FIRST IDENTIFICATION OF A NOVEL PROBIOTIC BACTERIUM *STREPTOCOCCUS PHOCAE* AND IT'S BENEFICIAL ROLE IN DISEASES CONTROL

*Paulraj Kanmani[1], R. Satish Kumar[1], N. Yuvaraj[1], K. A. Paari[1], V. Pattukumar[1], Venkatesan Arul[2]**

1. PhD scholars, Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry-605014, INDIA.
2. Dr. Reader, Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry-605014, INDIA.

Abstract

The strain PI80 which showed promising antimicrobial effect on different shrimp and fish pathogens was isolated from gut of shrimp Penaeus indicus. Based on 16Sr RNA gene sequence similarities and phenotypic characterization, strain PI80 was identified as a member of the genus Streptococcus. The G+C content of the partial DNA sequence was 53 %. *Streptococcus phocae* PI80 exhibited resistance against methicillin, kanamycin, neomycin and amikacin. Hemolytic and phenotypic results showed the differentiation of strain PI80 from other species within the genus Streptococcus. *S. phocae* PI80 showed broad spectrum of antagonistic activity against Gram-positive and Gram-negative pathogens by producing its own antimicrobial compound like bacteriocin. Moreover the culture growth parameters revealed that the PI80 having potential to grow at high temperature, pH and NaCl concentration.

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Introduction

The genus *Streptococcus* consists of a phenotypically diverse group of catalase-negative, Gram-positive, cocci shaped bacterium. Many novel species from human and animal sources have been described in this genus[1]. Though more than 60 species has been reported in the genus, only few species have been isolated from marine animals. Microbial strains isolated from marine mammals are *Streptococcus phocae*[2-4], *Streptococcus halichoeri*[5], *Streptococcus dysgalactiae subsp dysgalactiae*[4] and *Streptococcus marimammalium*[6].

***Corresponding author:**
Dr. Venkatesan. Arul,
Reader, Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry-605014, INDIA

Phone: 091-413-2654429, **Fax:** 091-413-2655212
E-mail: varul18@gmail.com

Streptococcus phocae was first isolated from seals[2] later in fur seals[3]. This organism was suspected to be associated with respiratory infection, starvation both in adults and offsprings of fur seals. Vossen et al. (2004)[4] isolated beta haemolytic units of *S. phocae* from harbor seals (*Phoca vitulina*) and Grey seals (*Halichoerus grypus*). Gibello et al. (2005)[7] first identified *S. phocae* in Atlantic salmon *Salmo salar*. All these strains were beta hemolytic *Streptococci*. Though *S. phocae* was isolated from diseased fish it was not proved, whether the disease was caused by the microorganism. Skaar et al. (1994)[2]; Henton et al. (1999)[3]; Vossen et al. (2004)[4]; Gibello et al. (2005)[7] reported that hemolytic *Streptococcus phocae* isolated from different species of seals and fish.

Till to date no one reported non hemolytic *Streptococcus phocae* isolated from shrimp. Here, first time we have reported a non hemolytic probiotic *S. phocae* isolated from gut of shrimp *Penaeus indicus*.

The present study was carried out to identify and characterize a novel non-hemolytic *Streptococcal* strain isolated from Indian white

prawn *Penaeus indicus. S. phocae* has been used as a probiotic to culture the fish *Cyprinus carpio* and post larvae of *Penaeus monodon* and to restrain the following fish pathogen such as *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *V. harveyi* [8].

Isolation and characterization of a heat stable bacteriocin (9.2kDa) from *S. phocae* and its usage as biopreservative in food preservation and probiotics in shrimp farming is in progress.

Material and Methods

Isolation of strain PI80

Under aseptic conditions, gut was removed from *Penaeus indicus* shrimp caught in the wild, homogenized with 0.8% (w/v) NaCl, plated on lactobacillus MRS agar plates (Himedia, MUMBAI, INDIA) and incubated at 37°C for 16 h. Separate bacterial colonies were picked and grown on fresh MRS agar plates incubated at 37°C for 16 h. Over top of these plates, 10 ml of soft agar containing indicator strains (10^5 $CFUml^{-1}$) were poured. After incubation for 16 h at 37°C, the plates were examined for antagonistic activity.

Biochemical characterization of strain PI80

The biochemical characterization such as Gram staining, motility, starch hydrolysis, nitrate reduction, oxidase, catalase, indole, H_2S production and carbohydrate profile tests were analyzed for bacterial strain PI80.

