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Probiotic Antagonism of *Sphingomonas* sp. against *Vibrio anguillarum* Exposed *Labeo rohita* Fingerlings

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Abstract Antagonistic properties of *Sphingomonas* sp. AsCh-P3 against fish pathogen *V. anguillarum* was investigated in vitro and in vivo assays. Out of twenty probiotic isolates, nine expressed antagonistic activity by cross streak and six gave prominent results by disc and well diffusion methods. Maximum growth inhibition zone (15 mm) was observed by *Sphingomonas sp.* AsCh-P3. Cross streaks of six isolates of known C.F.U./ml across different concentrations of pathogen indicated profound growth inhibition by low concentration. The six bacterial isolates were mixed in fish feed, dried and stored in refrigerator upto 4 days. *Sphingomonas sp.* AsCh-P3 showed 63.5% survival in dried feed under the storage condition and grew best with 30% inoculum size in 20 % fish feed extract. The *Labeo rohita* fingerlings were exposed to *V. anguillarum* intraperitoneally. Fish mortalities appeared in a dose dependant manner. Pathogen exposed fishes were provided with *Sphingomonas sp.* AsCh-P3 treated feed in different experimental groups as G1-soon after, G2- prior and after and G3-prior challenge. Percent mortalities of 33.33, 16.66 and 23.33 and RPS values of 16.67, 58.35 and 41.67 were recorded for G1, G2, and G3, respectively. Probiotic loads reduced the pathogens virulence in dose responsive manners.

Keywords Probiotics and fish health, Antagonistic bacteria to fish pathogen, Virulence, *Vibrio anguillarum,* Disease resistance, *Sphingomonas*, Vibriosis, *Labeo rohita*

1. Introduction

Intensive quaculture has led to higher outbreaks of disease encompassing an ever increasing range of pathogens [1, 2]. Efforts have been made to reduce infections caused by *Vibrio*, this genus has been associated with severe disease in fish culture [3, 4]. Species such as *Vibrio harveyi*, *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus* and *V. Vulnificus* have frequently been associated with mortalities both in hatcheries and grow out ponds [5-7].

Vibriosis occurs predominantly in marine and brackish waters and occasionally in fresh water fish species. *V. anguillarum* is the most commonly encountered fish pathogen [8-10]. *V. anguillarum* is a Gram negative bacterium that causes hemorrhagic septicemia in fish, a disease that leads to great economic losses in fish farming worldwide. Although this bacterium has been reclassified as *Listonella anguillarum* based on 5S rRNA gene sequence analysis, however, it is still commonly referred to as *V. anguillarum* [11].

Prevention of fish diseases by the application of live pathogen-antagonistic bacteria has received widespread interest and such probiotics are gaining much popularity [12-16]. Probiotics refer to beneficial (live) bacterial cultures whose applications improve health of the host. A probiotic must not be pathogenic or toxic to its host. Most probiotics are supplied as live supplements in food and they obviously have the ability to survive in intestinal tract where they break down toxic or innutritious components of the diet and/or may prevent potential pathogens from colonizing the gut by production of antimicrobial compounds, or by out competing them for nutrients or mucosal space [17-20].

In this work, first virulance of *V. anguillarum* to healthy *Labeo rohita* fingerlings has been established. Purpose of the present study was to determine antipathogenic potential of *Sphingomonas* sp. AsCh-P3 in *L. rohita* fingerlings following the vibriosis trial.

2. Materials and Methods

2.1. Source of Fish Pathogens

Virulent strain of *Vibrio anguillarum* (90-11-287; sero type 01) that carries the pJM1 plasmid was a generous gift from K. Pedersen, Royal Veterinary and Agricultural University, Copenhagen, Denmark [21].

2.2. Isolation and Selection of Probiotic Bacterial Isolates

Twenty bacterial isolates were secured from different

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home made yogurt and raw milk samples for studying their antagonistic activity against the two fish pathogens [22].

