Chapter 2A

Isolation and selection of probiotic microorganisms from the intestine of adult Clarias batrachus

2.A.1. Introduction

Clarias batrachus (Linn.) is widely recognized for its nutritional and economic significance. However, the species is presently at a deteriorating phase and the restoration has vastly been underestimated. The constraint in productivity is mainly attributed to the pathogenic infections (Ahmed et al. 2012). Antibiotics and other antimicrobial substances have been traditionally applied to counteract disease outbreak. However, indiscriminate use of antibiotics for cultivation of *C. batrachus* has resulted horizontal and vertical transfer of the antibiotic-resistant genes. The screening and application of autochthonous probiotic for the cultivation of indigenous *C. batrachus* is in major focus worldwide to retrieve the situation. Probiotics are biocompatible beneficial microorganisms which are in increased use to prevent and control aquatic diseases in recent decades. However, the selection of probiotics should be done in species-specific manner to overcome ineffectiveness (Hai et al. 2007). There is growing awareness on the influence of intestinal bacterial composition of fish for probiotic potentiality. The gastrointestinal flora of aquatic animals often secrete extracellular enzymes (e.g., amylases, proteases, lipases) or growth factors (e.g., vitamins, siderophores, fatty acids, amino acids) which serves both digestive and defensive function (Sissons 1989; Irianto and Austin 2002).Therefore, studies on the composition and characteristics of the dominant intestinal microflora are crucial to obtain effective probiotic strain.

Probiotics can substantially limit the growth of pathogenic bacteria through different approaches without any side effects. The biochemical criteria like antibiotic sensitivity, cell surface hydrophobicity, bile salt tolerance, bile salt hydrolase activity and genetic constituents like *bsh, uvr, slpA* are the most important prerequisite to establish a

microbe as a potential probiotic organism (Papadimitriou et al. 2015). *Bacillus* sp., *Lactobacillus* sp., and *Lactococcus* sp. were the most commonly used aquaculture probiotics (Zorriehzahra et al. 2016). *Pseudomonas aeruginosa* VSG-2; intestinal isolates of freshwater fish has shown to possess potential antagonistic activity against pathogenic *Aeromonas hydrophila* (Giri et al. 2012). Enhanced growth, survivability and feed utilization efficiency was observed in *C. gariepinus* juveniles by supplementing the feed with *Lactobacillus acidophilus* (Al-Dohail et al. 2009). Further, Banerjee et al. (2015) isolated potential enzyme-synthesizing bacterial isolate *Bacillus licheniformis* from the adult *C. batrachus*. Olayinka and Afolabi (2013) applied *Lactobacillus acidophilus* for the cultivation of *C. gariepinus* and observed improved fish health and haematological parameters. Falaye et al. (2016) reported enhanced growth, weight gain and FCR in *C. gariepinus* fingerlings through applying fortified diet infused with *Lactobacillus plantarum*. There are number of probiotics used for the cultivation of major carps but the report on probiotics of C. *batrachus* is meager. There is increased demand of developing autochthonous probiotic for the cultivation of the species.

In the present study, suitable probiotic strains have been successfully isolated from the intestine of indigenous *C. batrachus* and subsequently identified. Stepwise experiments were performed to validate their probiotic properties.

2.A.2 Materials and Methods

2.A.2.1 Collection of indigenous *C. batrachus*

Adult healthy indigenous *C. batrachus* were collected from a semi-intensive culture pond at Ramsagar (23.06° N and longitude 87.15° E); Bankura district, West Bengal, India. The perennial lentic pond was surrounded with paddy-rice field and was far away from chemical effluents. The fishes were brought to the laboratory in living condition and were acclimatized for 48 h in re-circulated water tanks.

2.A.2.2 Isolation of intestinal microflora

Catfishes were sacrificed by using Tricaine mesylate; an anesthetic licensed drug. The fish skin was surface sterilized with 70% ethanol (Al-Harbi and Uddin 2005). The ventral surfaces were horizontally dissected and the intestinal tracts were aseptically removed from the abdominal cavities. The fish intestines were thoroughly washed with 0.9% (w/v) chilled sterile normal saline solution (NSS) to remove feed materials, dirt and non-adherent microflora. Intestines were then soaked in filter paper to absorb moisture content. The length and weight of the intestines were measured. They were then macerated, homogenized and vortexed (Fig. 2.A.1) in NSS (Irianto and Austin 2002).

Fig. 2.A.1: Isolation of bacteria from the intestine of *C. batrachus*: (A) Dissection of the ventral surface; (B) Intestinal tract of *C. batrachus*; (C) Homogenization of the intestinal content.

2.A.2.3 Cultivation of microbiota

Each homogenate were then serially diluted (10^{-1} to 10^{-10}) with deionized water. The diluted samples (10^{-6} to 10^{-10}) were aseptically inoculated in some common and specific culture media (tryptone soya agar, nutrient agar, MRS agar, Bacillus agar and Pseudomonas agar) by spread-plate technique. The plates were then incubated at 30 °C for 24-48 h in BOD incubator (Barman et al. 2011). The work was done in triplicates.

2.A.2.4 Colony characterization of the isolates

The bacterial load of the intestines was enumerated through total plate count using Digital colony counter (Model- 362; Environmental & Scientific Instruments Co., India). The colony morphologies (configuration, elevation, margins, surface, pigments, and size) were studied under stereo microscope (Olympus, India) and tabulated. Pure cultures of the isolates were preserved at 4 °C for future analysis.

2.A.2.5 Staining of the bacterial isolates

The intestinal isolates were characterized by Gram's staining technique. The endospore forming ability of the bacterial isolates were further tested following the protocol of Schaeffer-Fulton (1933).

2.A.2.5.1 Gram's staining

Bacterial isolates were smeared on clean grease-free slides. They were then air dried, heat fixed and subsequently Gram-stained. The slides were allowed to air dry, blotted and observed under light microscope (CH20i; Olympus, India) through oil immersion objective at 1000X total magnification.

2.A.2.5.2 Endospore staining

Endospore staining of bacterial isolates were performed accordingly and observed under oil immersion objective at 1000X total magnification of light microscope (CH20i; Olympus, India).

2.A.2.6 Test of antagonism against pathogens

The antagonistic activity of the isolated bacterial strains was tested against common fish pathogens by agar well diffusion method (Gram et al. 1999). Pathogenic Gram negative bacterial strains *Vibrio vulnificus* (MTCC 1145), *V. harveyi* (MTCC 7954) and *V. parahaemolyticus* (MTCC451) were obtained from the Microbial Type Culture Collection and Gene Bank (Chandigarh, India). *Aeromonas hydrophila* SBK1 (HM802878.1) was collected from the departmental stock (Department of Microbiology, Vidyasagar University). In addition to that, intestinal bacterial isolates were also tested against two Gram positive bacteria *Bacillus cereus* and *B. subtilis* (departmental isolates). At first, 100 µl of test pathogens (12×10^3 cfu/ml) were inoculated and spread over the tryptone soya agar (TSA) plates and allowed for 3-5 min of drying. 50 µl aliquots of concerned bacterial strains (24×10^3 cfu/ml) were placed in specified bored wells (5 mm diameter) on those TSA plates. The plates were then incubated at 30 °C for 24 h. The test was done in triplicates. The intestinal bacterial isolates that produced significant inhibitory zones against target pathogens would be selected as potent antimicrobial substance producers or probiotic strains (Vignolo et al. 1993).

2.A.2.7 Selection of potent isolates

The colonies that produced maximum inhibitory zone against pathogens were primarily considered as potent isolates and selected for further analysis.

2.A.2.8 Cultivation of putative probionts

A specific screening media (tryptone soya broth) was selected according to Irianto and Austin (2002) with the following composition (g/l: Tryptone 17, Soya peptone 3, Sodium chloride 5, Dipotassium hydrogen phosphate 2.5, Dextrose 2.5; Hi-media, India). The selected isolates were cultivated at different temperature (10-65 °C), pH (5.0-11.0) and salt concentrations (0.1-10.0 %) to obtain their growth range as well as to optimize their growth requirement (Barman et al. 2011).

2.A.2.9 Determination of growth phase

The selected intestinal isolates were cultivated in tryptone soya broth (TSB) in shaker-incubator at 37 °C with 100 rpm for 48 h to study the different phases of bacterial growth. The growth of bacterial isolates was measured by spectrophotometer at 620 nm at regular time interval (Prosser and Tough 1991). The test was done in triplicates.

2.A.2.10 Motility test

Motility test of PKA17, PKA18 and PKA19 bacterial isolates was performed by hanging drop method. A drop of fresh bacterial suspension was hung from the centre of the cover slip to the cavity of a grooved slide. The slide was then inverted carefully and focused under phase contrast microscope (RXLr-4; RADICAL, India) at 400X total magnification.

2.A.2.11 Biochemical characterization of probiotic bacteria

The biochemical characteristics of the isolate PKA17, PKA18 and PKA19 were studied according to Bergey’s Manual of Determinative Bacteriology (Holt et al. 1994).

2.A.2.11.1 Sugar fermentation test

Phenol red broth (g/l: Peptone 10, Beef extract 1, Sodium chloride 5, Phenol red 0.018, pH 7.4±0.2; Hi-media, India) was prepared for determination of fermentation reactions of test

isolates. 5-10 % of desired carbohydrates were added separately in broth base medium. The media was then distributed in fermentation tubes (containing Durham's tubes in inverted condition) and sterilized at 15 lb/inch^2 pressure at 121 °C for 15 min. The tubes were then inoculated with the fresh culture of bacterial isolates (1%) and incubated at 37 °C for 48-72 h. The culture tubes were then observed for formation of acid (colour change from red to yellow) and gas (bubble in Durham's tube) that would indicate positive reaction.

2.A.2.11.2 Catalase test

The bacterial isolates were grown separately on nutrient agar (g/l: Peptone 5, Sodium chloride 5, Meat extract 1.5, Yeast extract 1.5, Agar 15, pH 7.4±0.2; Hi-media, India) slants for 48 h. After incubation, the slant cultures were flooded with 0.5 ml of 6% w/v H_2O_2 (Qualigens Fine Chemicals, India). The culture tubes were then observed for production of effervescence which indicates a positive reaction.