Polymerase Chain Reaction for 16SrRNA gene amplification

The total genomic DNA was isolated from *Streptococcus phocae* PI80 and used as template DNA for gene amplification. The 16SrRNA gene sequence was amplified using 16SrRNA gene specific primers (Forward primer 5'- AGA GTT TGA TCC TGG CTC AG-'3 and Reverse primer 5'- ACG GCT ACC TTG TTA CGA CTT-'3). The reaction mix was carried out totally in 25µl containing water (7.5µl), Taq buffer (3µl), dNTP (2.5µl), both forward and reverse primers (2µl), Taq polymerase (5µl) and template DNA (5µl). All chemicals were procured from GENEI (BANGALORE, INDIA). The reaction conditions were performed with initial denaturation temperature at 95°C (4min), denaturation temperature at 95°C (30 Sec), annealing temperature at 55°C (30 Sec), extension at 72°C (30 Sec) and final extension at 72°C (10 min). Amplification was carried out in Eppendorf Thermocycler, programmed for 30 cycles. The amplified DNA fragment was separated on a 1% agarose gel and purified using Quick PCR purification spin columns. The purified fragment was directly sequenced in MACROGEN, Korea. These sequences were then identified by homology search using BLAST program and sequences were submitted in Genbank.

Methods for analyzing the probiotic properties

1. Cell adherence and acid stability

S. phocae PI80 (5×10^{10}) was administered orally to the animal model and the cells attached to mucous layer were calculated as colony forming unit ($CFU\ ml^{-1}$). Moreover, acid tolerance was tested *in vitro* in gastric system model as described by Mouecoucou et al. (2004)[9]; Duc et al. (2004)[10] with some modifications. The total cell viability was recorded after exposure of strain PI80 to the above explained condition and was compared with control.

2. Assay of antibiotic susceptibility and hemolytic properties

The susceptibility of the strain *S. phocae* PI80 against Penicillin, Methicillin, Tetracyclin, Oxytetracyclin, Erythromycin, Ampicillin, Amoxycillin, Kanamycin, Gentamycin, Vancomycin, Neomycin, Amikacin, Chloramphenical, Nitrofurantoin and cefpodoxin were tested in Lactobacillus MRS agar medium. All antibiotics were purchased in HIMEDIA (MUMBAI, INDIA). Moreover, their hemolytic property was analyzed in sheep and human blood agar plates.

3. Assay of bacteriocin activity

Bacteriocin activity was estimated using the agar well diffusion method [11] and expressed in arbitrary units ($AUml^{-1}$), calculated as $a^b \times 100$, where "a" represents the dilution factor, and "b" represents the last dilution that produce an inhibition zone of 2 mm in diameter. One arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest two fold dilution showing a clear zone of inhibition of the indicator strains and its activity is expressed per ml after multiplication by 100. More than five pathogenic organism such as *Vibrio*

parahaemolyticus, *V. vulnificus*, *V. anguilarum*, *V. fischeri*, *A. hydrophila* and *Listeria monocytogenes* 657 was used for the assay of bacteriocin activity.

Effect of probiotic *S. phocae* treatment on control of *Vibrio harveyi*

Black tiger shrimps (*Penaeus monodon*) were procured from a commercial hatchery. After 40 days of acclimation period, shrimps were separated in triplicates (30 animals each) in to 500 L-capacity fiber tank. The control group was fed with regular diet for entire trial period and one group was fed with probiotic *S. phocae* PI80 and the third group was fed with probiotic *S. phocae* PI80 after *Vibrio* infection. *V. harveyi* (MTCC 3435) were used as disease causing agent in the experiment. These strains were grown in TSA broth (Himedia, MUMBAI, INDIA) at 30^{o}C for 16hr. At end of 15 days feeding trial, treatment and control group were exposed to *V. harveyi* at 10^{6} CFUml^{-1} for 20 – 30 min. Infection was ensured by inoculating the diseased shrimp on TCBS agar (Himedia, MUMBAI, INDIA) and verified by biochemical tests.