2.3. Inhibition of Fish Pathogens by the Bacterial Isolates

Antagonism of *V. anguillarum* by the bacterial isolates was assessed by cross streaking method [23]. For this purpose, suspension of the pathogen in phosphate buffer saline (O.D 0.5±0.05) was streaked at right angles across streaked inocula of the bacterial isolates (O.D. 0.5±0.05) to be tested on nutrient agar. The plates were incubated at room temperature (~27-30°C) for 24-48 hours. Antagonism was indicated by an interruption in the growth of the pathogens. From the twenty bacterial isolates tested, nine isolates were selected on the basis of their prominent antibiosis against the pathogen for further validation through filter paper disc and well diffusion methods.

2.4. Antagonism Assay

Antagonistic activity of the nine select bacterial isolates against *V. anguillarum* was assessed by disc and well diffusions methods [24]. The pathogen and the probiotics were grown in nutrient broth for an overnight period at 37ºC, centrifuged and filter sterilized. One hundred micro liters of each filtered sterilized culture fluid was loaded on 9 mm diameter sterile disc made from Whatman filter paper No. 1. These discs were placed on nutrient agar plates seeded with 50 *µl* of *V. anguillarum* suspension of 0.5±0.05 O.D. The plates were incubated at room temperature (~27-30ºC) for 24-48 hours and the zones of growth inhibition around the discs were then measured.

For well diffusion method, *V. anguillarum* inoculums was mixed in nutrient agar medium (kept molten around 50ºC) at 3% (v/v) ratio and poured in sterilized petri plates. Wells of 9 mm diameter were punched into the solidified agar with the help of a metal borer and then 100 *µl* of filtered sterilized culture fluid of a given bacterial isolate was introduced in a well. The plates were incubated at room temperature (~27-30ºC) for 24-48 hours and growth inhibition zones (GIZ) were then recorded. The five bacterial isolates which yielded GIZ of higher than 11 mm diameter were selected and proceeded for further study.

2.5. Inhibition of the Probiotics against Different Concentrations of the Fish Pathogen

To find antibiosis of the probiotics against different concentrations of the pathogen, cell suspensions of 0.05, 0.1, 0.25, 0.5, and 1.0 O.D. at 600 nm were prepared in phosphate buffer saline (PBS) of pH 8. Cell densities of the probiotics were adjusted at 0.5±0.05 in the buffer. Viable counting of the bacterial suspensions was performed on nutrient agar. The suspensions of the bacterial isolates were streaked at right angles across streaked inocula representing different concentrations of the pathogen on nutrient agar. The plates were incubated at room temperature (~27-30ºC) for 24-48 hours. Interruption and its intensity in the growth of the pathogen indicated the level of antibiosis of test bacterial isolates [23].

2.6. Inoculation of Probiotics Isolates in Fish Feed

The select bacterial isolates were grown overnight in nutrient broth, centrifuged and the pellets suspended in PBS up to cell density of 0.5 ± 0.05 at 600 nm. The bacterial suspensions (20% v/w) were mixed with autoclaved formulated fish feed and dried in a laminar flow cabinet, kept at room temperature (~27-30ºC) for three days and then freezed for one week period. Serial dilutions of the processedvfeed samples were prepared in saline water (0.89%) and analyzed for viable counting on nutrient agar plates. The percent survival of each of the bacterial isolate was calculated as follows; % survival = (Ao/A1) × 100. Where; Ao = C.F.U. of bacteria per gram after drying and A1 = C.F.U. of bacteria per gram before drying.

On the basis of the antagonistic and survival potentials, the probiotic isolate later identified as *Sphingomonas sp.* AsCh-P3 was selected for inoculum optimization in the formulated fish feed.