2.A.2.11.3 Indole production test

The bacterial isolates were grown in sterile tryptone (1%, w/v) broth (g/l: Casein enzymic hydrolysate 10, Sodium chloride 5, pH 7.5±0.2; Hi-media, India) for 24-48 h. After incubation, few drops of Kovac's reagent (5 g p-dimethylamino benzaldehyde, in a mixture of 75 ml amyl alcohol and 25 ml HCl; Hi-media, India) were gently added to the culture tubes and slightly agitated. The tubes were then allowed to stand for 5-10 min. The formation of cherry-red ring at the top of the tube would indicate the formation of indole whereas yellow colour indicates negative result.

2.A.2.11.4 Methyl red and Voges-Proskauer test

Glucose phosphate broth (g/l: Buffered peptone 7, dextrose 5, dipotassium phosphate 5, pH 6.9±0.2; Hi-media, India) was distributed in 10 ml quantities in culture tubes and sterilized at 15 lb/inch^2 pressure at 121 °C for 15 min. The tubes were then incubated at 37 °C with fresh bacterial inoculums for 48-72 h. After incubation, 1-2 drops of methyl red indicator (0.2 g methyl red in a mixture of 60 ml ethyl alcohol and 40 ml distilled water; Hi-media, India) was added to the culture broth. For Voges-Proskauer test, 2 drop of Barritt Reagent A (5% α-napthol in absolute ethanol; R029, Hi-media, India) and 2 drop of Barritt Reagent B (40% potassium hydroxide; R030, Hi-media, India) were added to each culture tube. The tubes were gently shaken for 30-60 seconds to oxidize the acetoin (acetylmethylcarbinol) and allowed to stand for 15 min to obtain colour reaction. The development of red colour indicates positive reaction in both cases.

2.A.2.11.5 Citrate test

The citrate utilization test was carried out to determine the ability of test isolates to utilize citrate as sole source of carbon. Bacterial isolates were inoculated into sterile Simmons Citrate agar (g/l: magnesium sulphate 0.2, ammonium dihydrogen phosphate 1, dipotassium phosphate 1, sodium citrate 2, sodium chloride 5, bromothymol blue 0.08, agar 15; pH 6.8±0.2; Hi-media, India) slants and incubated overnight at 37 °C. The utilization of citrate can be detected by the appearance of blue colour.

2.A.2.11.6 Urea hydrolysis

The urea broth (g/l: urea 20, monopotassium phosphate 0.091, disodium phosphate 0.095, yeast extract 0.1, phenol red 0.010, pH 6.8±0.2; Hi-media, India) was inoculated with the

test bacterial isolates and precisely incubated. The hydrolysis of urea can be detected by the appearance of cerise colour.

2.A.2.11.7 Starch hydrolysis

The test was performed by growing the isolates on sterile starch agar (g/l: peptic digest of animal tissue 5, sodium chloride 5, yeast extract 1.5, beef extract 5, starch soluble 2, agar 15, pH 7.4±0.2; Hi-media, India). Clear hollow zones around the colonies upon addition of iodine solution would indicate starch hydrolysis (Dhawale et al. 1982).

2.A.2.11.8 Protease test

Bacterial isolates were streaked on casein hydrolysate agar (g/l: casein enzymic hydrolysate 5, beef infusion 150, peptic digest of animal tissue 5, yeast autolysate 1.5, sodium phosphate 2.5, sodium chloride 5, agar 15, pH 7.8±0.2; Hi-media, India) to assess the ability of synthesizing extracellular protease enzyme (Gomaa 2013). The appearance of distinct clear zone around the respective colonies would indicate a positive reaction.

2.A.2.11.9 Cellulase test

Bacterial strains were streaked on carboxymethylcellulose agar (g/l: carboxymethylcellulose sodium salt 2, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, peptone 0.2, Agar 17, pH 7.0; Hi-Media, India) plates and incubated at 37 °C for 24-48 h. The plates were then flooded with Gram's iodine solution (Iodine 1g, Potassium iodide 2 g, Distilled water 300 ml; Hi-Media, India) for 3-5 min. The stain was then drained off and the plates were observed for appearance of zone of clearance around the colony (Kasana et al. 2008).

2.A.2.11.10 Xylanase test

The Xylan media (g/l: $(NH4)_2SO_4$ 1; $MgSO_4$ 0.2; K_2HPO_4 0.2; $CaCl_2$ 0.2; $MnCl_2$ 0.02; yeast extract 0.1, xylan 10.0, Agar 20; pH 7; Hi-Media, India) was used for determination of xylanase activity (Mandal 2015). Prior to sterilization, xylan was completely dissolved in water by sonication (7 kHz, 2 min). The bacterial isolates were grown on respective xylan agar plates and incubated at 37 °C for 24-48 h. The plates were then observed for the formation of precise transparent hollow zones surrounding the colonies.

2.A.2.11.11 Phosphate solubility test

Bacterial strains were inoculated on pikovskayas agar (g/l: Yeast extract 0.5, dextrose 10, calcium phosphate 5, ammonium sulphate 0.5, potassium chloride 0.2, magnesium sulphate 0.1, manganese sulphate 0.0001, ferrous sulphate 0.0001, agar 15; Hi-Media, India) by continuous streaking method and incubated at 37 °C for 24-48 h. The solubilization of phosphate is indicated by the manifestation of clear zone around the colonies and hence, confirms the extracellular secretion of alkaline phosphatase enzyme by bacterial isolates (Behera et al. 2017).

2.A.2.11.12 Nitrate reduction test

The efficacy of bacterial isolates to synthesize nitrate reductase in order to reduce nitrate to nitrite or nitrogenous compounds was assessed by culturing in sterile nitrate broth (g/l: peptic digest of animal tissue 5, beef extract 3, potassium nitrate 1, pH 7.0±0.2; Hi-media, India) at 37 °C for 48 h. The formation of nitrite is detected through the development of red colour (prontosil) upon addition of few drops of sulphanillic acid (R015; Hi-media) followed by alpha-napthylamine (R009; Hi-media). A pinch of zinc dust was further added in negative tubes.

2.A.2.12 Molecular identification of bacterial strains

The putative probionts were subjected to 16S rDNA molecular sequencing.

2.A.2.12.1 Isolation of chromosomal DNA

The bacterial isolates were grown overnight at 37 °C in Luria Broth (g/l: Casein enzymic hydrolysate 10, Yeast extract 5, Sodium chloride 5; Hi-media, India). 2 ml of this aliquot was aseptically transferred to the respective Eppendorf tube and centrifuged at 10000 g for 10 min. The supernatant was discarded and 875 µl of Tris-EDTA (10 mM Tris HCl, 1 mM EDTA; pH 8.0) buffer was added to the cell pellet. The cells were gently dissolved in TE buffer. 100 µl of 10% w/v Sodium dodecyl sulfate (10 g SDS in 100 ml deionized water) followed by 5 µl of Proteinase K (10 mg of Proteinase K in 1 ml deionized water) was added to the cells. The preparation was then vortexed and incubated at 37 °C for 1 h. Equal volume of phenol-chloroform (pH 7.0) extraction was added to the content, mixed thoroughly by inverting the sample and incubated at room temperature for 5 min. The contents were further centrifuged (10000 g for 10 min at 4 °C) and the upper phase (aqueous layer) was carefully transferred to a fresh Eppendorf tube avoiding mechanical disruption. The process was repeated once again. 100 µl of 5M sodium acetate (41 g of sodium acetate in 100 ml distilled water) was added to it and mixed thoroughly. 2 ml of isopropanol was further added to precipitate the DNA. The preparation was centrifuged (5000 g for 10 min), supernatant was discarded and 1ml ethanol (70%) was added to rinse the pellet. These tubes were spin at 10,000 rpm for 2 min and subsequently air-dried. The DNA was resuspended in TE buffer. 5 µl RNase A solution was added to the sample to obtain pure DNA. The sample was then subjected to agarose gel electrophoresis and observed through UV transilluminator (Bangalore Genei Pvt. Ltd., India).

2.A.2.12.2 Amplification and sequencing of 16S rDNA

Fragments of 16S rDNA genes of the selected strains were amplified in Thermal cycler (Veriti™ 96-Well; Applied Biosystems®, USA) using universal forward and reverse primers. The quality of PCR products were further evaluated on 1.2% agarose gel. Excess primers and nucleotides were enzymatically hydrolyzed by ExoSAP-IT™ (Thermo Fisher Scientific Inc.). The purified products were subsequently subjected to Sanger sequencing using BDT v3.1 Cycle sequencing kit on ABI Genetic Analyzer (3730XL, Thermo Fisher Scientific Inc., USA).

2.A.2.12.3 Phylogram construction

Nucleotide BLAST search was conducted using National Centre for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on maximum identity scores nearest sequences of selected strains were obtained. Multiple sequence alignment were performed using ClustalX2 and ClustalW software (Larkin et al. 2007). Phylograms were created by Phylip 3.69 software and the phylogenetic trees were represented by Dendroscope software through neighbor-joining method (Tuimala 1989; Huson et al. 2007).

2.A.2.13 Scanning electron microscopic study

The scanning electron microscopy of the bacterial isolates was carried out following the protocol of Prabhurajeshwar and Chandrakanth (2018) with some modification. The bacterial strains were grown overnight in respective Luria Broth and harvested through centrifugation at 6000 rpm for 10 min at 4 °C. The cells were then washed (3000 rpm for 10 min) twice with PBS and resuspended in it. The fixation (2% glutaraldehyde in 0.1 M phosphate buffer) and post-fixation (1% osmium tetroxide solution) were performed

gradually. The specimens were then air-dried to critical point using series of graded alcohols. The samples were put on small glass slides and gold-coated by sputter coater (SC7620; Quorum Technologies Ltd, UK). The final preparation was observed under scanning electron microscope (EVO 18; Carl Zeiss, Germany).

2.A.2.14 Antibiotic susceptibility test

Antibiotic susceptibility tests were conducted on TSA by placing discs containing defined concentrations of specific antibiotics against probiotic strains (Bauer et al. 1966). The antibiotics that were used included inhibitors of the protein synthesis: streptomycin, clindamycin, gentamycin, chloramphenicol, ciprofloxacin, tetracycline; cell wall synthesis: vancomycin; nucleic acid synthesis: nalidixic acid. The effectiveness of a particular antibiotic was measured from the diameter of the zone of inhibition around the discs after incubation at 37 °C for 24 h. The disc diffusion method thus provided the basis for a sensitivity spectrum of test organisms.

