response, break down the indigestible compounds, and improve production of vitamins and

* Corresponding author: qazi.zool@pu.edu.pk
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enzymes (Gomez-Gil *et al*., 2000; Abraham and Banerjee, 2007; Welker and Lim, 2011; Divya *et al*., 2012; Arig *et al*., 2013; Golic *et al*., 2017). According to Ishibashi and Yamazaki (2001) probiotics are food or drug containing live microbes improving physiological effects of animal host when ingested.

In aquaculture, feed/water adjusted with probiotics is administered. The usage of feed supplemented with probiotics is an improved technique ensuring the efficacy of the probiotic microorganisms for healthier fish production (Irianto and Austin, 2002). Typically, the probiotics are supplemented to the feed of animals as freeze-dried culture (Nikoskelainen *et al*., 2001). It is essential to adequately conserve the viability of probiotics throughout its processing, storage and consumption (Linders *et al*., 1996; Wouters *et al*., 1998).

Probiotics stimulate the growth and immune response by different types of Gram positive and Gram negative bacteria, yeast, unicellular algae and certain bacteriophages in wide range of fishes when administered in fish feed, water and as disease resistant as was evident in literature such as lactic acid bacteria in larva and *Lactobacillus rhamnosus* (JCM 1136) in rainbow trout (Panigrahi *et al*., 2004), *Bacillus* S11 to shrimp gut (Rengpipat *et al*., 2000), yeast Zymosan in shrimp (Panigrahi *et al*., 2004), Different lactobacilli such as *Lactobacillus thermophillus*, *L. plantarum*, *L. acidophilus*, *L. casei*, *L. bulgaricus* and *L. rhamnosus* are used frequenly in fish feed to improve growth and survival as well as immunomodulant to be considered as supplement to vaccines to resist infectious diseases (Nayak, 2010; Esteban *et al*., 2014; Muñoz-Atienza *et al*., 2014; Sahoo *et al*., 2015; Dawood, 2016; Gobi *et al*., 2016). Probiotics in form of mono culture or multicultures stimulate innate immunity by expressing cytokines, lysozyme and phagocytic activities. They are hypothesized to promote immunoglobulin cells and granulocytes (Nayak, 2010).

The pathogen inhibiting potential of both Gram positive and negative bacterial organisms to overcome infections has been reported in the *in vitro* and *in vivo* studies (Itoh *et al*., 1995; Authira *et al*., 2011; Gobinath and Ramanibai, 2012; Tabak *et al*., 2012). The genus *Bacillus* is considered as a biocontrol agent in feed and water (Abraham and Banerjee, 2007). *Bacillus* sp. is producing siderophores, hydrogen peroxide, lysozyme, proteases and bacteriocin that inhibit pathogen in the fish intestine (Jones, 2002; Mohideen *et al*., 2010; Seenivasan *et al*., 2012). Ghosh *et al*. (2007) reported antagonistic potential of B. subtilis SG4 from *Cirrhinus mrigala* against *Aeromonas hydrophyla*, *Psedomonas fluorescens*, and *Edwardsiella tarda.*

Rohu (*Labeo rohita*) is renowned as the most popular among freshwater fish due to good taste in Pakistan and other Asian countries. It is being reared extensively in fresh water (rivers and ponds) to meet the food requirement of the area. In this study, the mode of transmission of pathogenic *P. fluorescens* to *L. rohita* fingerlings have been investigated *in vivo*. The purpose of the present work was to evaluate the anti-pathogenicity of two bacterial isolates in *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Source of fish pathogen

A virulent strain *Pseudomonas fluorescens* was isolated on Pseudomonas agar P and Pseudomonas agar F from *Labeo rohita* having ulcerative disease collected from the Research fish ponds, University of the Punjab, Lahore. The strain was characterized phenotypically and biochemically up to the species level along with the production of the pigment pyocyanin and fluorescein on Pseudomonas P and F base media. The results were compared with *Pseudomonas* species as described in Bergey's Manual of Systematic Bacteriology (Uğur *et al*., 2012)

Screening of potential probiotic bacterial strain

Twenty bacterial isolates (from different homemade yogurt and raw milk samples) as selected from previous study was further processed for evaluation of their antagonistic activities against fish pathogen (Chaudhary and Qazi, 2011).

Probiotic inhibition of the fish pathogen by isolated bacteria

Antagonism of *P. fluorescens* by the isolated bacteria was evaluated by cross streak technique (Austin *et al*., 1992). Fish pathogen suspension, prepared in phosphate buffer saline (PBS) (O.D. set at 600 nm at 0.5±0.05) was streaked perpendicularly across already streaked bacterial isolate (with cell densities set as 0.5±0.05 spectrophotometrically) on a solidified nutrient agar plate. After 48 h incubation period at room temperature (RT), antagonism as visible growth interruption of the fish pathogen was observed. On the basis of visible antibiosis, ten isolates were screened against the fish pathogen for subsequent experimentation.

Antagonism assay

Screened ten isolates were further assayed for antagonistic activities against fish pathogen *P. fluorescens* by filter paper disc and well diffusions methods (Olsson *et al*., 1992).

