Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 12292 - 12305
Received 25 April 2021; Accepted 08 May 2021.

Identification and Screening of Probiotics as a Biocontrol Agent against Pathogenic Vibriosis in Shrimp Aquaculture

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ABSTRACT:
The beneficiary bacteria isolated from healthy shrimp gut were found to be active against the growth of *Vibrio* pathogens and executed maximum zone of inhibition. The main objective of the research is to identify and screen probiotic isolates from healthy shrimp gut as biocontrol measures against *Vibrio* pathogenic species. The probiotics isolated from the shrimp gut were analyzed for different parameters including Gram staining, morphology, IMViC, acids, bile, NaCl, and antimicrobial activity against *Vibrio* pathogens. A total of 36 isolates were isolatedout of which 9 were screened as best microbes. By using MHA media swabbed with *Vibrio* pathogen species in the replica method, 3 isolates showed a maximum zone of inhibition against test pathogens. These test pathogen *Vibrio cholera, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio harveyi* were collected and grown on TCBS media. The three isolates identified as*Lactobacillus rhamnosus,Lactobacillus paracasei,Pediococcus acidilactici*exhibited reproducible probiotic activity against test pathogen by well diffusion method. This study indicated that probiotic foods in shrimp farming will help them to fightagainst pathogenic organisms.
Key words: Probiotics, Identification, *Lactobacillus, Vibrio* pathogens, Antagonistic activity.

INTRODUCTION:

World's fastest-growing food production sector is the Aquaculture, mainly with the prawns and shrimp culturing (1). Shrimp culture has become commercially important farming in many parts of the world which was placed in second traded seafood commodity after salmon. In international traded seafood, roughly 15% usage is shrimp, remaining over half-emerge from aquaculture (2). During 1970 to 2008, FAO observed that an average annual growth rate of shrimp culture is 18% (3), the rising worth of $ 19.4 billion in 2012 (4). Despite the annual growth rate of aquaculture will reduce from 5.7 percent in 2003-2016 to 2.1 percent in 2017-2030 due to slowdown in growth of Chinese aquaculture production, in recompense by other countries increase in production (5).

Along with it annual global loss is also mainly due to the severe damage with the pathogenic agents. The Global Aquaculture Alliance estimated that 22% per year loss is observed in shrimp culture due to disease outbreak, amounting to approximately $ 1 billion annually (6). Vibriosis disease caused by the *Vibrio*species is a destructive disease that reduces the aquaculture shrimp population in Asian countries. *Vibrio cholera*, *Vibrio alginolyticus*, *Vibrio cholera*, and *Vibrio harveyi* are some species responsible for necrosis in shrimps. Up to now, there is no adequate treatment to control the vibriosis in shrimps. Previously antibiotics are used to reduce the disease but due to adverse effects on public health and pathogen acquiring antibiotic resistance lead to stop the practice (7, 8). Researchers suggested the usage of non-pathogenic bacteria, probioticsrather than usage of antibiotics (9, 10, 11). Single or mixed culture of probiotic

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microbes of probiotic food, improves the host health by enhancing intestinal microbial balance (12). Lactic acid bacteria have been extensively used as probiotics against shrimp pathogenic agents (10). Shrimp intestine tract, skin, and gills constitute a major part of *Bacillus* microflora which has been used as biocontrol probiont against fish and shellfish pathogenic bacteria (13, 10, 14). The present work aims to isolate the probiotic bacteria that can be used as a biocontrol agent against pathogenic bacteria in shrimp and to test bacterial isolate with antagonistic activity against *Vibrio* pathogen *in-vitro*.

MATERIAL AND METHODS:

Isolation of Probiotic Bacteria:

We have collected the healthy *Litopenaeus vannamei* (White leg shrimp) from Krishna river beds. Bacterial samples were isolated from the gut of the shrimp.By using MRS media we isolated *Lactobacillus* species from the shrimp gut. Approximately 5-6 grams of the gut of the shrimp were collected and it is made up of 50ml proliferation broth and vortex it. After 24h of incubation at normal temperature serial dilution was done and 0.1ml of the sample was spread on MRS agar plates and incubated at 37^{o}C from 24-48h. Later after incubation time, morphological characterization of *Lactobacillus* species was observed and noted. Pure colonies are collected and stored for further work.