2.A.2.15 Cell surface hydrophobicity test

The putative probionts were grown overnight at 37 °C in tryptone soya broth media. The cultures were then centrifuged (Cooling micro centrifuge CM-12 Plus; REMI, India) at 4000 rpm for 10 min. The supernatants were discarded and the pellets were washed twice with phosphate-buffered saline (PBS) and finally resuspended in PBS to an optical density of 1.00 (A_0) at 620 nm. 3 ml of this aliquot was overlaid by 0.6 ml of xylene (Merck Life Science Pvt. Ltd., India), vortexed vigorously for 1 min and incubated at 37 °C to allow the immiscible solvent and aqueous phase to get separated. The absorbance (A_f) of the aqueous phase was subsequently measured. The percentage of hydrophobicity was precisely calculated (Savage 1992):

$$\textbf{Hydrophobicity}\ (\%) = \frac{A_0 - A_f}{A_0} \times 100$$

Where, A_0 = initial absorbance, A_f = final absorbance

2.A.2.16 Bile salt tolerance test

The intestinal bacterial isolates were tested for their bile salt tolerance ability at different concentration of bile salts in tryptone soya broth (Gilliland et al. 1984). TSB media containing 0%, 0.037%, 0.075%, 0.15% and 0.3% bile salt were prepared and thoroughly sterilized. Fresh bacterial culture (1%) were inoculated into those respective sterile TSB medium and incubated at 37 °C for 24 h at 100 rpm. The test was done in triplicates. The absorbance was measured by spectrophotometer at 620 nm and growth was evaluated by spreading of test cultures on TSA (Jin et al. 1998).

2.A.2.17 Bile salt hydrolase activity

Bile salt hydrolase activity was detected by slight modification of Shehata et al. (2016). The intestinal bacterial isolates were grown on TSA plates containing 0.5% (w/v) sodium taurodeoxycholate hydrate (TDCA; Hi-media, India) and 0.037% $CaCl_2$. Plates were incubated at 37 °C for 48-72 h. The visible zone of precipitation surrounding the colonies ensures bile salt hydrolase activity of the test isolates.

2.A.2.18 Haemolysis test

The test of haemolysis was done with both defibrinated sheep and fish blood. 5% (v/v) sterile defibrinated sheep/fish blood was added to the sterile blood agar base (45 °C <). It was then shaken gently avoiding formation of air bubbles and petriplated. Bacterial isolates were streaked on blood agar plates and incubated at 37 °C for 24-48 h. The plates were then observed for the appearance of any clear zones (β-haemolysis) or greenish hemolytic

zone (α-haemolysis) around the vibrant bacterial colonies. The absence of such zone (γ-haemolysis) would indicate non-pathogenic nature of the test isolates (Forbes et al. 2002).

2.A.2.19 Study of mutual compatibility

Three selected bacterial isolates were cross-streaked against each other to study their mutual interaction pattern on tryptone soya agar medium. After overnight incubation at 37 °C, the growth and interaction patterns of the bacterial isolates were observed.

2.A.3 Results and Discussion

2.A.3.1 Isolation of bacteria from the intestine of *C. batrachus*

The bacterial content of the intestines (length: 14.5±2.72 cm; weight: 2.9±0.62 g) of indigenous *C. batrachus* (length: 27.11±3.77 cm; weight: 270.5±11.76 g) were assessed both quantitative and qualitatively. The viable bacterial count of catfish intestines was $1.6\pm2.9 \times 10^{10}$ cfu/g whereas the bacterial count of the pond water ranged from $8.3\pm2.75 \times 10^{3}$ to $2.8\pm1.45 \times 10^{4}$ cfu/ml. The much higher number of bacterial count in the intestine of *C. batrachus* than the surrounding culture water indicated that the digestive tracts of fish provided a favorable ecological niches for these microorganisms.

A total of thirty-two autochthonous bacteria were isolated from the intestinal tract of healthy *C. batrachus* on the basis of distinct colony morphology and Gram characterization (Table 2.A.1). The Gram positive bacteria (84.38 %) have significantly dominated the intestinal population. The study also revealed the presence of spore-forming rod-shaped bacteria in considerable number. The presence of such diverse profile of autochthonous microflora might play a major role in providing protection against pathogenic microorganisms. Jimoh et al. (2014) obtained a total plate count (TPC) of 6.5×10^{5} cfu/g from the gastro-intestinal tract of *Clarias gariepinus*. Ramesh et al. (2015)

studied the gastrointestinal flora of healthy *L. rohita* and obtained twenty-six types of bacterial isolates. Sedláček et al. (2016) studied the composition of culturable enteric bacteria from the intestine of Antarctic fish. Sakata et al. (1978) reported the dominance of aerobic heterotrophic bacteria in the gastrointestinal tract of yellowtail (*Seriola* sp.).

Table 2.A.1: Colony morphology and Gram nature of bacterial isolates isolated from intestines of *C. batrachus*.

Isolates	**Colony Characters**							
	Configuration	**Elevations**	**Margins**	**Surface**	**Pigments**	**Diameter (mm)**	**Media**	**Gram Character/ Shape/ Arrangement**
PKA1	Circular	Convex	Undulated	Smooth, glittering	Off White	5-8	NA	Gram positive spore-forming rod; single and clusters
PKA2	Irregular	Umbonate	Curled	Rough	White	5	NA	Gram positive rod; single
PKA3	Irregular	Umbonate	Curled	Rough	White	5	NA	Gram positive rod; capsuled; single
PKA4	Circular	Raised	Entire	Smooth	Off white	2	NA	Gram positive rod; single
PKA5	Circular	Raised	Entire	Smooth	Glittering	2	MRS	Gram positive rod; single
PKA6	Circular	Raised	Entire	Smooth	Off white	3	MRS	Gram positive rod; single and chain
PKA7	Small circular	Slightly raised	Undulated	Smooth	Off white		NA	Gram positive spore-forming rod; chain form
PKA8	Irregular	Umbonate	Curled	Rough	White	5	NA	Gram positive rod; single
PKA9	Circular	Flat	Entire	Smooth	Yellow	2	NA	Gram negative cocci-shaped; clusters
PKA10	Circular	Raised	Entire	Smooth	Off white	2	Pseudomonas	Gram negative small rod; single
PKA11	Small circular	Slight raised	Entire	Smooth	Off white	2	Bacillus	Gram positive rod; single
PKA12	Irregular	Flat	Lobate	Smooth	Off white	3	TSA	Gram positive rod; single
PKA13	Circular	Raised	Entire	Smooth	Yellow	1.5	NA	Gram negative cocci-shaped; clusters
PKA14	Circular	Raised	Entire	Smooth	Brown	1.5	NA	Gram positive cocci-shaped; single
PKA15	Circular	Slight raised	Entire	Smooth	Off white	2	MRS	Gram positive cocci-shaped; single
PKA16	Irregular	Flat	Undulate	Smooth	Transparent	2	Bacillus	Gram positive rod; single
PKA17	Circular	Flat	Undulate	Smooth	Off white	2	TSA	Gram positive spore-forming rod; single
PKA18	Circular	Raised	Erose	Smooth	Middle-Off white	5	TSA	Gram positive rod; single

					Outer-Transparent			
PKA19	Circular	Raised	Erose	Smooth	Off white	3	TSA	Gram positive spore-forming rod; single
PKA20	Circular	Raised	Entire	Smooth	Off white	2	NA	Gram positive rod; single
PKA21	Circular	Raised	Entire	Glittering smooth	Off white	1.8	NA	Gram positive long-rod; single
PKA22	Circular	Raised	Undulate	Smooth	Off white	4	NA	Gram negative rod; single
PKA23	Circular	Raised	Entire	Smooth	Off white	2	Pseudomonas	Gram positive rod; single
PKA24	Circular	Raised	Undulate	Smooth	Off white	2	Pseudomonas	Gram positive rod; single or chain
PKA25	Circular	Raised	Entire	Smooth	Off white	3	Pseudomonas	Gram positive rod; single
PKA26	Irregular	Raised	Lobate	Smooth	Off white	5	Pseudomonas	Gram positive rod; single
PKA27	Circular	Raised	Entire	Smooth	Off white	2	TSA	Gram positive rod; single
PKA28	Circular	Slight Raised	Undulate	Smooth	Transparent	2	Pseudomonas	Gram positive rod; capsuled; single
PKA29	Irregular	Raised	Entire	Smooth	Off white	7	MRS	Gram positive rod; single and clusters
PKA30	Irregular	Raised	Undulate	Smooth	Off white	6	MRS	Gram positive rod; single and clusters
PKA31	Irregular	Raised	Undulate	Smooth	Off white	4	MRS	Gram negative rod; single and clusters
PKA32	Irregular	Raised	Entire	Smooth	Off white	3	MRS	Gram positive rod; single and clusters

2.A.3.2 Selection of probiotic bacteria

The antagonistic activity of intestinal bacterial isolates of *C. batrachus* was evaluated against pathogenic *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus* and *A. hydrophila* strains. The potent isolates manifested clear and translucent zone of inhibition around the well (Table 2.A.2). Five intestinal isolates PKA1, PKA2, PKA17, PKA18 and PKA19 showed significant antagonism against target pathogens (Fig. 2.A.2). PKA1 was effective only against *V. harveyi* and *V. vulnificus*. PKA17 showed significant antagonism (24.93±0.12 mm) against *V. vulnificus* followed by *V. parahaemolyticus* and *V. harveyi*. PKA18 elicited considerable inhibition (15±5 mm) against *V. vulnificus*. PKA19 showed inhibitory activity against *V. parahaemolyticus* and *V. harveyi*. However, the bacterial isolates didn't exhibit any antagonism against pathogenic *A. hydrophila* and non-pathogenic *B. subtilis* and *B. cereus* strains. The antagonistic activity of intestinal isolates may be attributed to the release of chemical substances with bactericidal or bacteriostatic effect. Verschuere et al. (2000) stated that the inhibitory effects of bacterial isolates may be due to the production of bacteriocins, siderophores or enzymes. Kato et al. (2016) isolated *Lactobacillus* and *Lactococcus* from the surface of *C. gariepinus*, and observed antimicrobial activity against aquaculture pathogens. The isolates of *Cirrhinus mrigala* resulted considerable antagonism against the pathogen *Pseudomonas fluorescens* (Ghosh et al. 2007). Abraham et al. (2007) stated that the ability of intestinal isolates to control pathogenic infections has direct influence on aquatic production.