The twenty four h young cultures of a pathogen

as well as probiotic isolates were prepared in a liquid nutrient medium, incubated at 37 ºC, centrifuged and filter sterilized. Each filter-sterilized inoculated culture fluid (100 µl) was loaded on the sterilized filter paper disc (Whatman No. 1) of 9 mm diameter. Subsequently, the discs were positioned on solidified nutrient medium plates, pre-inoculated with 50 *µl* of fish pathogen *P. fluorescens* suspension (cell densities set at 0.5±0.05) by the spread plate method. Growth inhibition zones (GIZ) around each disc were measured after 24-48 h of incubation at RT. For well diffusion method, fish pathogen *P. fluorescens* suspension was loaded in solidified nutrient medium (at 50ºC in 3% (v/v) ratio) and dispensed in sterile petri plates.

Wells having diameter 9 mm, were punched into nutrient agar plates employing metal borer. Successively, 100 *µl* of filter sterilized bacterial culture was loaded in each well. The nutrient agar plates with punched wells were incubated at RT (~27-30 ºC) up to 48 h and growth inhibition zones (GIZ) were recorded. Six most efficient bacterial isolates yielded GIZ ≥10 mm in diameter were screened and used for further experimentation.

Antibiosis of probiotic isolate against varying doses of bacterial fish pathogen

To evaluate the antibiotic prospective of probiotics against varying doses of fish pathogen, liquid cultures of probiotic isolates (cell density set as 0.05, 0.1, 0.25, 0.5, at 600 nm wavelength) were made in PBS (pH 8). Probiotic cell suspensions were set at an absorbance of 0.5±0.05 spectrophotometrically in PBS. The probiotic suspensions were enumerated by viable counting and cross streaked perpendicularly across varying cell densities of fish pathogen on solidified nutrient plates. The inoculated plates were then incubated for 24-48 h at RT (~27-30 ºC). Interruption in the growth pattern of the fish pathogen illustrated the antibiosis potential of test probiotic isolates as already established (Austin *et al*., 1992).

Probiotic supplementation in formulated fish feed

The selected probiotic isolates were cultivated for 24 h in a liquid nutrient medium. Bacterial culture was then centrifuged and suspended in PBS of 0.5 ± 0.05 cell density at 600 nm. Cell suspensions (of 20% v/w) were amalgamated with sterilized formulated fish feed and air dried for three days in the laminar air flow chamber at RT (~27-30 ºC) and kept frozen for a week. Serially diluted processed fish feed samples made in PBS (0.89%) were enumerated by viable counting on solidified nutrient medium. Evaluation of probiotic bacterial survival (%) was made by following calculation.

$$\% \text{ Survival} = \frac{\text{C. F. U. of probiotic bacteria/g of amalgateamd feed after drying}}{\text{C. F. U. of probiotic bacteria/g of amalgamated feed before drying}} \times 100$$

Based on the relatively high antagonistic properties and greater survival potentials, the probiotic isolates *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 (later identified through 16S rRNA gene sequencing) were screened for performing inocula optimization in formulated feed.

Optimization of probiotic inocula in formulated feed

To optimize probiotic inocula, feed extract was used. For feed extract preparation, dried feed (20 g) was boiled for 10 minutes in 100 ml distilled water and autoclaved for 15 minutes under standard conditions. The sterilized feed was centrifuged to obtain the clear extract. The probiotic isolates, suspensions of AsCh-P3 and AsCh-A7 in PBS were inoculated with different percent v/v inocula (10, 20, 30) in the sterilized feed extract and incubated at RT (30±0.2°C). Growth was evaluated at constant intervals of twenty four h up to three consecutive days in form of C.F.U.ml^{-1}.

Characterization of probiotic isolates

Cell and colony morphology tests as well as biochemical assays were employed to all the probiotic bacterial isolates for taxonomic categorization (Collins *et al*., 1995) and subsequent identification. Selected probiotic strains AsCh-P3 and AsCh-A7 were also subjected to molecular characterization through 16S rRNA gene sequencing.

Fresh bacterial culture was used to extract DNA by heating in 50 mM NaOH (45µl) for 5 minutes 95ºC. Subsequently 5 µl of Tris HCl (1M, pH 8) was added. Bacterial pellet was obtained after 10 minute centrifugation. 16S rRNA gene was amplified using DNA polymerase (KOD FX). PCR Reaction mix consisted of dNTP (2 mM), both primers (50 uM) 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as well as 1492R (5′-AGGCTACCTTGTTACGACTT-3′) and 2 µl of probiotic bacterial DNA. PCR set for 35 cycles was accomplished, each comprising of denaturation (10-seconds at 98 °C), annealing (30-second at 53 °C) and extension (1-minute at 72 °C). PCR products were purified and partially sequenced employing automated sequencer.

The sequences were assayed for homology determination using BLAST-GenBank database (http://www.ncbi.nlm.nih.gov/blast) and procured for the accession numbers.

Pathogenicity of P. fluorescens *in* L. rohita *fingerlings*

L. rohita fingerlings were got from the fish farm at Muridkey, Pakistan in August. Initially, they were acclimatized to the experimental conditions for the first 10 days in Environmental Microbiology Laboratory,

University of the Punjab, Lahore. Fish health status was keenly observed under experimental conditions during the experimental period.