Antagonistic activity against *Vibrio* Culture:

The antagonistic activity of the isolated species against the *Vibrio* pathogens was done as per the protocol of the agar well diffusion method. Four strains were used as target agents for the present antagonistic activity which includes *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio cholera* which were collected from the Department of Zoology, Acharya Nagarjuna University. The collected strains were identified by growing in TCBS and TSA media and by biochemical analysis. Initially, *Vibrio* culture was inoculated in TSA and Zobell marine broth and incubated at 32^{o}C for overnight. After 48h 10ml culture of isolates in MRS broth at 37^{o}C was centrifuged at 10,000 rpm for 15min and supernatant was collected. To the Muller Hinton agar plate, with the help of gel borer wells are made to this 50µl of cell-free supernatant was loaded. The plates were previously swabbed with overnight *Vibrio* pathogen culture. The zone of inhibition was measured after 24h incubation time.

Molecular Characterization:

On screening with the antagonistic activity with the *Vibrio* pathogens, bacterial isolates were further confirmed and identified by sequencing the partial 16S ribosomal RNA (rRNA). The universal primers were used for amplifying the 16S rRNA sequences. The fragments were amplified in an Eppendorf under the following conditions: 94 _C, 30 cycles of 94 _C for 45 s, 55 _C for 30 s and finally 72 _C for 10 min. Amplified PCR products were run through agar gel, and the sequence was sequenced by Bioport Delhi. The sequence of the three isolates was run through the BLAST and compared with EMBL, GENBANK and DDBJ databases at NCBI. The sequence was sent to the GENBANK for the accession number, and by using neighbor-joining method the Phylogenetic tree was constructed using MEGAX software (15).

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Biochemical Characterization:
Based on the maximum zone of inhibition against *Vibrio* pathogen only three isolates were selected as the promising strains. Genus level of isolates was identified through morphological and biochemical analysis using Bergy's manual of systematic Bacteriology (16). And further molecular identification was done by using 16S rRNA sequencing. Microscopic observation and Gram staining were performed following the protocol of Pelczar (17). Biochemical parameters included hydrogen sulfide, catalase, oxidase, nitrate reduction, Indole, Methyl red, Voges Proskauer and Simmons Citrate (IMVIC) tests are done by following the protocol of Holt (16). Carbohydrate Utilization tests such as Glucose, Lactose, Sucrose, Fructose, Galactose, Maltose, Salicin, Sorbitol, Mannose, and Raffinose sugars are done by following the protocol of (17).

Optimization and Probiotic Characterization:

Growth Curve:
In MRS broth, potent isolates were inoculated and maintained at 37^{o}C, at 180 rpm shaking incubator. At regular intervals of time for every 5h sample was measured at 600nm optical density (5h to 36h).

Temperature Tolerance:
In MRS broth isolates were inoculated and maintained at different temperature i.e., 30^{o}C,33^{o}C,37 ^{o}C,40^{o}C, and 45^{o}C. After incubation time at 12h and 24h optical density was measured.

pH Tolerance:
Tolerance to acid and basic condition was estimated by adjusting the broth at different pH. MRS broth was adjusted with a pH of 4, 5, 6, 7, 8, 9 and 10 by using 1% HCl and 1N NaOH. Entire glassware and MRS broth with different pH used in the assay was autoclaved. Overnight cultures of isolates were inoculated and incubated at 37^{o}C. Optical density at 600nm and pH were measured at regular intervals of time 30min, 60min, 90 min and 120 mins and finally after 24 hours (19,20,21).

Bile Tolerance:
Bile tolerance determination is as important as acid tolerance because, through the host species in a delivery pathway, the isolate need to sustain at different bile salts concentrations which include 0.3, 0.5, and 0.8% (w/v) Oxgall bile salt. MRS broth was adjusted with different bile concentration and overnight culture of probioticswere inoculated and incubated at 37^{o}C. Optical density at 600nm and pH were measured at regular intervals of 30min, 60min, 90 min and 120 mins and finally after 24 hours (19, 20, 21).

Effect of Salinity:
Probiotic isolates were inoculated in MRS broth with different NaCl concentrations (3, 6, 9, and 12%) under shaking incubator at 37^{o}C for 24-48h. Optical density at 600nm was measured at a regular interval of time 0, 12,24h.

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Results and Discussion:

Isolation of Probiotics from shrimp gut:

A total of 36 isolates were isolated from the shrimp gut and we named them as, SGM(Shrimp gut MRS media) and SGKM (Shrimp gut Krishna MRS media). Out of 36 isolates, nineisolates showed maximum and optimum results when tested with *Vibrio* culture, on screening with probiotic parameters, biochemical tests and based on all tests three best isolates were molecularly identified as lactic acid-producing bacteria.