2.7. Optimizing Bacterial Inoculum for the Fish Feed

For the inoculum optimization, instead of solid feed, its extract was used. For this purpose, 20 g of the feed were boiled in 100 ml distilled water for 10 minutes and then autoclaved, routinely, for 15 minutes. The autoclaved feed was centrifuged to get clear extract. Cell suspension of the bacterial isolate, AsCh-P3 in PBS was inoculated at 10%, 20% and 30% (v/v) in the feed extract. The cultured feed extracts were incubated at room temperature (30±0.2^{o}C) and the growth was assessed daily up to 3 days post incubation as C.F.U./ml.

2.8. Identification and Biochemical Characterization of the Bacterial Isoltes

For taxonomic identification, all the bacterial isolates were subjected to colonial, cell morphology and biochemical tests [25-27] and identified accordingly. However, identification of the select strain AsCh-P3 screened through its 16S rRNA gene sequencing.

2.9. DNA Extraction, 16 S rRNA Gene Amplification and Sequencing

Total DNA of the isolate was extracted from its fresh culture. A loopful of the culture from a colony on nutrient agar was heated in water bath (95ºC) in 45µl of 50 mM NaOH followed by addition of 5 µl of 1M Tris HCl (pH 8) and the eppendorff was centrifuged for 10 minutes. Amplification of the 16S rRNA gene was then accomplished by PCR with DNA polymerase (KOD FX). The reaction mixture comprised of 2 mM deoxynucleoside triphosphates (dNTP), 50 uM of each 27F (5□-AGAGTTTGATCCTGG CTCAG-3□) and 1492R(5□-AGGCTACCTTGTTACGAC TT-3□) primers and 2 µl of the template DNA (bacterial supernatent). PCR was performed for 30 cycles, each consisting of a 10-sec denaturation step at 98℃, a 30-sec

annealing step at 53℃ and a 1-min extension step at 72℃. PCR product was purified and partially sequenced on automated sequencer.

The sequence was subjected to homology search using BLAST-querying the GenBank database http://www.ncbi.nlm.nih.gov/blast (last accessed March, 2011) of the National Center for Biotechnology Information (NCBI).

2.10. Pathogenecity of *Vibrio anguillarum* in *Labeo rohita* Fingerlings

Labeo rohita fingerlings were obtained from the fish farm at Muridkey in the month of August. They were acclimated to the experimental conditions for 10 days in the laboratory. Health status of the fish was kept under examination throughout the acclimatization period.

The fingerlings were initially exposed to *Vibrio anguillarum* in bath challenge but neither disease symptoms nor mortality were observed. Therefore, intraperitoneal (*i.p*) inoculation was accomplished according to the methods described by Romalde et al. [28] and Robertson et al. [29]. Accordingly, overnight fresh culture of *Vibrio anguillarum* was centrifuged, suspended in PBS at cell densities of 1.0, 0.5, 0.25, 0.1, 0.05 which were marked out for C.F.U./ml as 50×10^9, 243×10^5, 56×10^5, 267×10^3, 97×10^3 respectively.

To examine the virulence of the fish pathogen, groups of 10 *Labeo rohita* fingerlings received i.p injections of different concentrations of the bacterium. All fishes in each group were injected intraperitoneally with 0.1 ml of PBS suspension representing a given concentration of fish pathogen. Control group was injected with sterile PBS in the same manner. Each group was housed in a separate aquarium at 27-30ºC containing 10 liter static fresh water and continuously aerated through an air stone. One third water of aquarium was daily changed and the feces were siphoned off. The fish groups were monitored up to 30 days and fed sterilized formulated fish feed. Mortalities were recorded daily. Pathogen dose of 56×10^5C.F.U./ml with 40% mortality was selected for next experiment.

2.11. Probiotic Treatment of Infected Fish

The bacterial isolate *Sphingomonas* sp. AsCh-P3 showing higher antagonistic activity for *V. anguillarum* in vitro assay was employed for the in vivo study. These experiments were conducted after consulting the methods described by Gram et al. [30] and Spanggaard et al. [31]. For in vivo experiment, the experimental set up is described in the table 1.