The *L. rohita* fingerlings were challenged to *P. fluorescens* bath initially, but it resulted neither in the appearance of any disease nor mortality. Hence, intraperitoneal (*i.p*) injection of the pathogen was done by following the methods established by Romalde *et al*. (1996) and Robertson *et al*. (2000). For this purpose, overnight grown young culture of *P. fluorescens* was centrifuged and suspended subsequently in PBS to obtain O.D. of 1.0, 0.5, 0.25, 0.1, 0.05 spectrophotometrically that were subjected to viable counting for evaluating the C.F.U.ml^{-1} as 61 ×10^{9}, 181 ×10^{5}, 45×10^{5}, 268×10^{3}, 170×10^{3} respectively.

To inspect the pathogenicity of the fish pathogen *P. fluorescens*, ten *L. rohita* fingerlings in each group were injected intraperitoneally with varying doses of virulent bacterium. All groups of fishes were given *i.p* injections of 0.1 ml pathogen suspended in PBS representing a set dose of a pathogen. The control fish group was given *i.p* injection of sterile PBS without fish pathogen similarly. Every group was kept in a separate aquarium at RT (27-30 ºC) having 10 liters of fresh water and was aerated continuously employing air stone. One third of the water in each aquarium was routinely changed every twenty four h and the debris/feces were also siphoned off. All fish groups were observed up to 30 days. They were given sterile fish feed and checked for mortalities daily. Pathogen concentration with of 268×10^{3} C.F.U.ml^{-1} causing 50% mortality was chosen for subsequent experiment.

Probiotic treatment of pathogen challenged fish

The probiotic bacterial isolates *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 with greater antagonistic activities for *P. fluorescens* in *in vitro* studies were further scrutinized for the *in vivo* assays. The experiments were performed by following the methods established by Gram *et al*. (1999) and Spanggaard *et al*. (2001).

For *in vivo* assay, the negative (C1) and positive control (C2) groups were fed with control feed 15 days and 30 days after i.p pathogenic injections. In the experimental groups G1a, G2a control feed was administered for 15 days prior pathogenic dose followed by *Sphingomonas* sp. AsCh-P3 as well as *Bacillus aerius AsCh-A7* treated feed for 30 days, whereas G1c, G2c was provided with the respective probiotic supplemented feed prior (15 days) and control feed after (30 days) pathogenic dose. Experimental groups G1b, G2b received *Sphinogomonas* sp. AsCh-P3 and *Bacillus aerius AsCh-A7* enriched feed prior (15 days) and after (30 days) pathogenic dose.

All the control as well as experimental fish group consisted of triplicates with 10 fishes per replicate. At the experimental start, different groups were given simple sterilized or corresponding probiotic amalgamated feed for fifteen days. Every experimental fish was given i.p injections with a pathogen suspension of 0.1 ml. The positive control fish group was given fish pathogen whereas the negative control group was subjected to *i.p* injection with 0.1 ml of PBS only. Mortalities in all the aquaria (at RT of 24 to 27 ºC) were routinely noted every 24 h, whereas the dead fishes were removed continuously from each aquaria. Further, dead fishes were investigated clinically for pathogen infection. Following calculations were made for evaluation of relative percent survival (RPS) as established by Amend, 1981.

$$\text{RPS} = 1 - \left(\frac{\text{experimental group mortality \%}}{\text{control group mortality \%}}\right) \times 100$$

Statistical analysis

Experimental data were analyzed employing one way analysis of variance (ANOVA) following Duncan's multiple range test (SPSS ver.18.0, SPSS, Chicago, IL, USA).

RESULTS

In vitro antagonistic potentialof antibiotic bacterial isolates against pathogen P. fluorescens

Initially 20 bacteria were scrutinized for their antibiotic potential against fish pathogen *P. fluorescens* using cross streak method (Table I). As shown in Table I, bacterial isolates exhibited antagonism against the fish pathogen while remaining 10 isolates could not express any growth antagonism to the fish pathogen.

Based on the GIZ by cell-free broth of the positive probiotic isolates, six bacterial isolates were selected for further study. The best inhibition zone was 15 mm, 12 mm by *Sphingomonas* sp. AsCh-P3 and 17 mm, 13 mm by *Bacillus aerius* AsCh-A7 against *P. fluorescens* as recorded by disc and well diffusion methods respectively. Whereas the remaining selected bacterial isolates showed inhibition zones (10-12 mm) as shown in Table II.

Growth antagonism of the selected probiotic bacteria against varying doses of fish pathogen

Six probiotic bacterial isolates of known enumeration (C.F.U.ml^{-1}) were streaked perpendicularly to the inocula of different concentrations of fish pathogen (Table III). Antibiosis was shown by *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 against *P. fluorescens* at all strengths. A general antibiosis trend reflected reasonable growth inhibition for the fish pathogens cross streaked with inocula containing a less number of C.F.U.ml^{-1}.

Table I. Antagonistic activity of different probiotic isolates against fish pathogen *Pseudomonas fluorescens* by cross streak method.