Screening of Antagonistic Strain:

It was noted that all the 9 strains were found to inhibit all the four *Vibrio* pathogens, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio cholera* which were collected from Department of Zoology and AquacultureFrom the gut of the *L.vannamei*, three probiotic isolates were isolated and molecularly identified as lactic acid-producing bacteria *lactobacillus spp*., named as SGM4, SGM7, and SGKM-9. The antagonistic activity was performed for the bacterial isolates, the zone of inhibition was recorded, and the results are depicted in the table-1, 2.

All the three isolates showed a maximum zone of inhibition against *Vibrio* pathogens, comparatively, results showed similar activity with the previous research. Several studies have concluded that *lactobacillus* species can produce a bactericidal protein which acts on pathogenic agents by antagonistic activity (22, 23). One research reports that *Lactobacillus acidophilus* shows a maximum zone of inhibition up to 16mm against the *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (24). Similar results were also identified by Balcazar and Rojas with the Bacillus spp. (25).

Many research concluded that the production of peptide antibiotics like bacteriocins by Lactobacillus spp. are effective against the bacteria of Gram-positive and Gram-negative (26). Similar reports identified that LAB strains isolated from the gut of *Mugil cephalus* showed a 10-15mm zone of inhibition against the *V.harveyi* and *V. parahaemolyticus* (27). Several studies reported that *Lactobacillus* strains isolated from the gut of the shrimp were analyzed for antimicrobial activity *in-vitro* showed maximum zone of activity 16mm and minimum 8-13mm against the *V.harveyi* pathogen. In present investigation SGM-4, SGM7 ad SGKM-9 strains isolated from the *L.vannamei* gut showed zone of inhibition 23-24mm against the four pathogens *V.cholera*, *V.harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* gave similar results of 16mm zone of inhibition by Sivakumar et al. (24) and Ghosh et al. (27) worked with LAB isolates against *V.harveyi* and *V. parahaemolyticus*. On-field analysis in P. monodon adult by using *Bacillus subtilis* probionts against the *V.harveyi* has recorded a 90% reduction in mortality (28). Abraham (2004) has recorded a 59% decline in lethality of Penaeus monodon against *Vibrio harveyi* at 10^6CFUml^{-1} when trail test with the *Alteromonas spp.*(29)

Some aquatic animal reports: In *Scophthalamus maximus* on probiotic trail showed maximum zone of inhibition 23mm against the pathogenic agents on *in-vitro* analysis and it was failed in *in-vivo* analysis (30). In rainbow trout 25% mortality decrement was observed with *Pseudomonas fluorescens* against *Vibrio anguillarum* (31). Additionally, related results have been identified with probiotic isolates in protecting the shrimp aqua culturing against the *Vibrio* pathogens.

Table: 1 Zone of inhibition (mm) of antagonistic activity of probiotics against *Vibrio* strains

Pathogens	SGM-7	SGM-4	SGKM-9	Mean ±SD	Coefficient of variance

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V.alginolyticus	22.00	21.67	23.33	22.33±0.88	0.78
V.parahaemolyticus	24.33	24.67	22.33	23.77±1.26	1.59
V.cholera	16.00	14.33	15.67	15.33±0.81	0.78
V.harveyi	21.00	18.00	18.67	19.22±1.57	2.48

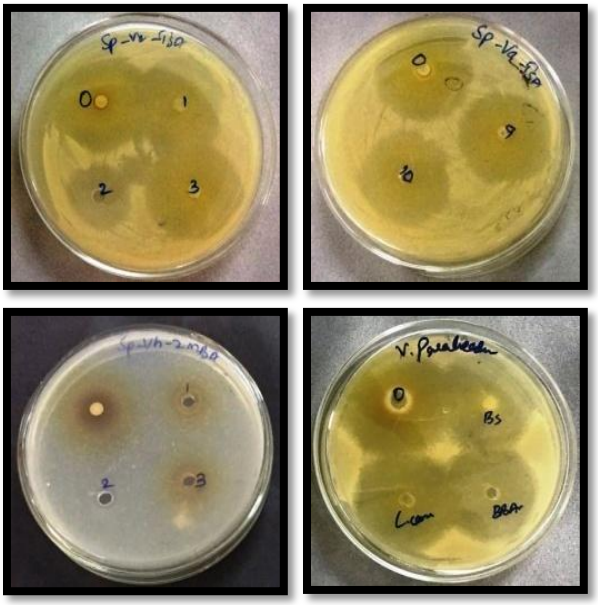
Table: 2 Statistical analysis of Antagonistic activity

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	125.96	3.00	41.99	30.10	0.00
Columns	2.89	2.00	1.44	1.04	0.41
Error	8.37	6.00	1.40		
Total	137.22	11.00			

P-value (0.00) < 0.05 (95% of significance level) , Which means there is significant difference among 4 Isolate diameters(mm) when tested with 4 Pathogens.
P-value (0.41) > 0.05 (95% of significance level) , Which means there is no significant difference among 4 Isolates when compared to each other.