Each of control and experimental group comprised of triplicates with 10 fish/replicate. At start of the experiment, different groups of the fishes were provided simple sterile or corresponding probiotics treated feeds for fifteen days. Then each experimental fish was injected intraperitoneally with 0.1 ml of the pathogen suspension. The positive control group received the pathogen whilst the negative control was administered *i.p* with 0.1 ml of PBS. Aquaria water's temperature was maintained at 24 to 27ºC. Mortalities were recorded daily and the dead fish removed out from aquaria. All the dead fish were checked for clinical signs of vibriosis. Relative percent survival (RPS) was calculated according to Ellis [10] as follows: RPS = 1-(% mortality of experimental group / % mortality of control) × 100.

2.12. Statistical Analysis

All the experimental data were analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple range test (SPSS ver.16.0 software, SPSS, Chicago, IL, USA).

3. Results

3.1. In Vitro Bacterial Antagonism against the Fish Pathogen *Vibrio anguillarum*

Twenty bacterial isolates were tested for their antibiosis against the fish pathogen *Vibrio anguillarum* by cross streak method (Table 2). As can be seen from this Table, only 09 bacterial isolates expressed antagonism against the pathogen, while 11 could not exert any growth inhibiting effect to the pathogen. Based upon the growth inhibition zones of the cell free by filtered sterile broths of the probiotic isolates were selected for further study. *Sphingomonas sp.* AsCh-P3 showed highest GIZ of 15 mm against the pathogen whereas the remaining select isolates showed GIZ in the range 11-13 mm against the pathogen (Table 3).

Table 1. Details of the experimental set up for probiotic application to the infected fish

Sr. No.	Groups	Sub groups	Feeding (15 days)	*i.p.* injection (0.1 ml)	Feeding (30days)
1	Negative Control	C1	Sterile feed	PBS	Sterile feed
2	Positive Control	C2	Sterile feed	*V. anguillarum*	Sterile feed
3	Experimental Groups	G1	Sterile feed	*V. anguillarum*	*Sphingomonas sp* AsCh-P3 treated feed
		G2	*Sphingomonas sp* AsCh-P3 treated feed	*V. anguillarum*	*Sphingomonas sp* AsCh-P3 treated feed
		G3	*Sphingomonas sp* AsCh-P3 treated feed	*V. anguillarum*	Sterile feed

Table 2. Antagonistic activity shown by different probiotic isolates against fish pathogens *Vibrio anguillarum* and *Pseudomonas fluorescens* by cross streak method

Probiotic Isolates	*V. anguillarum*
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. aeroginosa 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 subtilis 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 3. Growth inhibition shown by cell free culture fluid (nutrient broth) of different probiotic isolates against the fish pathogens *Vibrio anguillarum* and *Pseudomonas fluorescens*

Probiotic Isolates	*V. anguillarum*	
	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	12	12
P. pseudoalcaligenes AsCh-A4	10	10
Kurthia gibsonii AsCh-A5	11	11
Enterobacter agglomerans AsCh-A6	11	10
Bacillus subtilis AsCh-A7	12	12
Bacillus cereus AsCh-A9	13	12
Sporolactobacillus inilunis AsCh-L6	10	10

Values represent diameter of growth inhibition zones in mm
a diameter of each disc required = 9mm
b diameter of each well = 9mm

3.2. Growth Inhibition of the Select Probiotic Bacterial Isolates against Different Concentrations of the Fish Pathogen

Six probiotic isolates of known C.F.U./ml were streaked across inocula of strength of the fish pathogen (Table 4). Best antibiosis was shown by *Sphingomonas sp.* AsCh-P3 against *V. anguillarum* at all concentrations.

However, a general trend indicated profound growth inhibition for the pathogen streaked with inocula of test organisms containing less number of C.F.U./ml.