Probiotic isolates	P. fluorescens
P. mallei AsCh-P1	−
Sphingomonas sp.AsCh-P3	++
P. pseudomallei AsCh-P4	−
Enterobacter sakazaki AsCh-P6	−
Edwardsiella hoshinae AsCh-P8	−
P. pseudomallei AsCh-P9	−
P. gladioli AsCh-P10	+
P. putida AsCh-P13	−
P. pseudomallei AsCh-P14	+
P. aeruginosa AsCh-P15	−
Sporolactobacillus inilunis AsCh-L6	+
Aeromonas caviae AsCh-L14	−
Bacillus cereus AsCh-A2	−
Listeria murnyi AsCh-A3	±
P. pseudoalcaligenes AsCh-A4	+
Kurthia gibsonii AsCh-A5	+
Enterobacter agglomerans AsCh-A6	±
Bacillus aerius AsCh-A7	++
Enterobacter aerogenes AsCh-A8	−
Bacillus cereus AsCh-A9	+

+, Pathogens inhibited by probiotic isolates; ++, Pathogens inhibited strongly by probiotic isolates; ±, Pathogens inhibited weakly by probiotic isolates; −, Pathogens not inhibited by probiotic isolates.

Table II. Growth inhibition of different probiotic bacteria by cell free culture against the fish pathogen *Pseudomonas fluorescens.*

Probiotic Isolates	Disc[a] diffusion method	Well[b] diffusion method
Sphingomonas sp. AsCh-P3	15	12
P. gladioli AsCh-P10	10	10
P. pseudomallei AsCh-P14	11	11
Listeria murnyi AsCh-A3	11	10
P. pseudoalcaligenes AsCh-A4	10	10
Kurthia gibsonii AsCh-A5	11	10
Enterobacter agglomerans AsCh-A6	10	10
Bacillus aerius AsCh-A7	17	13
Bacillus cereus AsCh-A9	11	10
Sporolactobacillus inilunis AsCh-L6	10	10

Values represent diameter of growth inhibition zones in mm; Isolates were selected based on antagonism against both pathogens and were optimized for further parameters. a, diameter of each disc required = 9mm; b, diameter of each well = 9mm.

The viable counting of the probiotic isolates in formulated fish feed

Intervening period involved in storage and transportation, etc. of feed enriched with probiotics is regarded very critical for effectual administration to an animal. This study was conducted to evaluate the viability of the bacterial strains in formulated fish feed, followed by drying at room temperature (3 days) and storage at 4 °C (4 days) (Table IV).

The selected probiotic isolates *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 showed the maximum survival rate up to 63.5 and 70.17% in fish feed respectively (Table V). Both bacterial isolates exhibited the highest growth with 30% inocula at RT (Fig. 1).

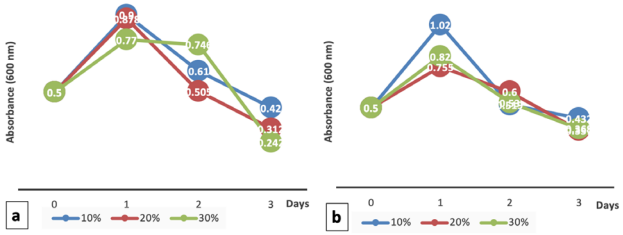

Fig. 1. Effect of inocula sizes on growth of the probiotic isolates *Sphingomonas* sp. AsCh-P3 (a) and *Bacillus aerius* AsCh-A7 (b) in fish feed extract (20% w/v) at room temperature for different incubation days.

Identification and characterization of select probiotic isolates

Conspicuous colonial growth of bacterial isolate AsCh-P3 on a solidified nutrient plate showed an irregular round, raised, creamy white with wavy margins, 2.5 mm in diameter, opaque and butyreus in consistency while colonies of probiotic isolate AsCh-A7 on solidified nutrient medium were round, convex, yellowish off white with entire margins, 1-1.5 mm in diameter, transparent and semi viscous in consistency.

Bacterial isolate AsCh-P3 (G-negative) and AsCh-A7 (G-positive) were facultative anaerobe, rod shape with rounded ends with size of 1.5×1 µm and 2×1 µm containing 2 and tuft of polar flagella respectively. Both isolates did not necessitate NaCl to grow, but tolerated salt concentration (up to 6%). The bacteria were found to grow at 25, 37 and 45 °C, reduced nitrate salt with no gas emission, positive catalase, oxidase and Voges Proskauer tests. Both strains showed good growth at MacConkey agar medium but cannot grow on cetrimide agar. Test for urease is negative for AsCh-P3 and positive for AsCh-A7. AsCh-P3 showed positive methyl red test while negative by AsCh-A7. AsCh-P3 cannot grow, but AsCh-A7 showed good growth at Simmon's citrate agar medium.

Table III. Bactericidal activity of selected probiotic isolates against different inocula of *Pseudomonas fluorescens* by cross streak method.

Probiotic isolates	Inoculum C.F.U.ml^{-1}	Different concentrations of *P. fluorescens*				
		61×10^9 (1.0)	181×10^5 (0.5)	45×10^5 (0.25)	268×10^3 (0.1)	170×10^3 (0.05)
Sphingomonas sp. AsCh-P3	193×10^5 (0.5)	±	+	+	+	+
Pseudomonas pseudomallei AsCh-P14	282×10^5 (0.5)	±	±	+	+	+
Listeria murnyi AsCh-A3	217×10^5 (0.5)	−	±	±	±	±
Kurthia gibsonii AsCh-A5	174×10^5 (0.5)	−	±	±	±	±
Bacillus aerius AsCh-A7	185×10^5 (0.5)	±	+	+	+	++
Bacillus cereus AsCh-A9	207×10^5 (0.5)	−	−	±	±	±

Figures in parenthesis showed the bacterial concentrations suspended in phosphate buffer saline at 600 nm. +, Pathogens inhibited by probiotic isolates; ±, Pathogens inhibited weakly by probiotic isolates; −, Pathogens not inhibited by probiotic isolates.