Fig 1Illustrates the zones of inhibition of probiotic isolates against the *Vibrio* pathogen



Molecular characterization of the selected three isolates:
Isolation of DNA:
From potential probiotic strains chromosomal DNA was isolated and the purity of the chromosomal DNA was concluded by using agarose gel electrophoresis. In this study agarose of 1.5% used for gel run through electrophoresis. UV trans-illuminator was used to study the molecular level matching of the DNA purity with the help of 50bpLadder.

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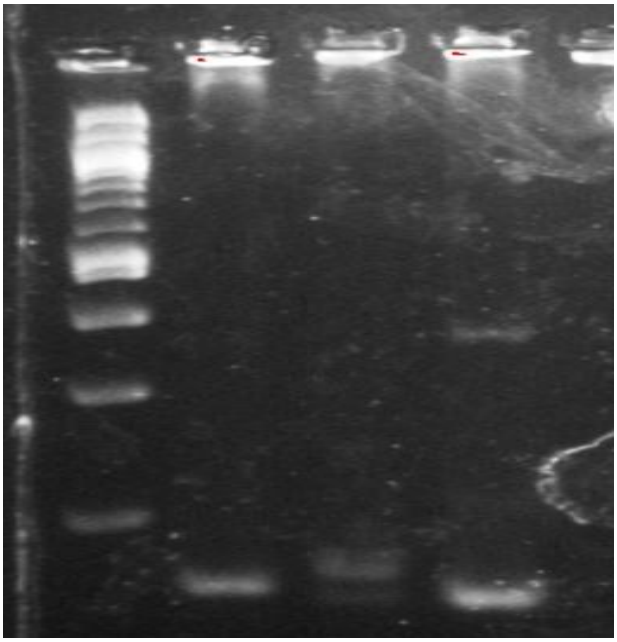
Figure: 2 PCR amplification product of 3 isolates

Ladder 1 2 3



A Phylogenetic tree was constructed using the sequence of three isolates (16SrRNA sequence) and a representative sequence from the databases. Phylogenetic analysis utilizing a neighbor-joining dendrogram showed the results that SGM-4, SGM-7, SGKM-9 belongs to *Lactobacillus paracasei*, *Pediococcus acidilactici*,*and Lactobacillus rhamnosus*(Table 3).

Table: 3 NCBI GENBANK Accession numbers of three isolates:

S.No	Isolates name	Molecular level identification	NCBI Accession number
1	SGM-4	*Lactobacillus paracasei*	MT125880
2	SGM-7	*Pediococcus acidilactici*	MT125882
3	SGKM9	*Lactobacillus rhamnosus*	MT125886

Phylogenetic relationship of SGM-4, SGM-7, and SGKM-9:

Based on the 16S rRNA gene sequence SGM-4 shows the Phylogenetic relationship between the strain SGM-7 and SGKM-9 and identified as a member of genus *Lactobacillus paracasei*. The percentage is expressed in the dendrogram to state the relation between the identified strains (Fig: 3).

Same as the above SGM-4 on the basis of 16S rRNA gene sequence SGM-7 shows the Phylogenetic relationship between the strain SGM-4 and SGKM-9 and identified as a member of genus *Pediococcus acidilactici*. The percentage is expressed in the dendrogram to state the relation between the identified strains (Fig: 3).

As like two sequence SGM-4, and SGM7, another sequence of the SGKM-9, on the basis of 16S rRNA gene sequence SGKM-9 shows the Phylogenetic relationship between the strain SGM-4

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and SGM-7 and identified as a member of genus *Lactobacillus rhamnosus*. The percentage is expressed in the dendrogram to state the relation between the identified strains (Fig: 2).