Table 4. Antagonistic activity shown by the selected probiotic isolates against different concentrations of the fish pathogen *Vibrio anguillarum* by cross streak method

Probiotic Isolates	Inoculum CFU/ml	Different concentrations of *V. anguillarum*				
		50×10^9 (1.0)	243×10^5 (0.5)	56×10^5 (0.25)	267×10^3 (0.1)	97×10^3 (0.05)
Sphingomonas sp. AsCh-P3	193×10^5 (0.5)	±	+	+	+	++
P. 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 subtilus AsCh-A7	185×10^5 (0.5)	±	±	+	+	+
Bacillus cereus AsCh-A9	207×10^5 (0.5)	−	−	−	−	±

Figures in parenthesis shown 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.

3.3. Viable Counts of the Probiotics in the Formulated Feed

Provision of food loaded with probiotic to an animal is generally characterized by an intervening period required for transporting and storage etc. This experiment was designed to observe the viability of the probiotic in the formulated feed, followed by drying at room temperature for 3 days and then storing in refrigerator for 4 days (Table 5, Figure 1). The select bacterial isolate *Sphingomonas sp.* AsCh-P3 showed highest survival rate up to 63.5% in the feed (Table 6). The bacterium grew best at 30% inoculum at 27-30ºC (Table 7, Figure 2).

On the basis of results of antagonistic experiments and best survival rate in fish feed, Sphingomonas *sp.* AsCh-P3 was selected for in vivo study.

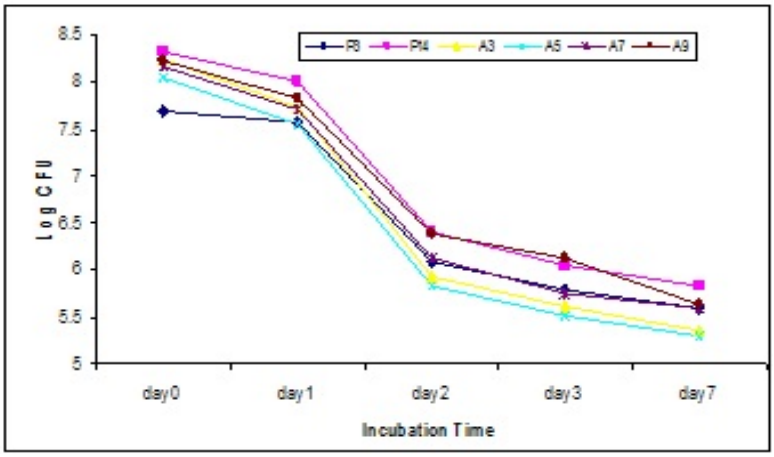

Figure 1. Viable counts of the selected probiotic bacterial isolates in fish feed during drying at room temperature (day 1-3) and storage in refrigerator (upto day 7)

Table 5. Viable counting (C.F.U./g) of fish feed mixed with select bacterial isolates suspensions (0.5 O.D. at 600 nm) following drying in laminar flow at room temperature 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 subtilis 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 and CFU/g was measured

Table 6. Percent survival rate of the probiotics in fish feed following drying in laminar flow at room temperature up to 3 days and 4 days storage in refrigerator

Probiotic Isolates	Days				
	0[a]	1	2	3	7
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 subtilis AsCh-A7	100	39.52	3.77	43.18	70.17
Bacillus cereus AsCh-A9	100	33.33	2.64	53.81	32.09

a 10% (w/v) of each bacterial suspension was mixed in sterilized fish feed and CFU/g was measured
% survival rate was calculated as; (CFU/g in feed after drying/ CFU/g in feed before drying at previous day)×100

Table 7. Effect of inocula sizes on growth of the probiotic *Sphingomonas sp.* AsCh-P3 in fish feed extract (20% w/v) at different incubation days

Days of incubation	*Sphingomonas sp.* AsCh-P3		
	10%	20%	30%[a]
0	101×10^5		
1	208×10^7	32.80×10^7	51.35×10^9
2	119×10^7	36.54×10^7	44.51×10^9
3	82×10^7	66.97×10^7	51.05×10^9

a , *Sphingomonas* sp. AsCh-P3 with 30% inoculum was selected for in vivo study; Values represent C.F.U/ml of the feed extract