In the present study, PKA1, PKA2, PKA17, PKA18 and PKA19 exhibited strong antagonistic activity against target fish pathogens and hence were considered for further study.

Table 2.A.2: Inhibitory effects of bacteria isolated from the intestine of *C. batrachus* against target pathogens.

Bacterial Isolates	**Zone of Inhibition (mm)**			
	V. harveyi	***V. vulnificus***	***V. parahaemolyticus***	***A. hydrophila***
PKA1	11±0.5	11.5±0.5	-	-
PKA2	15±2	8±1	13.67±1.53	-
PKA17	12.25±1.86	24.93±0.12	17.33±2.52	-
PKA18	11.33±9.86	15±5	-	-
PKA19	15.66±13.65	-	19±1	-

The isolates didn't exhibit any antagonism against non-pathogenic B. subtilis and B. cereus strain

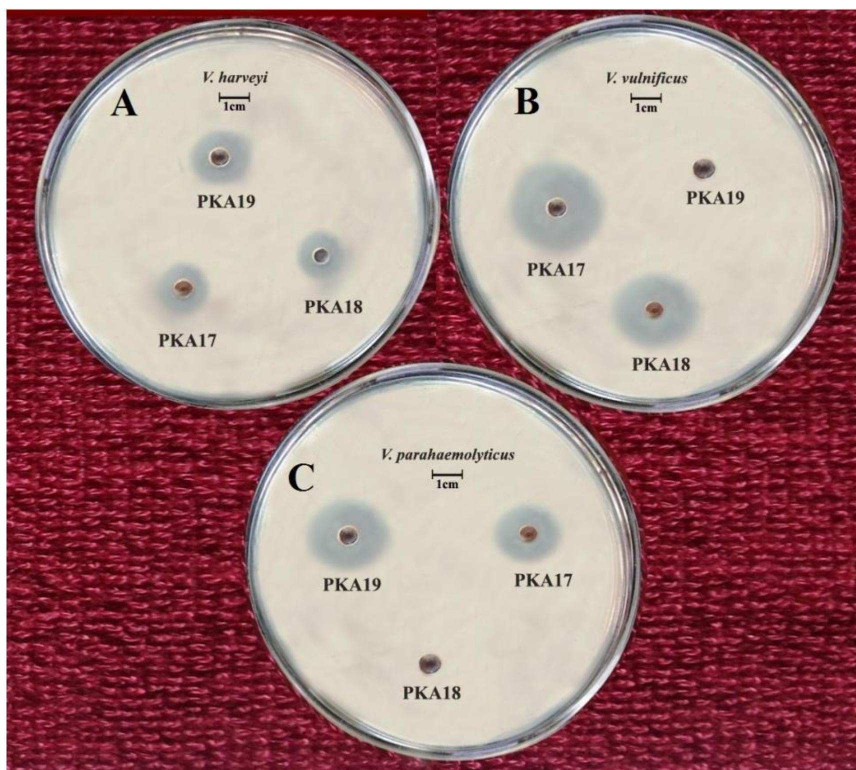

Fig. 2.A.2: Antagonistic activity of bacterial isolates PKA17, PKA18 and PKA19 against pathogenic (A) *V. harveyi* (B) *V. vulnificus* (C) *V. parahaemolyticus*.

2.A.3.3 Cultivation of putative probionts

The growth of the major bacterial isolates was observed in a wide range of pH, temperature and NaCl concentration. PKA1, PKA17, PKA18 and PKA19 grew best at 37 °C whereas PKA2 at 30 °C temperature (Fig. 2.A.3). PKA1, PKA2 and PKA19 required pH 7.0 and PKA17, PKA18 required pH 6.0 for optimum growth (Fig. 2.A.4). All isolates showed optimum growth at 0.1% salt concentration (Fig. 2.A.5). Finally, three bacterial isolates (PKA17, PKA18 and PKA19) were selected for the further study on the basis of higher zone of inhibition against fish pathogens.

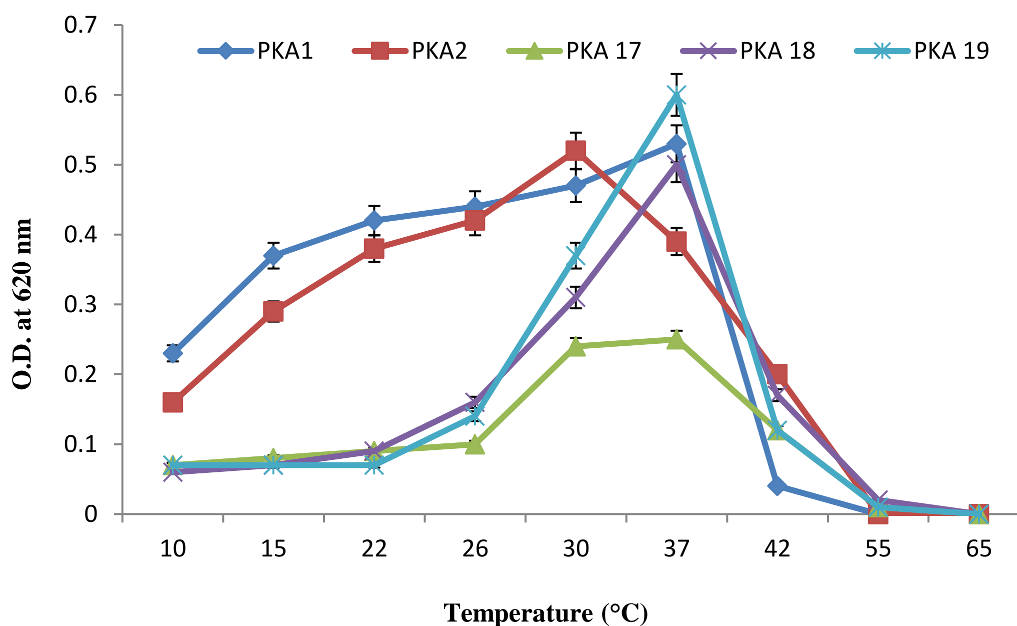

Fig. 2.A.3: Growth of selected bacterial isolates of *C. batrachus* at various temperatures in tryptone soya broth media at pH 7 under shaking (100 rpm) condition.

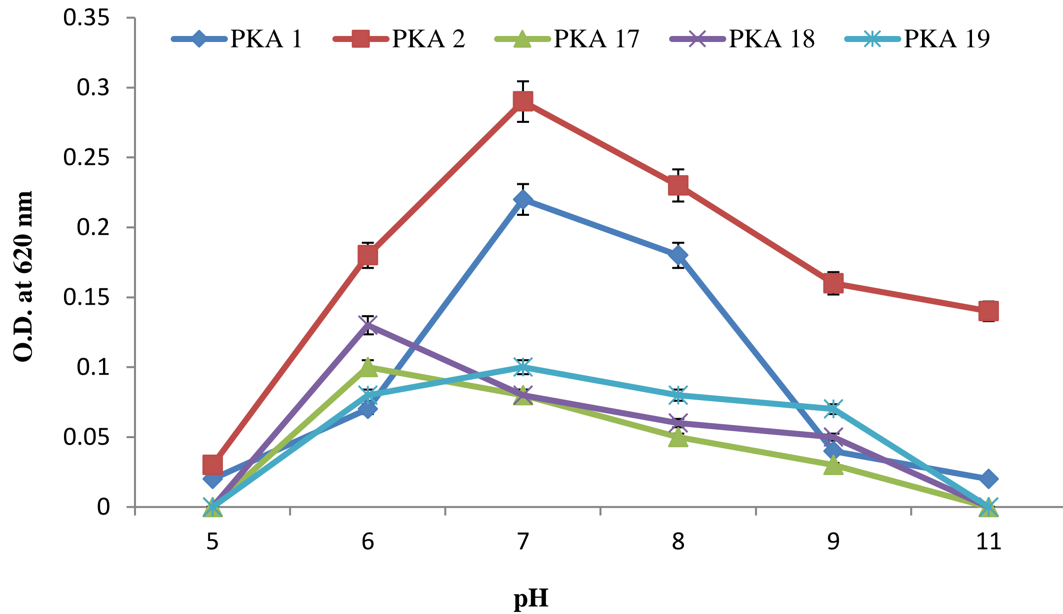

Fig. 2.A.4: Growth of selected bacterial isolates of *C. batrachus* at various pH in tryptone soya broth media at optimized temperature under shaking (100 rpm) condition.

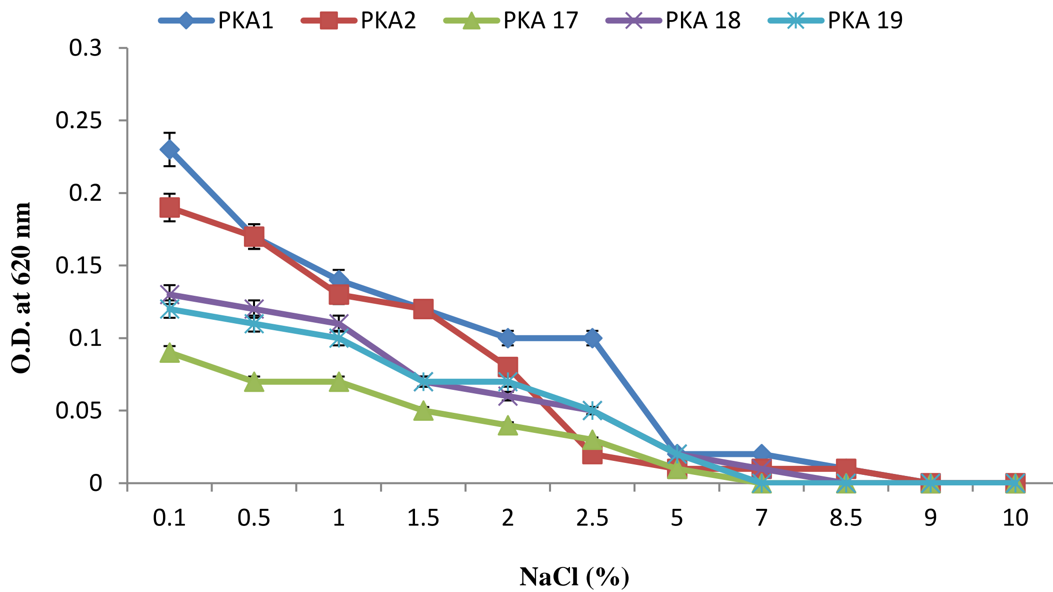

Fig. 2.A.5: Growth of selected bacterial isolates of *C. batrachus* at various salt concentrations in tryptone soya broth media at optimized temperature and pH under shaking (100 rpm) condition.