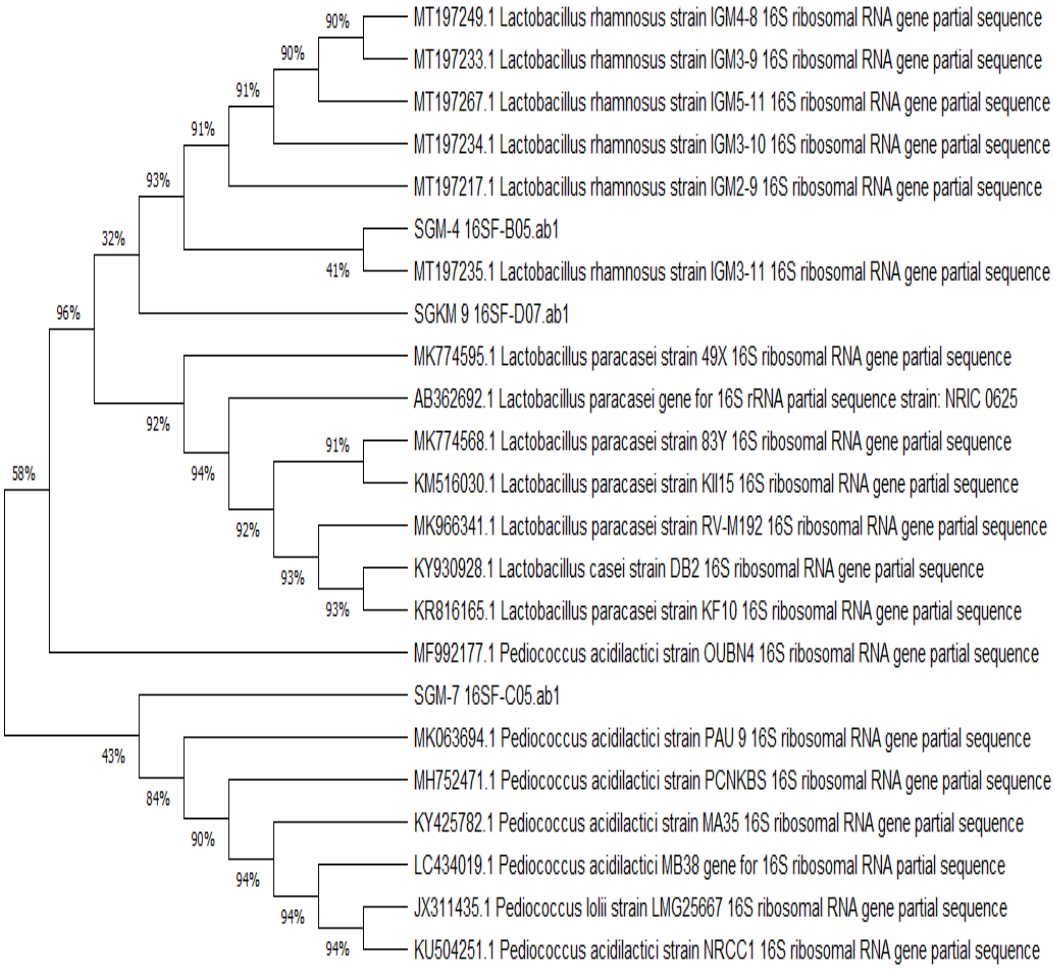

Fig3 Illustrates the Phylogenetic relation between the isolated and members of its genus

Biochemical Characterization of selected three isolates

All three isolates were found -ve for catalase--ve which is the first criterion for the probiotic parameter and found +ve for urease, starch hydrolysis, and Simmons citrates only SGM-4 showed -ve for the voges proskauer test. For hydrogen sulfide, Gelatin liquefaction test and Indole test all the isolates showed -ve, However, SGM-7 and SGKM-9 showed a +ve result for oxidase test, methyl red test (Table 5).

Utilization of carbohydrate by Probiotic:All the isolates showed +ve result with the monosaccharide and oligosaccharide sugar such as Glucose, Fructose, Sucrose, Galactose, Maltose (except SGKM-9), Salicin, Sorbitol (except SGKM-9, mannose and raffinose (except SGM-7 and SGM-4 (Table: 3). Based on the phonological characters the strains were identified as Lactobacillus species, further molecular level characterization were performed for further identification

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Table 4: Morphological characterization, biochemical characterization and carbohydrate utilization of probiotic isolates