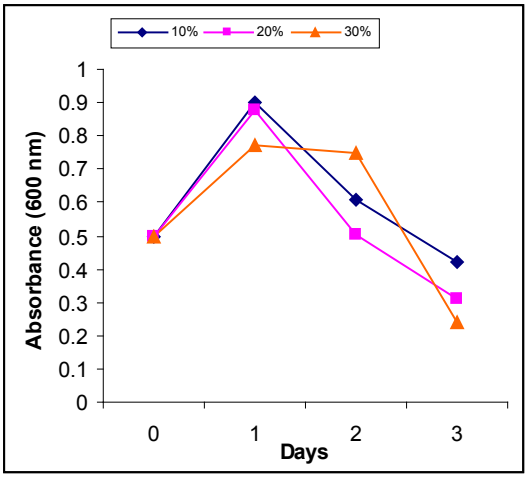

Figure 2. Effect of inocula sizes on the growth of the probiotic isolate *Sphingomonas sp.* AsCh-P3 in fish feed extract (20% w/v) for different incubation days

3.4. Characterization and Identification of AsCh-P3 Isolate

Colonies of bacterial isolate AsCh-P3 on nutrient agar appeared irregular, raised, creamy white with wavy margins, opaque and butyreus in consistency and measured up to 2.5 mm in diameter.

Bacterial isolate AsCh-P3 was Gram-negative slender rod shaped cells with rounded ends measured up to 1.5×1 µm. The facultative anaerobe was motile by means of 2 polar flagella. The isolate could not require sodium chloride for growth but tolerated up to 6% salt concentration grew at 25, 37 and 45ºC, reduced nitrate with no gas production. The urease negative bacterium yielded positive results for catalase, oxidase, Voges Proskauer, and methyl red tests. The strain showed good growth at MacConkey agar, no growth at cetrimide agar.

On the basis of morphological, biochemical tests and 16S rRNA gene sequencing, the isolate AsCh-P3 exhibited 99% similarity to 16S rRNA gene sequence of *Sphingomonas sp.*

3.5. Pathogenecity of *V. anguillarum* and Effects of the Probiotic Isolate

Mortalities in *Labeo rohita* fingerlings infected with *V. anguillarum*, mortalities appeared in a dose dependant manner (Table 8). The pathogen dose which resulted into 40% mortality was selected for further investigation. The fishes exposed to *V. anguillarum* showed symptoms of reddening of body, swelling and reddening of belly, hemorrhage and erythma in eyes (Figure 3).

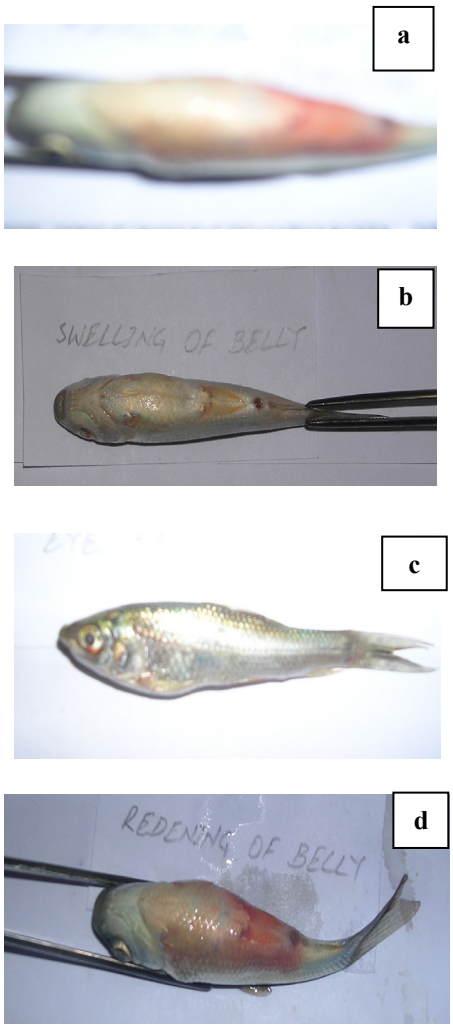

Figure 3. Typical vibriosis symptoms *i-e* reddening of body (a), swelling of belly (b), hemorrage/erythma in eye (c) and reddening of belly (d). following *i-p* administration of *V. anguillarum* (56×10^5 C.F.U/ml)