Table IV. Viable counting (C.F.U.g^{-1}) of probiotic supplemented fish feed (suspension at 0.5 O.D. 600 nm) followed by drying up to 3 days and 4 days refrigerator storage.

Probiotic isolates	Days				
	0[a]	1	2	3	7
Sphingomonas sp. AsCh-P3	93×10^6	38×10^6	125×10^4	63×10^4	40×10^4
P. pseudomallei AsCh-P14	212×10^6	102×10^6	256×10^4	112×10^4	70×10^4
Listeria murnyi AsCh-A3	177×10^6	53×10^6	85×10^4	42×10^4	23×10^4
Kurthia gibsonii AsCh-A5	114×10^6	36×10^6	70×10^4	33×10^4	20×10^4
Bacillus aerius AsCh-A7	150×10^6	50×10^6	132×10^4	57×10^4	40×10^4
Bacillus cereus AsCh-A9	167×10^6	66×10^6	249×10^4	134×10^4	43×10^4

a, 20% (v/w) of each bacterial suspension was mixed in sterilized fish feed.

Table V. Percent survival rate of probiotics in bacterial enriched, dried and stored fish feed.

Probiotic isolates	Days				
	0*	1*	2[a]	3[a]	7[a]
Sphingomonas sp. AsCh-P3	100	40.86	3.29	50.4	63.5
P. pseudomallei AsCh-P14	100	48.11	2.5	43.75	62.5
Listeria murnyi AsCh-A3	100	29.94	16.03	49.41	54.76
Kurthia gibsonii AsCh-A5	100	31.58	1.94	47.14	60.6
Bacillus aerius AsCh-A7	100	39.52	3.77	43.18	70.17
Bacillus cereus AsCh-A9	100	33.33	2.64	53.81	32.09

* % survival rate was calculated as; (CFU/g in feed after drying/ CFU/g in feed before drying at previous day)×100

Table VI. Mortality of *L. rohita* fingerlings challenged with intraperitoneal *P. fluorescens* pathogen within one month.

Groups	Bacterial Conc (O.D)	C.F.U. ml^{-1}	Days post inoculations							
			1	2	3	4[c]	5[c]	10[c]	20[c]	30[c]
Control	PBS[a]		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
P. fluorescens	1.00	61×10^9 [b]	10/10							
	0.5	181×10^5	10/10							
	0.25	45×10^5	8/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
	0.1	268×10^3	2/10	2/10	1/10	0/10	0/10	0/10	0/10	0/10
	0.05	170×10^3	2/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10

a, Control were injected with 0.1 ml of sterile PBS /fish; b, Experimental groups were injected with 0.1 ml of respective dose / fish; c, No mortality was recorded in control and experimental groups.; PBS, Phosphate buffer saline.

Morphological identification, biochemical tests as well as the molecular characterization involving 16S rRNA gene sequencing were performed on both probiotic isolates. It was found that isolate AsCh-P3 and AsCh-A7 exhibited 99% and 98% similarity index to 16S rRNA gene sequence of *Sphingomonas* sp. and *Bacillus aerius* respectively. The accession numbers MF543123 and MF543123 were assigned to the isolates *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7, respectively.

Pathogenecity of P. fluorescens and effect of probiotics

Mortalities of *Labeo rohita* fingerlings injected with *P. fluorescens* in 30 day experiment showed a dose dependent manner (Table VI). Pathogen concentration that consequent in 50% mortality was scrutinized for subsequent study. The fish groups exposed to *P. fluorescens* manifested symptoms like swollen body cavity, protrusion and reddening of the anus, damaged scales, erythma of eyes and inflated belly as presented in Figure 2.

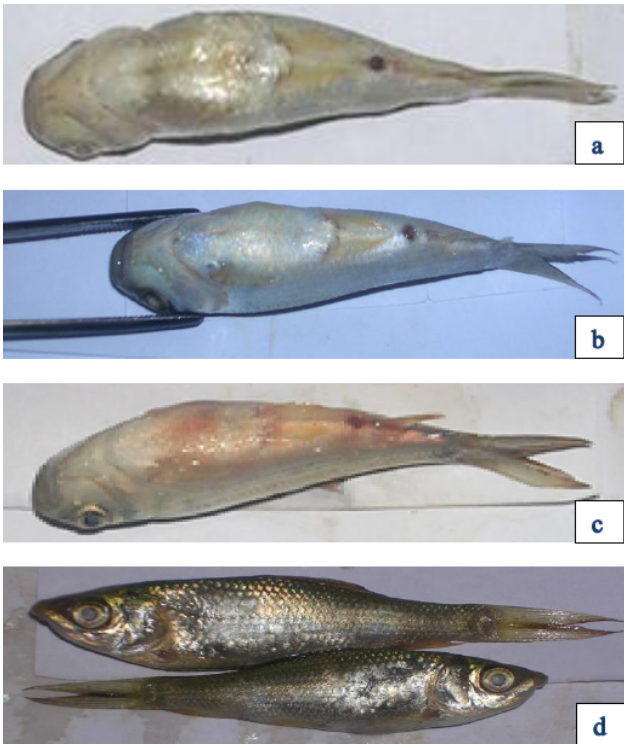

Fig. 2. Symptoms of Pseudomonas infection *i-e s*welling of belly (a), swelling of anus (b), reddening of belly (c) and damage of scales (d) following *i-p* administration of *P. fluorescens* (268×10^3 C.F.U.ml^{-1}).