Characters	Isolated probiotic bacteria		
	SGM4	SGM7	SGKM9
Form	circular	circular	Circular
Size	medium	punctiform	Medium
Surface	rough	Rough	Rough
Texture	mucoid	Mucoid	mucoid
Color	creamy	Creamy	White
Elevation	entire	Entire	entire
Margin	flat	Raised	Flat
Gram staining	+bacilli	+bacilli	+bacilli
Catalase	-	-	-
Urease	+	+	+
Oxidase	-	-	+
Hydrogen sulfide	-	-	-
Nitrogen reductase	+	-	+
Gelatin liquefaction	-	-	-
Starch hydrolysis	+	+	+
Indole	-	-	-
Methyl red	-	-	+
Voges-Proskauer	-	-	-
Citrate	+	+	+
CHO Utilization			
Glucose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Sucrose	+	+	+
Maltose	+	-	+
Lactose	+	+	+
Salicin	+	+	+
Sorbitol	+	+	-
Mannose	+	+	+
Raffinose	+	-	-

+= Positive result, -= Negative result, +bacilli= Gram-positive bacilli, -cocci= Gram-positive cocci.

Probiotic parameters of three isolates:

Acid Tolerance: The main criteria for probiotic is should habituate to acidic pH where the optimal condition of intestinal condition; therefore the probiotic should viable in gastrointestinal tract of the host intestine. Hence probiotic posses acidic pH that suggests the probiotic have the capability of acid resistance activity. Therefore acid resistance or tolerance test was main line of selecting promising isolate. Isolated cultures were inoculated in the selective media which was

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adjusted to different pH 4, 5, 6, 7, 8, and 9. Based on the feeding time, digestion and growing stage or adopted condition of the host the pH of the gastric juice may differ (32).

Following table explains the viability of isolates in different pH and survival rate of the isolates may vary from 77-95% of the cells up to 120min at acid and alkaline nature and after 24 hours also. This explains the identified isolates have the capability to grow and tolerate under duffer pH conditions. Out of 16 isolates only 9 isolates show tolerance of pH activity but these 3(SGM-4, SGM-7,SGKM-9) showed best tolerable based on the viability up to 120min at low and high pH. Following table and graph at 24 hours explains the pH variations. Previous reports are similar to the obtained results that were reported by Lin et al.,(33) was observed that viability of probiotic bacteria at low pH 2,3 and 4 up to 3 hours. Our three isolates were also showing similar results of viability up to 120min at low pH this suggests that our isolates are express acid tolerance activity.

Table:5 Optical density of the isolates at different pH at different time intervals

		Viability of bacteria at OD 600nm			
Isolates	pH	30min	60min	90min	120min
SGM-4	4	0.003±0.00	0.003±0.002	0.009±0.003	0.004±0.001
	5	0.005±0.004	0.003±0.001	0.005±0.002	0.004±0.00
	6	0.009±0.001	0.009±0.001	0.010±0.001	0.012±0.002
	7	0.004±0.004	0.001±0.00	0.004±0.001	0.003±0.001
	8	0.003±0.00	0.003±0.001	0.003±0.001	0.007±0.002
	9	0.001±0.00	0.009±0.001	0.011±0.00	0.001±0.001
SGM-7	4	0.003±0.00	0.001±0.00	0.004±0.001	0.003±0.001
	5	0.005±0.002	0.003±0.001	0.004±0.00	0.005±0.001
	6	0.003±0.005	0.004±0.00	0.005±0.001	0.006±0.001
	7	0.009±0.00	0.003±0.00	0.013±0.001	0.015±0.005
	8	0.00±0.00	0.004±0.00	0.007±0.00	0.007±0.002
	9	0.00±0.00	0.004±0.00	0.006±0.001	0.002±0.00
SGKM-9	4	0.005±0.00	0.006±0.00	0.006±0.001	0.005±0.001
	5	0.004±0.001	0.004±0.001	0.006±0.001	0.003±0.00
	6	0.007±0.001	0.004±0.00	0.007±0.001	0.007±0.001
	7	0.006±0.002	0.009±0.001	0.015±0.001	0.015±0.001
	8	0.008±0.001	0.0±0.0	0.011±0.003	0.009±0.002
	9	0.003±0.00	0.004±0.00	0.005±0.002	0.005±0.00

OD Values are expressed in mean ±Standard deviation

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Bile Tolerance:

Another defense mechanism of the host following the acid for selection of potent probiotic and for inhibiting the pathogen was the bile salt tolerance activity, which indicates the isolates should have the ability to survive under bile salt inhibition in the intestine of the host. Generally these bile salts are given as sterols source for the shrimp as an essential nutritional requirement. For the production of moulting hormone, shrimps require steroids, which allow the immediate passage during larval growth phases. Consequently steroids are required to supplement through the diet to obtain normal size and were supplied in two different concentrations 0.1% and o.5%.