Table 8. Mortality of *L. rohita* fingerlings within one month challenged by the pathogen *V. anguillarum* administered intraperitoneally

Groups	Pathogen Conc (O.D)	CFU/ml	Days post inoculation							
			1	2	3	4	5	10	20	30
Control	PBS[a]		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
V. anguillarum	1.00	50×10^{9}[b]	10/10							
	0.5	243×10^5	10/10							
	0.25	56×10^5	2/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10
	0.1	267×10^3	1/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10
	0.25	97×10^3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a, Control were injected with 0.1 ml of sterile PBS /fish (PBS-Phosphate buffer saline)
b, Experimental groups were injected with 0.1 ml of respective dose / fish.

Table 9. Mortality of *L. rohita* fingerlings within one month challenged by the fish pathogen *V. anguillarum* administered intraperitoneally and fed with *Sphingomonas* sp. AsCh-P3 augmented feed at 3% wet body weight under different experimental conditions

Groups	Days post challenge							
	1	2	3	4	5	10	20	30
Negative control-PBS (C1)	a 0±0.00	a 0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Positive control- *V.anguillarum* (C2)	b 2±0.00	b 1±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
V.anguillarum+ *Sphingomonas* AsCh-P3 (G1)	b 2±0.00	a 0.66±0.33	0.66±0.33	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Sphingomonas AsCh-P3+*V. anguillarum* + *Sphingomonas* AsCh-P3 (G2)	a 0.66±0.33	a 0.66±0.33	0.33±0.33	0±0.00	0±0.00	0±0.00	±0.00	0±0.00
Sphingomonas AsCh-P3+*V.anguillarum* +simple feed (G3)	c 1±0.00	a 0.66±0.33	0.66±0.33	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 each other at p≤ 0.5 at single factor analysis of variance

Table 10. Relative percent survival within one month experimental period of *L. rohita* fingerlings challenged with *V. anguillarum* intraperitoneally and fed with *Sphingomonas* sp. AsCh-P3 augmented feed

Groups	Subgroups	Mortality (%)	Survival (%)	RPS
Negative control-PBS (C1)	C1	a 0±0.00	a 100±0.00	-
Positive control-*V.anguillarum* (C2)	C2	b 4±1.15	b 60±5.78	-
V.anguillarum+*Sphingomonas* sp. AsCh-P3	G1	b 3.33±0.33	b 66.66±3.33	16.67
Sphingomonas sp. AsCh-P3 +*V.anguillarum* + *Sphingomonas* sp. AsCh-P3	G2	c 1.66±0.33	c 83.33±3.33	58.35
Sphingomonas sp. AsCh-P3+*V.anguillarum* + simple feed	G3	bc 2.33±0.33	bc 76.66±3.33	41.67

RPS: 1-(% Mortality of experimental group / % Mortality of positive control) x 100 All values represent means of three replicates ±S.E.M.
Values are significantly different at p≤ 0.5 at single factor analysis of variance
Values not sharing a common alphabet within a column differ significantly from each other.

V. anguillarum injected fingerlings were fed with control and probiotics containing feeds in various experimental designs the animals were kept under daily observation for mortalities up to 30 days (Table 9).

For positive control group exposed to pathogen (C2), 40% mortality was observed whereas in case of the group G1 (*Sphingomonas* AsCh-P3 treated after pathogen challenge), the value reduced to 33.33%. Mortality reduced down to 16.66% when the fish were fed the probiotic treated feed prior as well as after the pathogen injection in group G2. About 23% mortality was recorded for the group G3 that received probiotic containing feed before and sterile feed after the injection. It is important to note that the experimental groups provided with the probiotic augmented feed had significantly lower mortalities as compared to the positive control. Highest relative percent survival (RPS) of 58.35% appeared in G2 fishes fed with *Sphingomonas* AsCh-P3 containing feed before and after pathogen injection. Probiotic effect was manifested by increased RPS values in the experimental groups (Table 10).