2.A.3.4 Determination of growth phase

The growth phase of the three bacterial isolates PKA17, PKA18 and PKA19 have been studied in TSB media at 37 ºC for 36 h (Fig. 2.A.6). The PKA17 isolate showed prolonged (15 h) stationary phase compared to other putative probionts. PKA18 and PKA19 strains executed extended logarithmic phase. The lag phase of the bacterial isolates is relatively short (5±0.06 h) which indicated rapid physiological adaptation of the cells to the culture conditions. Bacterial growth is a dynamic process comprised of various anabolic and catabolic reactions. The elongated stationary phase of PKA17 may be correlated with the optimum production of biomass. Conversely, the extended logarithmic phase of PKA18 and PKA19 may promote synthesis of certain enzymes and growth factors. Lepercq et al. (2004) observed increased bile salts hydrolase activity in the cells harvested in exponential growth phase compared to the stationary phase.

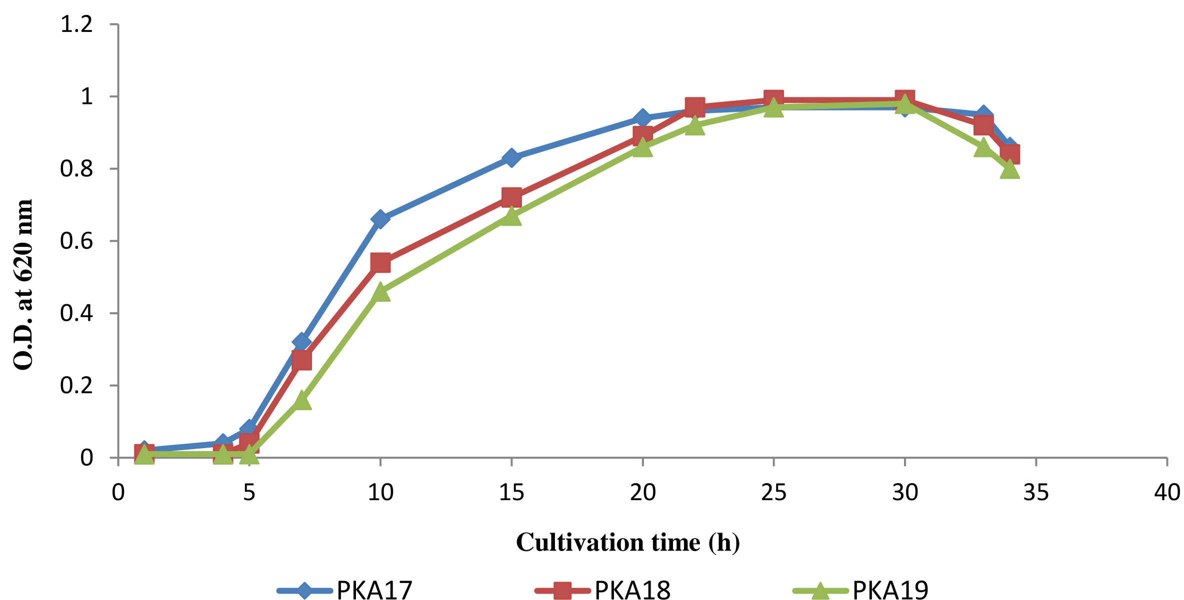

Fig. 2.A.6: Effect of cultivation period on growth of PKA17, PKA18 and PKA19 in TSB at 37 °C at optimized pH under shaking (100 rpm) condition.

2.A.3.5 Motility

The bacterial isolates PKA17, PKA18 and PKA19 were found to be motile in nature. The rod-shaped bacterial cells were observed with erratic movement in the surrounding medium.

2.A.3.6 Biochemical characterization of selected probiotic bacteria

The biochemical characteristics of the isolate PKA17, PKA18 and PKA19 were studied thoroughly (Table 2.A.3). The sugar fermentation profile exhibited the capacity of PKA17 strain to produce xylose, dextrose, mannose, L-arabinose and D-arabinose. The PKA18 strain was able to ferment maltose, fructose and trehalose whereas PKA19 strains fermented dextrose, maltose, fructose, trehalose, salicin and sucrose. Malonate-utilization efficiency was observed in PKA17 and PKA19 bacterial isolates. The PKA17, PKA18 and PKA19 isolates exhibited the ability to hydrolyze esculin and citrate. In addition, the PKA17 and PKA18 strains can also hydrolyze starch. All the strains were capable of synthesizing catalase. The isolates have also shown nitrate reduction ability. The production of extracellular enzymes protease, cellulase, xylanase and phosphatase were observed in bacterial isolates PKA17, PKA18 and PKA19 through the appearance of distinct clear zone around the respective colonies. The production of phosphatase was distinctly visible on pikovskayas agar medium (Fig.2.A.7).

The PKA17, PKA18 and PKA19 strains were morphologically identified as Gram positive spore-formers (Fig. 2.A.8). Cumulatively, the morphological and biochemical characteristics of PKA17 resembled mostly with *Lysinibacillus* sp. whereas PKA18 and PKA19 found homology with *Bacillus* sp. as per Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Table 2.A.3: Biochemical characteristics of the bacterial isolates PKA17, PKA18 and PKA19 isolated from the intestine of *C. batrachus*.

Serial No.	Test	PKA17	PKA18	PKA19
1	Lactose	–	–	–
2	Xylose	+	–	–
3	Maltose	–	+	+
4	Fructose	–	+	+
5	Dextrose	+	–	+
6	Galactose	–	–	–
7	Raffinose	–	–	–
8	Trehalose	–	+	+
9	Melibiose	–	–	–
10	Sucrose	–	–	+
11	L-Arabinose	D	–	–
12	Mannose	+	–	–
13	Inulin	–	–	–
14	Sodium gluconate	–	–	–
15	Glycerol	–	–	–
16	Salicin	–	–	+
17	Dulcitol	–	–	–
18	Inositol	–	–	–
19	Sorbitol	–	–	–
20	Mannitol	–	–	–
21	Adonitol	–	–	–
22	Arabitol	–	–	–
23	Erythritol	–	–	–
24	α-Methyl-D-glucoside	–	–	–
25	Rhammose	–	–	–
26	Cellobiose	–	–	–
27	Melezitose	–	–	–
28	α-Methyl-D-mannoside	–	–	–
29	Xylitol	–	–	–
30	ONPG	–	–	–
31	Esculin hydrolysis	+	+	+
32	D-Arabinose	D	–	–
33	Citrate utilization	+	+	+
34	Malonate utilization	+	–	D
35	Sorbose	–	–	–
36	Catalase	+	+	+
37	Protease	+	+	+
38	Cellulase,	+	+	+
39	Xylanase	+	+	+
40	Phosphatase	+	+	+
41	Starch hydrolysis	+	+	–

42	Urease Test	−	−	−
43	Nitrate reduction	+	+	+
44	Indole	−	−	−
45	MR (Methyl Red)	−	−	−
46	VP (Voges Proskauer)	+	+	+

+ = *Positive Reaction*, − = *Negative Reaction*, D = *Delayed positive reaction*

Fig. 2.A.7: Growth and clear zone production of bacterial isolates on Casein Hydrolysate agar medium.

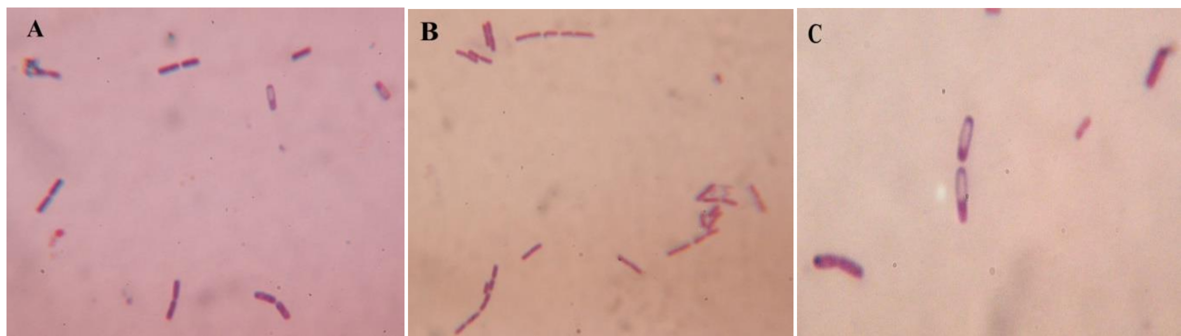

Fig. 2.A.8: Light microscopic view: (A) PKA17; (B) PKA18; (C) PKA19 at 1000X total magnification.

2.A.3.7 Molecular identification of bacterial isolates

The molecular identification of intestinal bacterial isolates of *C. batrachus* was performed through 16S rDNA sequencing. Fragments of 16S rDNA genes of PKA17 and PKA18 were amplified in Thermal cycler (Veriti™ 96-Well; Applied Biosystems®, USA) using universal forward (8F) and reverse (1492R) primers where as partial sequence of PKA19 rDNA was amplified by forward and reverse primer (27F and 1492R respectively). According to the sequencing result, the amplified products for PKA17 were 747 base pair, PKA18 were 677 and PKA19 were 1460 base pair long (Fig. 2.A.9) which showed corresponding single bands. The 16S rDNA partial sequences were subjected to nucleotide BLAST against non-redundant database. With respect to 100% query coverage and 94% identity from PKA17 BLAST result, twelve sequences were selected for phylogram construction. According to phylogeny constructed against 100 bootstrap value, PKA17 showed similarity with *Lysinibacillus sphaericus* OUG29GKBB (Accession No.KM972671.1) as shown in Fig. 2.A.10. On the other hand for the construction of PKA18 tree, thirteen sequences were selected having 98% query coverage and 96% identity values. PKA18 showed similarity with *Bacillus cereus* strain Gut16 (Accession No.KU156696.1) as shown in Fig. 2.A.11. According to the PKA19 BLAST result of 100% query coverage and 99% identity, ten sequences were selected for phylogram construction. According to phylogeny constructed against 100 bootstrap values, PKA19 showed similarity with *Bacillus thuringiensis* Bt 53 (Accession No. KY784654.1) as shown in Fig. 2.A.12. So, the above study indicated that the three isolated organism PKA17, PKA18 and PKA19 belongs to *Lysinibacillus sphaericus*, *Bacillus cereus* and *Bacillus thuringiensis* species and the accession numbers provided by GenBank were KX580190.1, KX826079.1 and MF139049.1 respectively.