Effect of growth parameters for *S. phocae* PI80

The effect of temperature, pH and salinity on the growth of *S. phocae* in MRS broth was determined by inoculation of early log phase culture and incubation at different temperatures (25°C, 30°C, 35°C, 40°C and 45°C), pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and NaCl (0%, 0.5%, 1%, 2% and 3%) for 48hrs. The samples were collected at every 6 hr intervals and absorbance at 600nm was read. Fresh MRS broth was taken as blank. Experiments were carried out in triplicates.

Results and Discussion

Identification and characterization of strain PI80

A total of 100 numbers of lactic acid bacteria were isolated from gut of shrimp (*Penaeus indicus*-PI) in MRS agar plates. These isolates were screened for antagonistic activity against fish pathogen *Aeromonas hydrophila* by cross streaking [12] and agar well diffusion method[13].

Finally, a single pinpoint isolate (PI80) was selected for *in vivo* study by maximum zone of inhibition against fish and shrimp pathogens such as *A. salmonicida, Vibrio anguillarum, V. fischeri, V. vulnificus* and *V. parahaemoliticus* which classifies it as a novel 'antagonistic probiotic bacteria' for shrimp larval rearing systems in hatcheries and farms. This was confirmed by testing the strain in common carp *Cyprinus carpio* and shrimp *Penaeus monodon* culture systems. LAB isolates were characterized on the basis of morphological, physiological and biochemical tests by Bergey's Manual of Systematic Bacteriology[14].

The biochemical characterization is commonly used technique to differentiate one bacterial strain to others[7]. In respect of biochemical identification, isolate belongs to the group of cocci, non motile, gram positive, oxidase and catalase negative (Table 1).

Characteristic	*S. phocae* PI80
Morphology	Cocci shaped
Gram reaction	+
Motility	Non motile
Hemolysis	Alpha hemolysis
Starch hydrolysis	-
Nitrate reduction	+
Oxidase	-
Catalase	-
Indole	-
H_2S production	-
Acid production from	
Lactose	+
Glucose	+
Adonitol	-
L-arabinose	-
Dulcitol	-
Galactose	-
Gelatin	-
Bile esculin	+
Sorbitol	-
Sucrose	+
Xylose	+
Isoleuin	-
Rafinose	-
Rhamnose	+

Negative (-), Positive (+)

Table 1. Biochemical characterization of newly isolated strain of *S. phocae* PI80.

The Lab isolate utilizes the sugar of glucose, lactose, xylose, rhamnose and sucrose. The strain PI80 was non hemolytic in human blood-agar plates where as α hemolytic in sheep blood agar plates. The 16S rRNA gene sequences are sufficient enough for the identification of bacterial isolates[15] and identification of bacterial isolate in molecular level was attempted by sequencing the 16S rRNA gene of streptococcal sp [16]. The identified sequences of our strain consisted of 847 base pairs and the BLAST program was used to compare the sequences with those of the gram positive cocci, oxidase and catalase negative species available in the Genbank database. The 16S rRNA gene sequence analysis revealed that PI80 displayed the highest sequence similarity (98.0%) with *Streptococcus phocae*. (GenBank accession number EU117220).

Probiotic characteristic properties

1. Mucous layer attachment and acid tolerant in invitro gastric system

The attachment of cells in mucous layer was analyzed after two days of cells administration. The concentration of cells was observed to be same and was compared with control group (data not shown). For analyzing the acid resistance properties, *S. phocae* PI80 was introduced into the artificially formulated gastric environment. The equal bacterial cell viability was recorded after exposure of *S. phocae* PI80 in gastric conditions.

2. Antibiotic susceptibility and hemolytic test

Antibiotic susceptibility test was performed for *S. phocae* PI80 against fifteen antibiotics. Among the antibiotic, only six antibiotics such as, erythromycin amoxicillin, nitrofurantoin, chloramphenicol, ampicillin, penicillin, oxytetracyclin, and tetracycline highly inhibited (20-37mm zone) the growth of culture PI80. Moreover, the isolate was less susceptible (10-19mm of zone of inhibition) to cefpodoxin and vancomycin. As well as, gentamicin also inhibited moderately about 6-7mm zone of inhibition. But no zone of inhibition was observed in antibiotics amikacin and methicillin, kanamycin and neomycin (Table 2).

The property of hemolytic activity was also tested for the culture isolate. The alpha and non hemolytic activities were observed in sheep and human blood agar plate. These results clearly differentiated that our isolate isn't similar to previously reported culture[2-4,7].