The *P. fluorescens* injected fingerlings were given control and probiotics amalgamated feeds in multiple experiments and animals were observed carefully for mortalities (up to 30 days) as recorded in Table VII. It appeared that provision of probiotics in feed before and after the pathogen injection (G1b, G2b) caused a significant decrease in the mortalities (20 and 10 %) as compared to respective positive control (C2). Similarly, the mortalities reduced significantly, *i.e.,* 26.67 and 13.34% for having a probiotic feed prior pathogen exposure (G1c, G2c). But 46.67 and 26.67% mortalities were recorded in the groups receiving the probiotics containing feeds soon after the pathogen administration (G1a, G2a). Highest RPS of 60 and 80% appeared in G1b and G2b fishes fed with *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 augmented feed before and after the pathogen injection. Probioticity of the isolates was exhibited by improved RPS in the experimental fish groups (Table VIII).

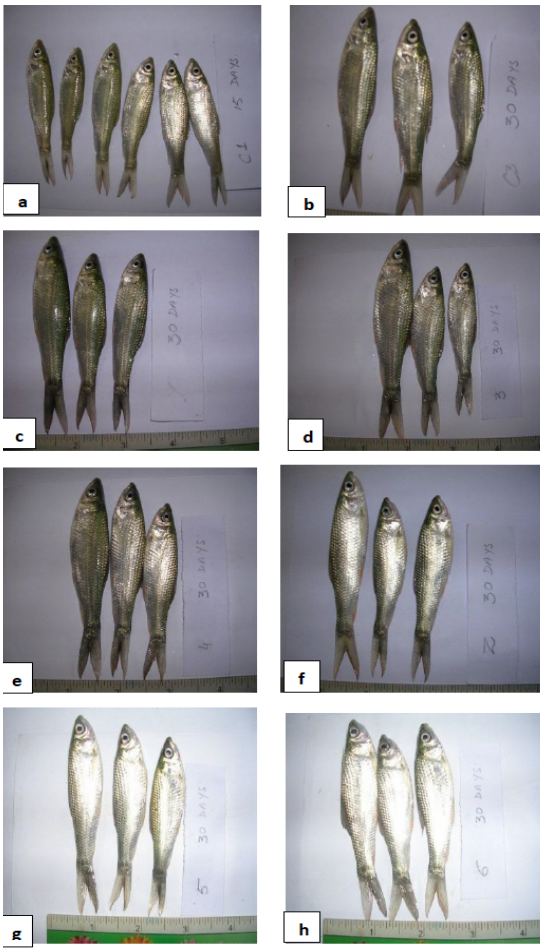

Fig. 3. Appearance of surviving fishes (asymptomatic) of negative control (a), positive control challenged with *P. fluorescens* (b), treated by *Sphingomonas* sp. AsCh-P3 (c-e) and *Bacillus aerius* AsCh-A7 (f-h) representing for different experimental groups G1a, G1b, G1c, G2a, G2b, G2c, respectively.

Table VII. Mortality of *L. rohita* fingerlings challenged with intraperitoneal *P. fluorescens* pathogen within one month and fed with probiotic augmented feed under different experimental conditions.

Groups	Group code	Days post challenge							
		1	2	3	4	5	10	20	30
Negative control-PBS	(C1)	a0±0.00	a0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Positive control- *P.fluorescens*	(C2)	b20.00	b2±0.58	1±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
P.fluorescens+ *Sphingomonas* sp. AsCh-P3	(G1a)	b2±0.00	Ab1.66±0.33	1±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Sphingomonas sp. AsCh-P3 + *P.fluorescens*+ *Sphingomonas* sp. AsCh-P3	(G1b)	a0.66±0.33	Ab0.66±0.33	0.66±0.33	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Sphingomonas sp.AsCh-P3 +*P.fluorescens* +simple feed	(G1c)	c1±0.00	Ab1±0.58	1±0.58	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
P.fluorescens + *B. aerius* AsCh-A7	(G2a)	c1±0.00	Ab1±0.00	ab0.66±0.33	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
B. aerius AsCh-A7+*P.fluorescens* + *B. s aerius* AsCh-A7	(G2b)	ac0.33±0.33	Ab0.66±0.33	a0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
B. aerius AsCh-A7+*P.fluorescens* + simple feed	(G2c)	ac0.66±0.33	Ab0.66±0.33	a±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00

All values represent means of three replicates ±S.E.M. Two values within a column not sharing a common alphabet differ significantly from both control and same experimental groups. Values are significantly different at p≤ 0.5 at single factor analysis of variance.

Table VIII. Relative Percent Survival in *P. fluorescens challenged L. rohita* fingerlings intraperitoneally within one month and fed with probiotics augmented feed.