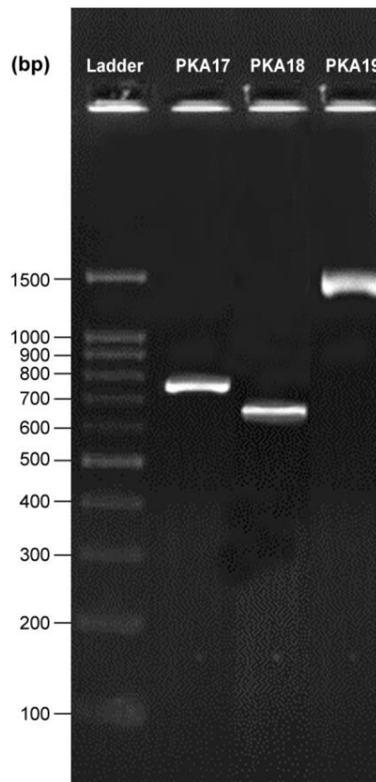

Fig. 2.A.9: Agarose gel analysis of molecular weight of PCR products of 16S rDNA of PKA17, PKA18 and PKA19 bacterial isolates of *C. batrachus* (The three amplified 16S rDNA products were run separately parallel to the ladder lane and ladder lane contained standard molecular weight marker DNA).

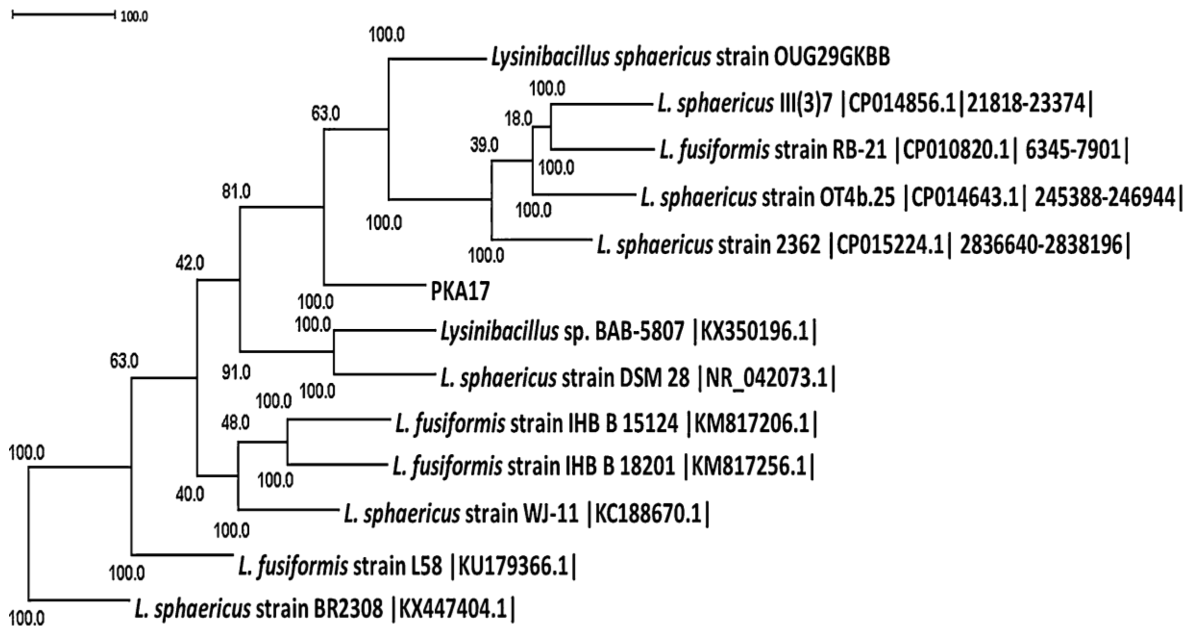

Fig. 2.A.10: Phylogenetic analysis based on 16s rDNA sequencing of isolate PKA17 and related bacteria showing similarity with *Lysinibacillus sphaericus* OUG29GKBB (AccessionNo.KM972671.1).

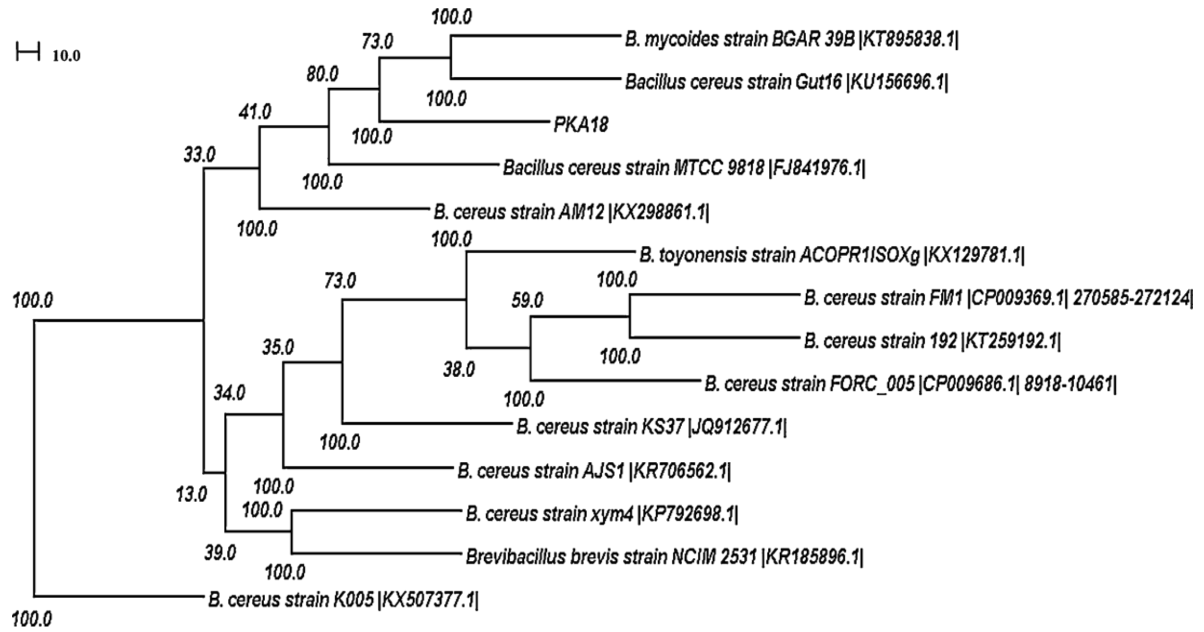

Fig. 2.A.11: Phylogenetic analysis based on 16s rDNA sequencing of isolate PKA18 and related bacteria showing similarity with *Bacillus cereus* strain Gut16 (Accession No.KU156696.1).

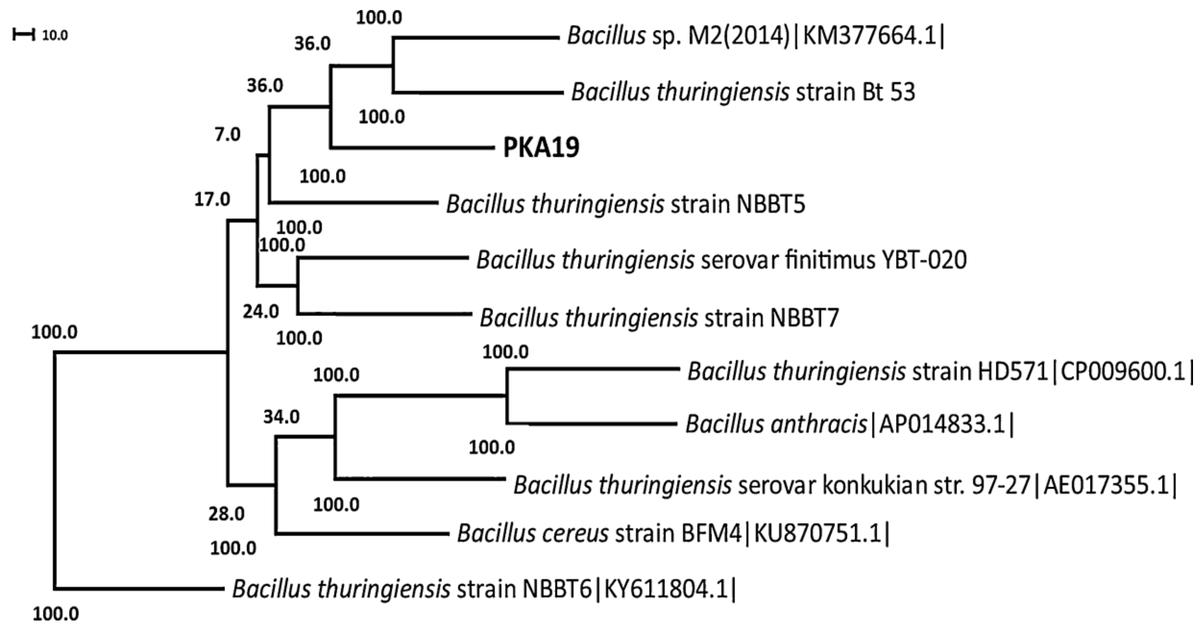

Fig. 2.A.12: Phylogenetic analysis based on 16s rDNA sequencing of isolate PKA19 and related bacteria showing similarity with *Bacillus thuringiensis* Bt 53 (Accession No. KY784654.1).

2.A.3.8 Scanning electron microscopic study

The scanning electron microscopy of putative probiotic isolates *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were carried out. The study revealed the shape, size and arrangement of bacterial cells (Fig. 2.A.13). The cell morphology of *L. sphaericus* PKA17 was found to be rod-shaped. The normal vegetative cells were slightly flattened in nature. In the time of cell division, each cell became elongated with a dumbbell like structure with narrow constriction at the middle. The daughter cells were round or elliptical in shape. Terminal spores were observed occasionally. During sporulation, the cell became tapering and pointed at the ends.