Antibiotics	*S. phocae* PI80	Zone (mm) diameter
Penicillin-G	S	+++
Ampicillin	S	+++
Methicillin	R	Nil
Kanamycin	R	Nil
Vancomycin	S	++
Chloramphenicol	S	+++
Erythromycin	S	++
Gentamycin	R	+
Amoxicillin	S	+++
Tetracyclin	S	++++
Oxytetracyclin	S	++++
Amikacin	R	Nil
Neomycin	R	Nil
Nitrofurantoin	S	+++
Cefpodoxime	R	+

(Nil) Absence of zone of inhibition,
(+) Zone of inhibition about 1-10 mm
(++) Zone of inhibition about 10-20 mm,
(+++) Zone of inhibition about 20-30 mm
(++++) Zone of inhibition about 30-40 mm

Table 2. Antibiotic susceptibility test of newly isolated strain of *S. phocae* PI80.

3. Bacteriocin activity

The isolate PI80 produce bacteriocin, which exhibited broad spectrum of inhibitory activity against closely related gram positive and gram negative bacterial strains. All indicator strains and their bacteriocin activity were listed in Figure 1. Among the pathogenic strains used *V. anguillarum* and *Listeria monocytogenes* 657 showed higher bacteriocin activity by the probiotic streptococcus. Agar well diffusion method was used to detect bacteriocin activity. Previously Skaar et al. (1994) [2], Henton et al. (1999) [3], Vossen et al. (2004) [4], Gibello et al. (2005)[7] reported that *S. phocae* didn't have any capability to produce the any antimicrobial compounds. Where as, Kabuki et al. (2007)[17] reported that *Streptococcus thermophilus* SBT1277 produce heat stable bacteriocin within the *Streptococcus* sp. Moreover, Satish kumar and Arul (2009)[18] reported the high molecular weight of bacteriocin was produced by *S. phocae* PI80 and its antilisterial activity.

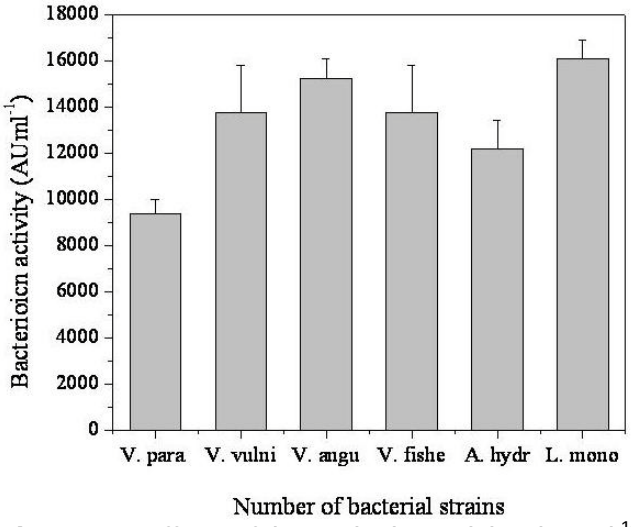

Figure 1. Effect of bacteriocin activity ($AUml^{-1}$) produced by probiotic *S. phocae* PI80.

Effect of probiotic *S. phocae* PI80 on survival of *P. monodon*

To investigate whether probiotic strain *S. phocae* are able to defend the shrimp *P. monodon* against vibriosis infection, shrimp were infected with *V. harveyi*. Supplementation of *S. phocae* (10 ml) showed 23 % mortality in shrimp exposed to *Vibrio harveyi* but not treated with probiotic whereas 0 % mortality was observed in shrimp exposed to *Vibrio harveyi* and treated with probiotic *S. phocae* PI80 (Figure 2).

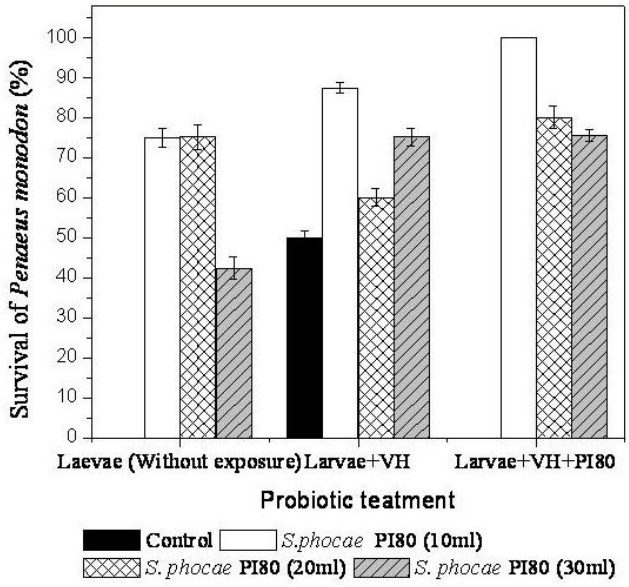

Figure 2. Accumulated mortality of *Penaeus monodon* infected with *V. harveyi* for 30 min with and without probiotic treatment.