Groups	Group code	% Mortality rate	% Survival rate	RPS (%)
Negative control-PBS	**(C1)**	**a0±0.00**	**a100±0.00**	
Positive control- *P. fluorescens*	(C2)	b50±5.78	b50±5.78	
P. fluorescens+ *Sphingomonas* sp. AsCh-P3	(G1a)	b46.66±3.33	b53.33±3.33	6.66
Sphingomonas sp. AsCh-P3 +*P. fluorescens*+ *Sphingomonas* sp. AsCh-P3	(G1b)	c20±0.00	c80±0.00	60.00
Sphingomonas sp. AsCh-P3 +*P. fluorescens* +simple feed	(G1c)	c26.66±3.33	c73.33±3.33	46.66
P. fluorescens + *B. aerius* AsCh-A7	(G2a)	c26.66±3.33	c73.33±3.33	46.66
B. aerius AsCh-A7+*P. fluorescens* + *B. aerius* AsCh-A7	(G2b)	a10±0.00	a90±0.00	80.00
B. aerius AsCh-A7+*P.f luorescens* +simple feed	(G2c)	ac13.33±3.33	ac86.66±3.33	73.32

All values represent means of three replicates ±S.E.M. Two values within a column not sharing a common alphabet differ significantly from both control and same experimental groups. Values are significantly different at p≤ 0.5 at single factor analysis of variance. RPS, Relative percent survival.

Asymptomatic surviving fish of negative and positive control as well as experimental groups challenged with the pathogen and treated with *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 were shown in Figure 3, respectively. Caudal fin deformation was observed in G2a group in which *Bacillus subtilis* AsCh-A7 supplemented feed was administered after pathogen challenge while muscle degeneration was recorded in group G1b and G2b provided with *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 treated feed before and after pathogen challenge (Fig. 4).

DISCUSSION

The present study investigated the bactericidal and bacteriostatic potential of probiotic isolates against *Pseudomonas fluorescens* (a pathogen of *Labeo rohita*) following cross streak, disc and well diffusion method. Production of inhibitory compounds in in vitro assay is helpful in the scrutiny of probiotic microbe (Gram *et al*., 1999). The possible mode of action of probiotics to exert antibacterial phenomenon may be due to antimicrobial peptide, bacteriocin, siderophore, lysozyme, proteases and production of organic acids to lower pH (Van Hai *et al*., 2009; Heo *et al*., 2013; Ferreira *et al*., 2015). The bactericidal activity of *Bacillus licheniformis* and *B. pumilus* was affected by high bile concentration and low pH (Ramesh *et al*., 2015). Pathogen growth and cell density was reduced by extracellular products (ECPs) which is the main virulent factor. *Lactobacillus plantarum* 44a and *Lactobacillus lactis* 18f inhibited *Aeromonas hydrophila*, *E. tarda* and *Staphylococcus aureus* with inhibition zones 7-12 mm (Rengpipat *et al*., 1998).

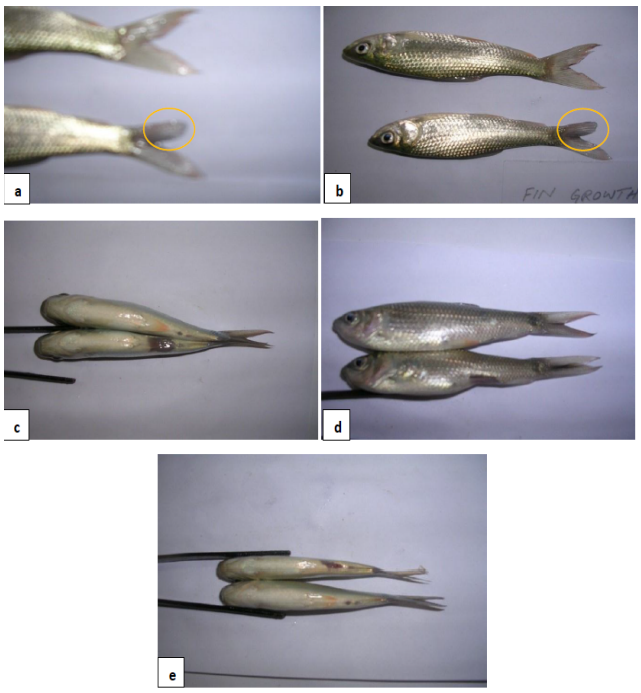

Fig. 4. Surviving fishes with varying degrees of caudal fin deformation by *P. fluorescens* in G2 group (a, b) treated with *B. aerius* AsCh-A7 and muscle degeneration by *P. fluorescens* in positive control C3 (c) and experimental groups G2b, G3b (d, e) treated with *Sphingomonas* sp. AsCh-P3 and *B. aerius* AsCh-A7, respectively.

P. fluorescens showed host specificity when the bacteria were given *i-p* injections in *L. rohita* fingerlings. Fishes were found susceptible to *Pseudomonas* septicemia. The majority of the dead fingerlings exhibited hemorrhages in the intestine, anal as well as body cavity inflammation and reddening of ventral body surface. In Pseudomonas septicemia, the observed symptoms were swollen body cavity, reddening of anus, belly inflated and erythma of eyes. It was inferred that the fish mortalities were concordant to the concentration of the fish pathogen in the intraperitoneal injections.