Probiotic exhibiting bile activity was concluded as natural bacterial growth inhibitors thus probiotics should resistant to bile salts of 0.1% and 0.5%. In present research we selected bile salt range of 0.3%, 0.5%, and 0.8%. All the 16 isolates were tested for bile salt tolerance only 9 isolates showed bile activity, but SGM-4, SGM-7 and SGKM-9 showed much better activity against the bile tolerance, therefore these three isolates were selected as bile salt tolerant isolates.

Generally only bile salt tolerant isolates were inhibitory for the Gram positive bacteria which lead to leakage of the intracellular contents of the cell by shrunken of the cell membrane finally cell death. Another mechanism involves oxidative damage, denaturation and misfolding of protein structure, DNA damage (34). As per the previous results 0.3% of bile salts are the detracting concentration for screening of bile resistant bacteria. This bile tolerance capacity was obtained by the enzyme vile salt hydrolase, which acts as a deconjugating the bile salts liberating the glycine/taurine moiety from the steroid core by hydrolysing the amide bond. Several studies reveal that resistance of potential bacteria to acid and bile secretion have performed both in vivo and in vitro and observe the same result but some discrepancy between them because of complicate position of the GIT of the host.

Table :6Optical density of the isolates at different bile concentration at different time intervals

		Viability of bacteria at OD 600nm			
Isolates	Bile	30min	60min	90min	120min
SGM-4	Control	0.015±0.001	0.033±0.004	0.067±0.000	0.09±0.001
	0.3	0.029±0.001	0.033±0.001	0.042±0.004	0.019±0.001
	0.5	0.036±0.002	0.036±0.003	0.042±0.000	0.016±0.015
	0.8	0.031±0.001	0.03±0.00	0.037±0.003	0.01±0.002
SGM-7	Control	0.022±0.002	0.027±0.000	0.051±0.001	0.058±0.001
	0.3	0.025±0.000	0.023±0.001	0.025±0.005	0.028±0.001
	0.5	0.035±0.002	0.036±0.001	0.028±0.002	0.028±0.000
	0.8	0.025±0.001	0.02±0.001	0.017±0.001	0.025±0.004
SGKM-9	Control	0.01±0.000	0.022±0.004	0.044±0.005	0.062±0.0031
	0.3	0.049±0.000	0.047±0.005	0.046±0.004	0.046±0.000
	0.5	0.045±0.004	0.044±0.005	0.044±0.004	0.38±0.001
	0.8	0.043±0.006	0.042±0.005	0.03±0.001	0.042±0.001

OD values are expressed in mean ± standard deviation.

Other Inhibitory Substance tolerance:

NaCl Tolerance:

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Probiotic strains mainly Lactic acid bacteria usually resistant to high NaCl concentration. Resistance to NaCl concentration is important parameters for opting good bacteria, on higher salt concentration, probiotic strain starts its metabolism and it produces acid which helps in inhibition of pathogen growth and development. SGM-4, SGM-7 and SGKM-9 were resistance to higher salt concentration up to 12%. Similar results were observed with the research of Aswathy (35) that isolates was resistance to 12% NaCl and remaining isolates few are tolerant to 9% and few are resistance up to 6%. When bacterial strains are exposed to medium with salt, it loses its turgor pressure which in turn affects the metabolism. By regulating the pressure inside and outside of the cell by inducing osmolytes such as glycine, betaine as an adaptive mechanism to withstand increased osmotic potential (36).

Table: 7 OD values of NaCl tolerance of the three isolates

		Viability of bacteria at OD 600nm	
Isolates	Nacl conc	0 hour	24 hour
SGM-4	Control	0.176±0.001	2.504±0.001
	3%	0.107±0.000	2.214±0.001
	6%	0.118±0.001	0.518±0.001
	9%	0.112±0.001	0.105±0.001
	12%	0.086±0.000	0.097±0.000
SGM-7	Control	0.066±0.001	2.434±0.001
	3%	0.057±0.002	2.471±0.001
	6%	0.050±0.002	0.061±0.001
	9%	0.048±0.002	0.035±0.002
	12%	0.031±0.003	0.032±0.002
SGKM-9	Control	0.034±0.001	1.464±0.002
	3%	0.034±0.001	0.836±0.004
	6%	0.033±0.001	0.083±0.002
	9%	0.028±0.002	0.027±0.001
	12%	0.011±0.001	0.012±0.001

OD values are expressed in mean ± standard deviation.