The mortality rate (25 %) was observed in control group. Similarly Balcazar et al. (2007)[19] observed 17-22% mortality in shrimp exposed to *V. parahaemolyticus* and 33% in shrimp not treated with probiotics. Results of this study reinforce the view that probiotic is a effective addition to disease restrain phenomenon in aquaculture system[20]. *Pseudomonas fluorescens* AH2 was able to reduce the mortality of rainbow trout infected with *V. anguillarum* and 47% of mortality was observed in control group inoculated with pathogenic strain [21]. In conclusion, the probiotic *S. phocae* PI80 was ability to restrain the mortality of shrimp while also improving the shrimp survival rate (100%).

Effect of temperature, pH and NaCl on growth of *S. phocae* PI80

The effect of temperature, pH and NaCl on growth of *S. phocae* PI80 was investigated in MRS broth. The log phase reached at very early stage in all culture parameters especially in pH 5.0. Among the growth parameters, increased growth (1.391± 0.073, 1.341± 0.028 and 1.351± 0.057) rate was observed at end of the incubation period at temperature 40°C, pH 7.5 and 0% NaCl (Figure 3, 4 and 5).

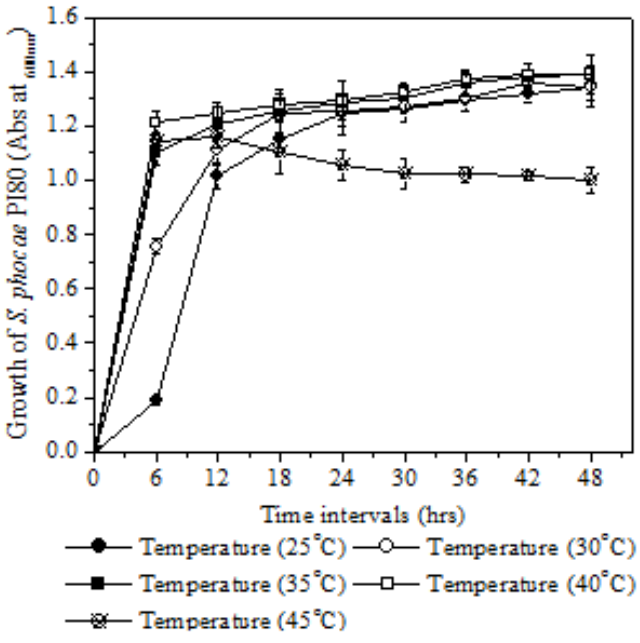

Figure 3. Growth profile of *S. phocae* PI80 in MRS broth at different temperature (25°C, 30°C, 35°C, 40°C and 45°C)

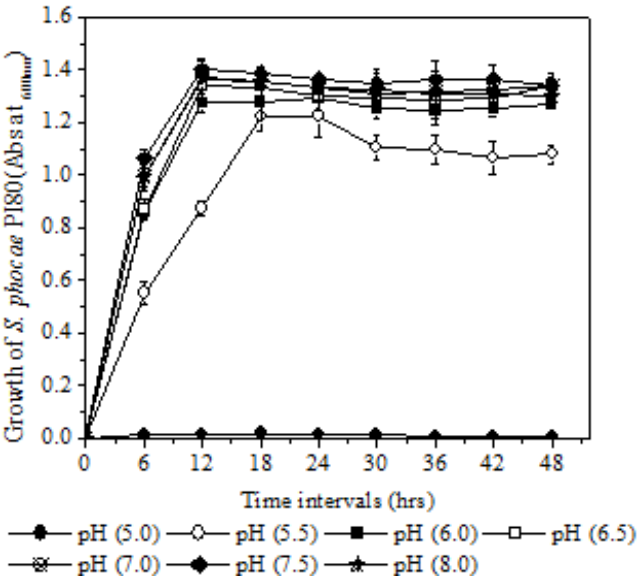

Figure 4. Growth of *S. phocae* PI80 in MRS broth at different pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0).