Regarding the bath exposure experiments performed with rohu fingerlings, no mortality was recorded even in groups with abraded skin for pathogen after 30 days post inoculation. Interestingly, the inoculated abraded fish for pathogen become normal within 30 days experimentation period. In a comparable study, Romalde *et al*. (1996) and Rasheed and Plumb (1984) were unable in reproducing the infection even in injured Gulf Killifish bathed in probiotic bacterial culture. Although here are numerous hypotheses related to the entry routes of pathogen in fish; as described in live feed by pathogens orally in turbot post larvae (Grisez *et al*., 1997). Also, skin, gills and anus were considered important entry portals of pathogen into eel (Chart and Munn, 1980), rainbow trout (Laurencin and Germon, 1987) and ayu (Kanno *et al*., 1989). Generally, failures of occurrence of disease in bathing experiments demonstrate the strong defensive mechanism of healthy fishes. Intraperitoneal inoculation of single cell protein of *B. pumilus* and *B. licheniformis* boosted the immunity against *Aeromonas hydrophila* infected *L. rohita* (Ramesh *et al*., 2015). However, bacterial pathogenic infections are most likely to happen in aquaculture. Interest in the use of fish probiotics has received increasing attention during the last decades.

Though fish mortalities in experimental groups that were given probiotics supplemented diet only after bacterial pathogen administration were higher than the fish group that was fed with probiotic enriched feed both prior and after pathogen administration but significantly lesser mortalities of the former group compared with the positive control group. This elucidated that probiotics might be competitive in colonizing their host, but they require more number of probiotic cells for enhancing their antipathogenic efficiencies. It is regarded that the presumed colonization of probiotics may have inhibited the growth and consequent virulence of the pathogen. Probiotics were proved as promising candidates in treating host GIT inflammation and preventing diseases (Azimirad *et al*., 2016; Modanloo *et al*., 2017). The presumed mechanisms may be nutrient competition (Ringo *et al*., 2016), adhesion to mucosal epithelium of GIT (Luis-Villaseñor *et al*., 2011), competitive exclusion of probiotics with intestinal epithelium and mucus to prevent pathogen colonization (Mahdhi *et al*., 2012; Sorroza *et al*., 2012) elevated feed digestibility by improving digestive enzymes (Zokaeifar *et al*., 2012), production of bacteriocin, fatty acids, organic acids and vitamin B_{12} (Vine *et al*., 2006).

Probiotics compete for adhesion receptor to reduce colonization of pathogen by antagonistic activity (Chabrillón *et al*., 2005; Luis-Villaseñor *et al*., 2011), hence proved to be as substitute for chemotherapeutants and antibiotics (Cheng *et al*., 2014). Probiotics have antagonistic activity by exerting competition of nutrients against pathogens (Ringo *et al*., 2016). Innate immunity was also be improved by probiotics by stimulating serum peroxidase, lysozyme and blood respiratory burst activities by *lactobacillus lactis* against *Streptococcus iniae* and *Pseudomonas fluorescens* in Nile tilapia and olive flounder (Heo *et al*., 2013; Merrifield and Carnevali, 2014; Beck *et al*., 2015).

Dietary administered *B. subtilis* and *P. aeruginosa* VSG-2 in concentrations of 1.5×10^7 and 10^7- 10^9 CFU $g^{-}1$ improve immune response against *A. hydrophyla* in *L. rohita* (Kumar *et al*., 2006; Giri *et al*., 2012) while *B. subtilis* AB1 supplemented diet in different ways (viable or sonicated or

cell free supernantant) *per se* protected rainbow trout against Aeromonas (Newaj-Fyzul *et al*., 2007). Bacillus genus contributes to promote growth, immune response and protect from diseases and reviewed for immunomodulation potential extensively (Mingmongkolchai and Panbangred, 2018).

Probiotics can be administered as multi strain or coculture to boost up the growth and immune response. *B. subtilis* L10 and G1 were resulted as immune stimulant and growth promoter in juvimile white shrimp against *Vibrio harveyi* when inoculated as combined culture (Zokaeifar *et al*., 2012). *B. subtilis* strain S12 inhibited *V. harveyi* in white shrimp, *Litopenaeus vannamei* in form of monoculture (Liu *et al*., 2014).

The literature evidenced the antagonistic potential of Bacillus sp. against aquatic pathogens as well as Acinetobacter sp. KX775221, *Acinetobacter tandoii* KX775222 and *Aeromonas hydrophila* KX756709 (Kaynar and Beyatli, 2012; Ramesh *et al*., 2015). *B. amyloliquefaciens* showed antagonism to control vibriosis in turbot *Scophthalmus maxima* (Chen *et al*., 2016a, b), Nile tilapia *Oreochromis niloticus* (Selim and Reda, 2015), catfish *Ictalurus punctatus* (Ran *et al*., 2012), European eel *Anguilla anguilla* (Cao *et al*., 2011) and Catla catla (Das *et al*., 2013).

CONCLUSION

The study was an attempt to evaluate the inhibitory effects of probiotic bacterial isolates *Sphingomonas* sp. AsCh-P3 and *Bacillus aerius* AsCh-A7 against fish pathogen *Pseudomonas fluorescens*. The outcomes revealed that the use of the probiotics augmented fish feed as demonstrated in this study will be highly beneficial for ensuring the fish health in future.

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Ethical approval

It is to certify that during experimental research, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Statement of conflict of interest

The authors declare that they have no conflict of interest.

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