4. Discussion

Nine out of the twenty bacterial isolates tested by cross streak method against the fish pathogen *V. anguillarum* showed positive results. Filtered sterilized supernatants of the isolates also manifested inhibitory effects on growth of the pathogen. This indicated that exo-products of the probiotics are involved in controlling the pathogen. It is well known that cell free supernatants of probiotic bacteria exert inhibitory activity against pathogens in aquaculture system with varying degrees of stability [30, 32-35]. It is also known that microbial populations may release chemical substances that have a bactericidal or bacteriostatic effect on other microbes. Production of antibiotics [36], bacteriocins [37], siderophores [38], lysozymes, proteases, and/or hydrogen peroxide and the alteration of pH values by the production of organic acids [39] are some of the most familiar processes which alter inter population relationships by influencing the outcome of competition for chemicals or available energy [38, 40] and show wide range of mechanisms underlying the antagonistic effects of probiotics.

Probiotic should be ingested in high enough numbers to beneficially affect the host's health. Good viability of probiotics in fish feed before its consumption is required for achieving their positive roles. Fish feed is usually formulated with a low water activity to prevent microbial deterioration over several months. This practice allows achieving a high

stability during the drying process, with final moisture content closer to the original value of the unprocessed feed [41, 42].

Vibrio anguillarum, a marine fish pathogen, could show host specificity when was inoculated intraperitoneally (*i-p*) in *Labeo rohita* fingerlings. The fresh water fish was susceptible to vibriosis and majority of the dead fingerlings showed hemorrhages of intestine, swelling of belly and reddening of ventral part of the body. Similar symptoms had been reported previously in flat fishes suffering from vibriosis [3, 43-45]. It was found that the mortalities were dependent on dose of the bacterial pathogen. Virulence of *V. anguillarum* became evident with a final mortality of 40% in fish challenged with 56×10^5 C.F.U. /ml. Hjelm et al. [46] reported 80% mortality in turbot expressed to *V. anguillarum* at concentration ranging from 10^3 to 10^7 C.F.U. /ml.

There are several hypotheses about the routes of entry of *V. anguillarum* in fish; oral delivery of the pathogen by live food has been reported in turbot post larvae [47, 48]. Skin, gills and anus are also important portals of entry of *V. anguillarum* into eel [49], rainbow trout [50] and ayu [51] respectively. Olsson et al. [52] proposed that *Vibrio* cells penetrate the intestinal mucus but epithelial cell penetration or endocytosis was not evident. However, bacterial infections are not uncommon in aquaculture.

Mortalities in groups fed with the probiotic supplemented feed only after pathogen administration turned out to be higher than the group which was fed probiotics containing feed both before and after the administration. This indicates that the probiotic might have been colonizing the host but they might had required further probiotic cells and/or time for increasing their antipathogenic potential. It may be speculated that the presumed colonization of probiotic earlier to the pathogen challenge might have prevented growth and virulence of the pathogen. Reduced percent mortality and increased RPS have been reported by medicinal plants extract supplemented fish feed [7, 53]. Conclusively, the probiotic *Sphingomonas* AsCh-P3 supplemented feed significantly decreased the fish mortalities following the exposure to the *V. anguillarum* under different experimental conditions as compared to positive control in *Labeo rohita* fingerlings. These results necessitate the provision of healthy microbial communities in aquaculture systems.

5. Conclusions

The study is an evaluation of inhibitory effects of bacterial strain *Sphingomonas* sp. AsCh-P3 against fish pathogen *Vibrio anguillarum* which appeared as a good probiotic candidate and will be used as additive in fish feed in future.

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