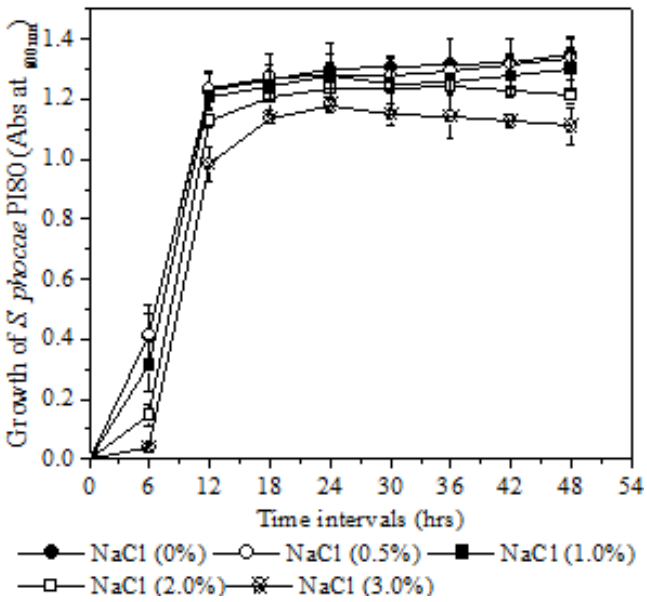

Figure 5. Growth of *S. phocae* PI80 in MRS broth containing different concentration of NaCl (0%, 0.5%, 1%, 2% and 3%).

Moreover, the log phase growth reached within 4hrs of incubation period. So, temperature 40°C, pH 7.5 and NaCl 0% are found to be optimum for only growth of *S. phocae* PI80. Also these results revealed that the probiotic bacterium *S. phocae* PI80 have the capacity to grow in higher temperature, pH and Na Cl. Because of this culture was isolated from marine shrimp intestine and it was proved by the production of bacteriocin at different Na Cl concentration. The higher amount bacteriocin profile was observed in 0.5-1% (Figure 6). Our results correspond to the report observed by kabuki et al. (2007)[17] in *S. thermophilus* SBT1277.

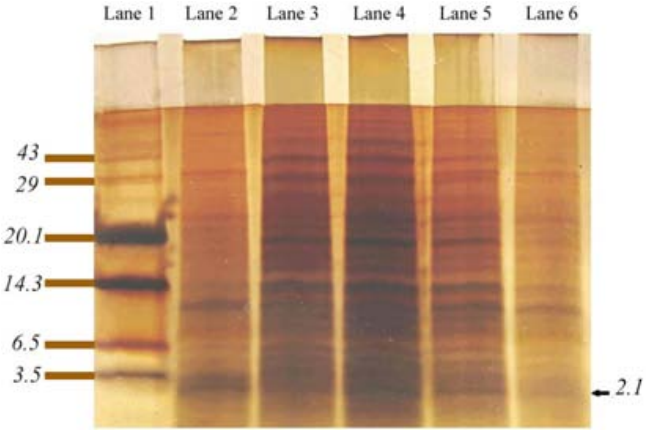

Figure 6. Tricine SDS -PAGE showing the effect of salinity on bacteriocin production by *S. phocae* PI80. Lane 1- MWM and Lane2 – Lane 6 shows the order of increasing concentration salinities (0, 0.5, 1, 2, and 3%).

Conclusions

The phenotypic and genotypic characterization revealed that strain PI80 was *Streptococcus phocae* and it has got ability to restrain gram positive and gram negative bacterial pathogenic strains by producing its antimicrobial compounds like bacteriocin. *S. phocae* PI80 survive well in higher environmental factors like temperature, pH and salinity. Hence, we conclude that *S. phocae* PI80 is able to displace bacterial pathogens by competitive process in the shrimp hatchery and it act as a better replacement than administering antibiotics and is now gaining acceptance for control of pathogens in aquaculture.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