*B. cereu*s PKA18 cells were identified with typical rod-shaped form. The cross-septation was observed distinctly during binary fission. However, dumbbell like structures was absent here. The size and shape of the daughter cells remain same as the mother cell. In some cells, single tetrahedral terminal sporangia were found which became separated from the mother cell by cross septum.

B. thuringiensis PKA19 cells also appeared in typical rod-shape. However, the width of the cell was found to be considerably higher than of *B. cereu*s PKA18. The binary fission occurred through the formation of a prominent cross septum at the middle portion of the mother cell creating two uniform daughter cells. Sporulation involved asymmetric cell division and occurred at a terminal end. The spores were much smaller and when separated were found spherical in shape.

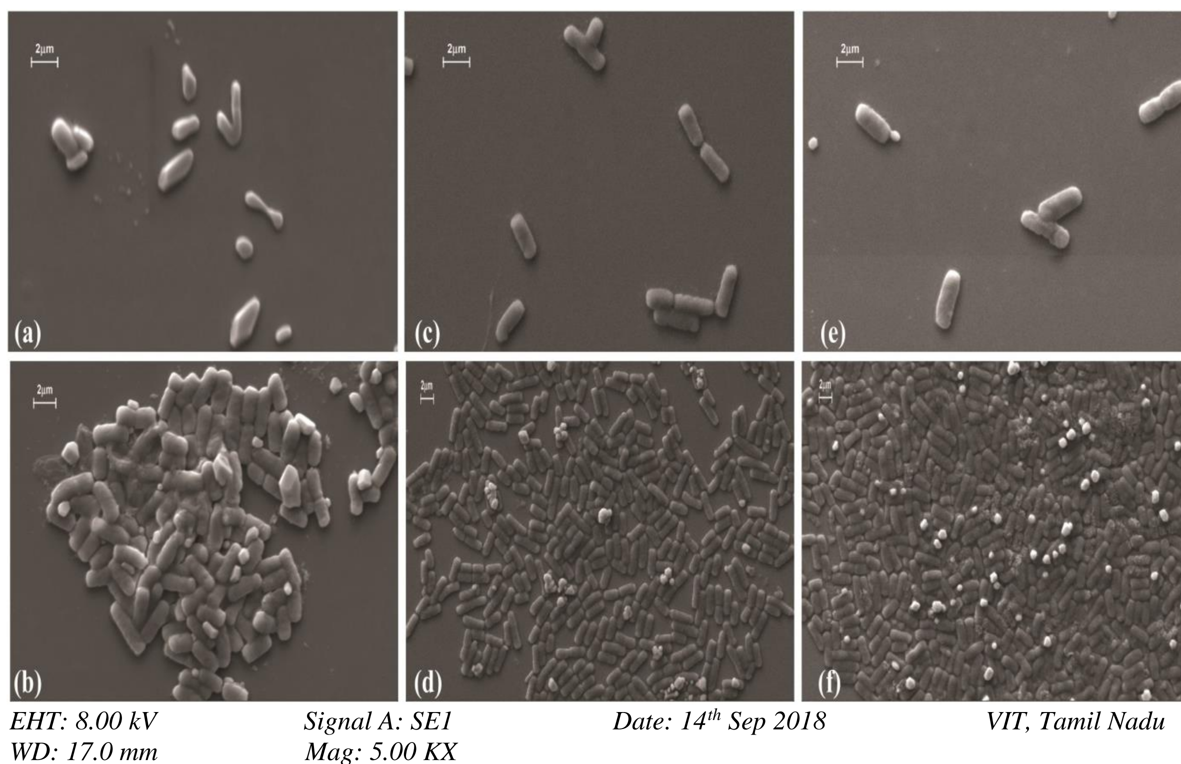

Fig. 2.A.13: Scanning electron micrograph: (a) Isolated form of *L. sphaericus* PKA17; (b) Colonial growth of *L. sphaericus* PKA17; (c) Isolated form of *B. cereus* PKA18; (d) Colonial growth of *B. cereus* PKA18; (e) Isolated form of *B. thuringiensis* PKA19; (f) Colonial growth of *B. thuringiensis* PKA19.

2.A.3.9 Antibiotic sensitivity

The antibiotic sensitivity test of putative probiotic isolates were conducted on tryptone soya agar against traditional antibiotics (Fig. 2.A.14). *L. sphaericus* PKA17 has shown maximum susceptibilities (Mean±S.E.) to streptomycin (2.07±0.03 cm), clindamycin (2.6±0.06 cm), ciprofloxacin (2.3±0.06 cm), chloramphenicol (1.8±0 cm), tetracycline (2.2±0.03 cm) and nalidixic acid (1.9±0 cm). *B. cereus* PKA18 was most sensitive to clindamycin (2.5±0.06 cm), vancomycin (1.6±0 cm), ciprofloxacin (2.2±0.03 cm) and nalidixic acid (1.9±0 cm). *B. thuringiensis* PKA19 showed maximum susceptibility to clindamycin (2.5±0.03 cm), chloramphenicol (1.8±0 cm) and nalidixic acid (1.9±0.03 cm). The antibiotics with different mode of action (inhibitors of the protein synthesis, cell wall synthesis and nucleic acid

synthesis) were sensitive to bacterial isolates. It has significant impact in aquaculture as antibiotic-resistant bacteria (ARB) often alter the nutritional landscape of the gut and expands pathogenic invasion (Bäumler and Sperandio 2016). As the test probiotics didn't exhibit toxicity or antibiotic-resistance property, these can be considered as candidate probiotic in aquaculture sector.

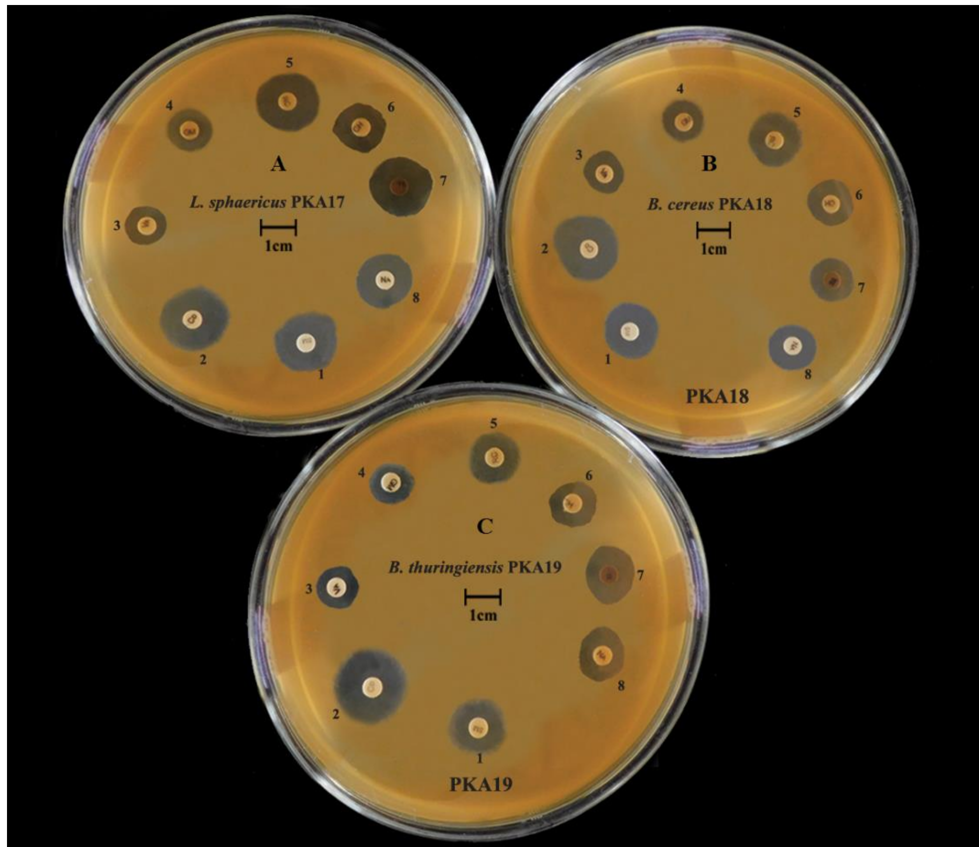

Fig. 2.A.14: Antibiotic susceptibility test of bacterial isolates: (A) *L. sphaericus* PKA17 (B) *B. cereus* PKA18 and (C) *B. thuringiensis* PKA19 against 1. Streptomycin (SM); 2. Clindamycin (CD); 3. Gentamycin (GM); 4. Chloramphenicol (CH); 5. Ciprofloxacin (RC); 6. Tetracycline (TE); 7. Vancomycin (VA); 8. Nalidixic acid (NA).

2.A.3.10 Cell surface hydrophobicity

The cell-surface hydrophobicity was measured to study the adhesive capacity of bacterial isolates to the intestinal mucus. The putative probiotic isolates exhibited high adherence towards non-polar solvent xylene (Table 2.A.4). The cell-surface hydrophobicity varied among the strains. *B. thuringiensis* PKA19 strain showed maximum cell-surface hydrophobicity (76.4%) which signifies its capacity of adherence to colonize the host intestine. Bacterial isolates *L. sphaericus* PKA17 (52.1%) and *B. cereus* PKA18 (46.3%) also have shown considerable amount of hydrophobicity. In the present study, all the test isolates exhibited high cell-surface hydrophobicity and ensured their capacity of adherence to the intestinal wall. Xu et al. (2009) observed high-cell surface hydrophobicity in probiotic isolates *Bifidobacterium longum* B6 and *Lactobacillus rhamnosus* GG. Balakrishna (2013) obtained high cell surface hydrophobicity in bacterial isolate MBTU_PB3 of *Poecilia reticulata*.

Table 2.A.4: Test of hydrophobicity of bacterial isolates showing high adherence towards xylene.