Effect of Temperature:

Three isolates SGM4, SGM7, SGMK-9 are thermophilic; the optimum temperature for isolates is 30-35^{o}C, whereas they are inactivated at 40^{o}C. Based on these results we have opted 37 ^{o}C for good growth of the isolates (Table-8).

Table: 8 OD values of Temperature resistance of three isolates

		Viability of bacteria at OD 600nm	
Isolates	Temp	0 hour	24 hour
	30^{o}C	0.037±0.001	0.022±0.002
SGM-4	35 ^{o}C	0.170±0.001	2.043±0.005
	45 ^{o}C	0.034±0.001	0.01±0.002
SGM-7	30^{o}C	0.023±0.001	0.024±0.001

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 12292 - 12305
Received 25 April 2021; Accepted 08 May 2021.

	35 °C	0.060±0.003	2.040±0.004
	45 °C	0.021±0.001	0.010±0.001
	30°C	0.018±0.000	0.046±0.001
SGKM-9	35 °C	0.002±0.000	2.485±0.001
	45 °C	0.012±0.001	0.033±0.000

Growth kinetics:
All the 3 isolates are examined for growth curve to observe the log phase of the culture. Maximum growth was observed from 8-28 h i.e. log phase is at 8-20h later stationary phase was observed. Finally, the decline phase was observed after 28 hours. The following figure clearly explains the growth kinetics (Fig: 7).

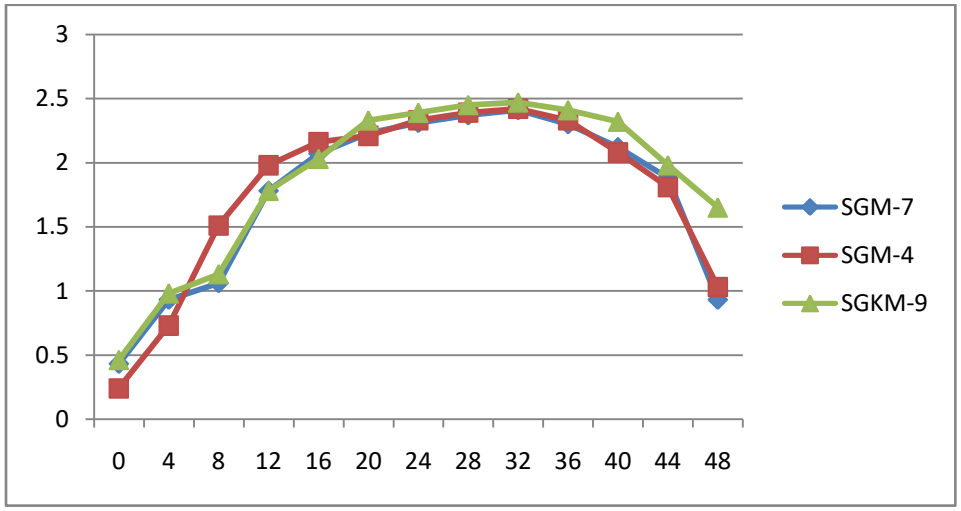

Fig 4 Graphical representation of the growth curve of three isolates, SGM-7, SGM-4, and SGKM-9 shows maximum growth at 8-28h, on x-axis 0 to 48h time is mentioned and on y-axis optical density value of probiotic isolates.

CONCLUSION:
Based on the above results we identified that three potent probiotic strains were isolated from the gut of the shrimp L.vannameiwere molecularly identified as *Lactobacillus paracasei, Lactobacillus rhamnosus, Pediococcus acidilactici*and all the three identified isolates showed maximum zone of inhibition against the*Vibrio* pathogens. These potent strains were showed optimum results with the probiotic characteristics and growth parameters. The final results suggest that probionts are potent in inhibiting the growth of the *Vibrio* pathogen in *the in-vitro* analysis. These probiotics can be used for controlling the shrimp larval pathogen which can be an alternative to the use of antibiotics in aquaculture.

Conflicts of interest
All authors have none to declare.

Acknowledgments
Authors are grateful to the Department of Science and Technology, KIRAN DIVISION Women Scientist Scheme-B, SR/WOS-B/467/2016 (G), 11-09-2017, New Delhi for the financial support.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 12292 - 12305
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Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 12292 - 12305
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