References

1. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclatural changes. Clin. Microbiol. Rev 2002; 15:613 - 630.
2. Skaar I, Gaustad P, Tonjum T, Holm B, Stenwig H. *Streptococcus phocae* sp. nov., a new species isolated from clinical specimens from seals. Int. J. Syst. Bacteriol 1994; 44:646 - 650.
3. Henton MM, Zapke O, Basson PA. *Streptococcus phocae* infections associated with starvation in cape fur seals. J. South Afr .Veter. Asso 1999; 70:98 - 99.
4. Vossen A, Abdulmawjood A, Lammler C, Wei R, Siebert U. Identification and molecular characterizaction of beta-hemolytic streptococci isolated from harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) of the German North and Baltic Seas. J. Clin Microbiol 2004; 42:469 - 473.
5. Lawson PA, Foster G, Falsen E, Davison N, Collins MD. *Streptococcus halichoeri* sp. nov., isolated from grey seals (*Halichoerus grypus*). Int. J. Syst. Evol. Microbiol 2004; 54:1753 -1756.
6. Lawson PA, Foster G, Falsen E, Davison N, Collins MD. *Streptococcus marimammalium* sp. nov., isolated from seals. Int. J. Syst. Evol. Microbiol 2005; 55:271-274.
7. Gibello A, Mata AI, Blanco MM, Casamayor A, Domı´nguez L, Ferna´ndez-Garayzabal JF. First Identification of *Streptococcus phocae* Isolated from Atlantic Salmon (*Salmo salar*). J. Clin. Microbiol 2005; 43(1):526 -527.
8. Gopalakannan A. Studies on the control of *Aeromonas hydrophila* infection and *Cyprinus carpio* and *Tilapia mossambicus* by immunostimulants and probiotics, Ph.D. Thesis, Department of Biotechnology, Pondicherry University, Pondicherry, India, 2006.
9. Mooecoucou J, Villaume C, Sanchez C, Mejean I, β-lactoglobulin /polysaccharide interaction during invitro gastric and pancreatic hydrolysis assessed in dialysis bags of different molecular weight cut-offs. Biochimi. Biophy. Acta 2004; 1670:105-112.
10. Duc LH, Hong HA, Barbosa TM, Henriques AO, Cutting SM. Characterization of Bacillus probiotics available for human use. Appl. Environ Microbiol 2004; 70:2161-2171.
11. Todorov SD, Dicks LMT. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against gram negative bacteria. Enz. Microbi. Technol 2005; 36:318-326.
12. Kekessy DA, Piquet JD. New method for detecting bacteriocin production. Appl Microbiol 1970; 20:282-283.
13. Lyon WJ, Glatz BA. Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium theoni*. Appl. Environ. Mcrobiol 1993; 59:83-88.
14. Holt JG, Krieg NR, Sneath PHA, Williams ST, Bergey's manual of determinative bacteriology, The Williams & Wilkins Co. Baltimore, Md. 9th eds.1994; p. 527-558.
15. Kolbert CP, Persing DH. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Cur. Opin. Microbiol 1999; 2:299 - 305.
16. Vela AI, Goyache J, Tarradas C, Luque I, Mateos A, Moreno MA, Borge C, Perea JA, Domı´nguez L, Fernandez-Garayzabal JF. Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in spain by pulsed-field gel electrophoresis. J.Clin Microbiol 2003; 41:2498-2502.
17. Kabuki T, Uenishi H, Watanabe Y, Seto Y, Nakajima H. Characterization of bacteriocin, Thermophilin 1277, produced by *Streptococcus thermophilus* SBT1277. J. Appl. Microbiol 2007; 102:971-980.
18. Satish kumar R, Arul V. Purification and characterization of Phocaecin PI80 an antilisterial bacteriaocin produced by *Streptococcus phocae* PI80 isolated from the gut of Indian White Shrimp (*Peneaus indicus*) J. Microbiol. Biotechnol 2009; 19(11): 1393-1400.
19. Balcazar JL, Luna TR, Cunninggham DP. Effect of the addition of four potential probiotic starins on the survival of pacific white shrimp (Litopenaeus vannamei) following immersion challenge with Vibrio parahaemolyticus. J. Invert. Pathol 2007; 96:147-150.
20. Balcazar JL, Decamp O, Vendrell D, de Blas I, Ruiz-ZarZuuela I. Health and nutritional properties of probiotics in fish and shelfish. Microb. Ecol. Health Dis 2006; 18:65-70.
21. Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen T. Inhibition of Vibrio anguillarum by Pseudomonas fluorescens strain AH2, a possible probiotic treatment of fish. Appl. Envion. Microbiol 1999; 65:969-973.