Bacterial isolates	A_0	A_f	Cell-surface hydrophobicity (%)
L. sphaericus PKA17	1	0.479	52.1
B. cereus PKA18	1	0.537	46.3
B. thuringiensis PKA19	1	0.236	76.4

2.A.3.11 Bile salt tolerance

Bile salt tolerance activity of the bacterial isolates was evaluated at different concentration of bile salts in TSB media. The bile salt (0.3%) exerted negligible inhibitory effect on the growth of bacterial isolates after 24 h of incubation (Table 2.A.5). *L. sphaericus* PKA17 strain tolerated bile salt (0.3%) more efficiently than other isolates. *B. cereus* PKA18 and *B. thuringiensis* PKA19 strains also have shown considerable growth and survivability at high

concentration of bile salt. Maximum growth of the isolates was observed at 0.037% bile salt concentration. The bacterial strains also have shown abundant growth on TSA after 24 h of incubation (Fig. 2.A.15). This bile salt tolerance property may subsequently enable the bacteria to survive and grow at intestinal tract of the host organism. Gilliland et al. (1984) stated that the tolerance to bile salts govern the colonization ability of bacteria at the host intestinal tract. Shehata et al. (2016) obtained high bile salt tolerance activity of lactic acid bacteria BO52.

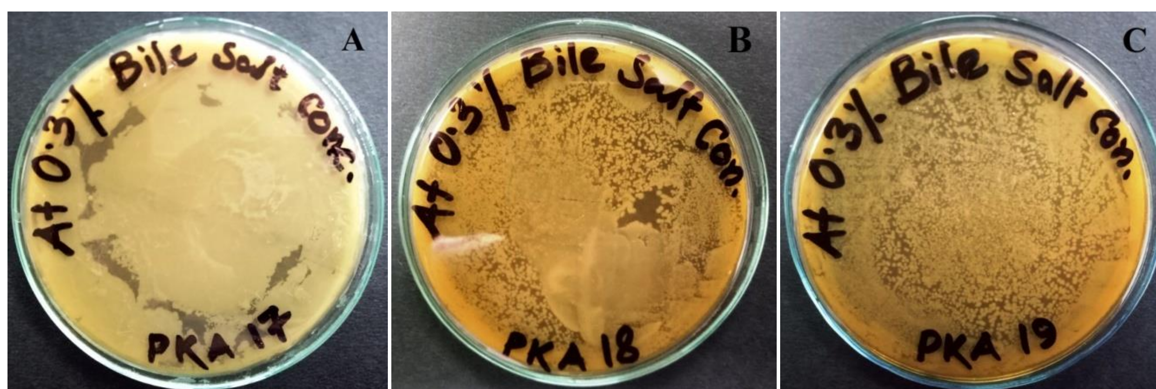

Fig. 2.A.15: Bile salt tolerance activity of (A) *L. sphaericus* PKA17; (B) *B. cereus* PKA18; (C) *B. thuringiensis* PKA19 at 0.3% bile salt concentration in TSB media after overnight incubation.

Table 2.A.5: Survival of test probiotics in tryptone soya broth supplemented with different concentrations of bile salts.

Isolates	0%	0.037%	0.075%	0.15%	0.3%
L. sphaericus PKA17	0.60	0.67	0.62	0.52	0.21
B. cereus PKA18	0.56	0.69	0.54	0.44	0.18
B. thuringiensis PKA19	0.52	0.58	0.50	0.31	0.15

2.A.3.12 Bile salt hydrolysis

Bile salt hydrolase (bsh) is a bile salt- or acid- hydrolysable enzyme synthesized by different autochthonous gastrointestinal microbiota to survive and colonize the intestinal lumen of the

host. The test isolates *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were capable of hydrolyzing bile salt (Fig. 2.A.16). The taurine-conjugated bile acid deconjugated to liberate deoxycholic acid which diffuses into the surrounding medium around vibrant colonies. The ability of probiotic strain to detoxify bile salt through production of bsh enzyme has often been considered as essential criteria for probiotic strain selection (Noriega et al. 2006).

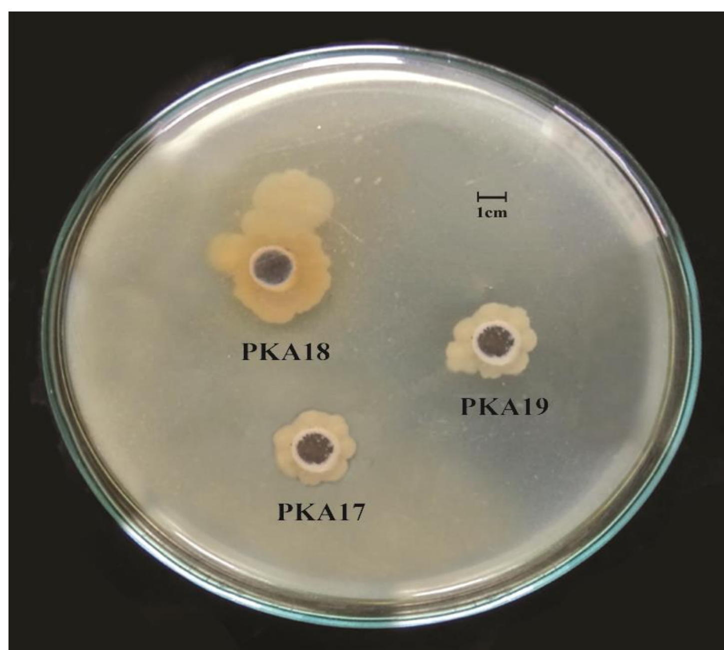

Fig. 2.A.16: The bile salt hydrolase (bsh) activity of bacterial isolates grown on bile salt-TSA medium as manifested by the formation of precipitation zone around the colony.

2.A.3.13 Haemolytic activity

The haemolytic activity of bacterial isolates *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were determined by inoculating the cultures on defibrinated sheep and fish blood agar. None of the test isolates have shown any transparent or greenish zone on blood agar plates, surrounding their colonies, irrespective of blood type (sheep, fish). The absence of haemolytic activity signifies the non-pathogenic nature of the bacterial isolates.

Similar type of observation was reported by Tallapragada et al. (2018) in *Lactobacillus fermentum* which showed γ-haemolysis on blood agar. Kavitha et al. (2018) studied the haemolytic nature of *Bacillus subtilis*, *B. cereus* and *B. amyloliquefaciens*; isolated from freshwater fish *Labeo calbasu*; and obtained similar kind of result. Haemolysis is an indicator of virulent nature of pathogenic organisms. The non-haemolytic activity is thus considered as a safety prerequisite for screening of probiotic organisms. The present study confirmed that the bacterial strains were non-haemolytic and hence can be considered as a probiotic-supplement.

2.A.3.14 Mutual compatibility

The bacterial isolates *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were selected to study their interactions among each other in tryptone soya agar medium. The growth pattern of the isolates after 24 h of incubation didn't show any antagonistic effect against other test bacteria (Fig. 2.A.17). It clearly indicated that, the three experimental bacteria have mutual compatibility and they can coexist in a microbiome.

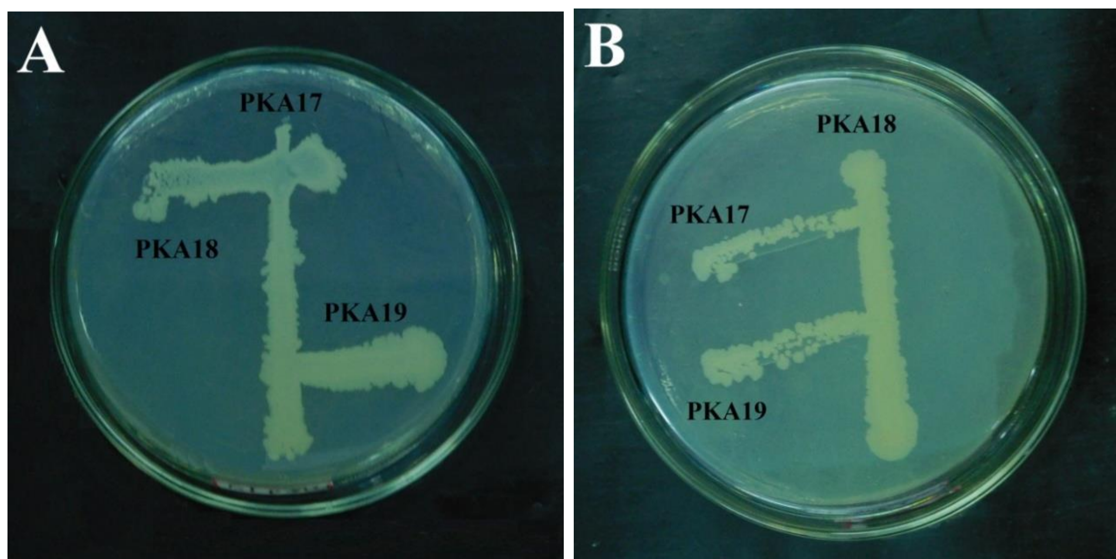

Fig. 2.A.17: The test of mutual compatibility: (A) PKA18 and PKA19 isolates were cross-streaked against PKA17; (B) PKA17 and PKA19 isolates were cross-streaked against PKA18 on tryptone soya agar media.

2A.4 Conclusion

The intestinal flora of *C. batrachus* were isolated and subsequently validated for probiotic properties. The total viable intestinal bacterial count was 1.6±2.9 × 10^{10} cfu/g. Thirty-two different autochthonous bacteria were isolated from the intestinal microflora of *C. batrachus* on the basis of distinct colony morphology. The Gram positive bacteria (84.38 %) dominated the population. Three intestinal isolates (PKA17, PKA18 and PKA19) have shown maximum antagonism against pathogenic *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus*. The biochemical characteristics and 16S rDNA sequencing identified the Gram positive rod-shaped motile strains (PKA17, PKA18 and PKA19) as *Lysinibacillus sphaericus*, *Bacillus cereus* and *Bacillus thuringiens*is respectively. The selected isolates were sensitive to common antibiotics. They have not shown any transparent or greenish zone on blood agar plates, surrounding their colonies and hence can be considered as non-pathogenic. They can also tolerate a high range of bile salt concentrations (0.3%) and exhibited significant cell surface hydrophobicity. The isolates *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were capable of hydrolyzing bile salt through the production of bsh enzyme. The ability of probiotic strains to tolerate and detoxify bile salt through the production of bsh enzyme has often been considered as the most important criteria to survive and grow at the intestinal tract of the host. Considering the importance of the bsh proteins to overcome the effect of bile salt for successful establishment in the gut environment the structural configuration of bsh protein was further predicted through *in silico* study.