Research Article

Synthesis of Antibacterial and Anticancer Substances by *Bacillus* sp. PRV3 and *Bacillus* sp. PRV23, an Intestinal Probiotics of Indian Fresh Water Fish

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ABSTRACT

Probiotics are "live microorganisms administered in adequate amounts that confer a beneficial health effect on the host." The host organisms and bacterial probiotic products are available for human and domestic animal consumption towards the stimulation of immune reactions. In this study, eight potent Probiotics were isolated from gut content of fishes namely *Hypselobarbus kolus*, *Channa murulius*, *Punitus melanampyx*, *Nemacheilus menoni* and *Oreochromis mossambicus* obtained from tribal's fish catchers of Periyar Lake, Kerala. Two probiotic bacteria, namely PRV3and PRV23 expressed maximum effect when allowed to undergo various tests namely acid tolerance, bile salt tolerance, hemolytic assay, auto-aggregation, antibacterial activity and antibiotic susceptibility tests. With this regard, the results by PRV3 and PRV23 strains and their bioactive compounds production was identified by FT-IR, HPTLC, HPLC and GC-MS. The antimicrobial and anticancer effects of bioactive compounds revealed that the bacteria derived compounds have maximum activity.

Keywords: Fish gut. Probiotics. Bioactive compounds. Anticancer activity. Anti-microbial activity.

INTRODUCTION

Aquatic animals in large-scale production facilities are exposed to stress, diseases and deterioration of the environmental conditions which lead to serious economic losses[1]. Among the numerous intestinal bacteria that beneficially affect the host intestine, some of them could be recognized as Probiotics[2]. During the last decade, chemical additives and veterinary medicines, especially antimicrobial agents were used to prevent and control diseases in aquaculture[3]. Bacterial infections are a major causal agent of mortality in fish hatcheries. Microorganisms are one of the most important authors of novel bioactive compounds such as antibiotics, immunosuppressant, antiparasitics, antitumor, hypocholesterolemic agents and enzyme inhibitors[4]. A promising alternative approach for controlling fish diseases is the economic consumption of probiotics or good bacteria, which control pathogens through a diversity of mechanisms. The function of probiotics, in human and animal nutrition[5] and has been applied to aquaculture[6]. *Bacillus subtilis* possess antitumor and immunomodulatory activities[7]. The production of antimicrobial substances by *Bacillus* species isolated from Japanese costal fish, and their use as a biocontrol agent [8]. There are a wide range of microalgae (*Tetraselmis*), yeast (*Debaryomyces*, *Phaffia* and *Saccharomyces*), gram positive (*Bacillus*, *Lactococcus*, *Micrococcus*, *Carnobacterium*, *Enterococcus*, *lactobacillus*, *Streptcoccus*, *Weissella*) and gram negative bacteria (*Aeromonas, Alteromonas, Photorhodo bacterium, Pseudomonas* and *Vibrio*). The antagonistic activity of *Pseudomonas* against a number of pathogens (e.g. *Aeromonas* and *Vibrio* sp.)[9]. Different probiotic effects addressed for *Bacillus* include production of antibiotics, providing protection against wide range of pathogenic bacteria [10]. Microbial-based therapy of cancer is one of the emerging cancer treatment modalities. Important advancements have been made to study and develop live bacteria or bacterial products such as proteins, enzymes, immunotoxins and secondary metabolites of bacteria and fungi which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrests or apoptosis induction[11]. Therefore, the aim of the present study is isolation and characterization of probiotic bacterial bioactive compounds and using them for biotherapeutic applications.

MATERIALS AND METHODS

Sample collection and screening of bacteria from fish gut

Live fish samples were collected from the Periyar lake, Kerala, South India. The collected samples were transferred to sterilized polyethylene bags containing habitat water. Among the selected fishes *Hypselo barbuskolus* (Koora), *Oreochromis mossambicus* (Tilapia) and *Punitus melanampyx* (Kudukonda) are carnivorous, while *Channa murulius* (Cherumeen) and *Nemacheilus menoni* (Ayira) are herbivorous. The samples were washed with sterile distilled water to remove any undesired dusty materials. Then, the supernatant was taken and serially diluted with sterile distilled water. The colonies were separated using quadrant streaking method.

The isolated microorganisms were characterized by physiological and biochemical tests such as Gram reaction, catalase test, oxidase test, Simmons citrate test, Indole test, amylase test and carbohydrate fermentation

test according to the criteria of Bergey's Manual of Systemic Bacteriology [12]

DNA isolation and 16S rDNA Sequencing

Genomic DNA was isolated by using the HIPURA Genomic DNA purification Kit. Later, bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal bacterial 16S rDNA primers, forward primer- (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer- (5' GGTTACCTTGTTACGACTT -3') (Lane et al.1991). PCR was performed on a 50-µl reaction mixture containing 1 µl (10 ng) of template, 0.5µg of each primer, 1.5 mM $MgCl_2$, and 50mM dNTP (deoxynucleoside triphosphate), 1U of Taq-polymerase and buffers as recommended by the manufacturer (Fermentas, Hanover, Germany) with the cycling parameters typically being 94°C for 60 sec, 55°C for 1min, and 72°C for 2 min (35 cycles) using Cyber-Lab® PCR system. PCR products were examined by electrophoresis in 1.5% (w/v) Agarose gel and sequenced. This sequence was then used for BLAST analysis. The 16S rDNA sequences were used for phylogenetic analysis using neighbor-joining method in MEGA7.0 [13].

Screening of Probiotic properties

Acid and bile salt tolerance

Therefore, in this study acid tolerance property of the isolate was determined by following the procedure described by [14]. The isolate was grown in nutrient broth for 24 hours at 30˚C. The growth of bacteria was measured at 560 nm and the survival percentage of strain to different pH was calculated. The bile salt tolerance of the isolate was determined as per [15]. Nutrient broth (100 ml) supplemented with different concentrations of bile salt (wt/vol. ox gall) was prepared and inoculated with one ml (3×10^7 cells ml^{-1}) of the isolate and incubated at 30 ˚C. After incubation the growth of bacteria was measured (A_{560} nm) at different time intervals and the survival percentage of the isolate was calculated.

Auto-aggregation assay

Auto-aggregation assay was performed as per the procedure described by [16]with certain modifications. Isolates were grown in nutrient broth for 24 h at 30 °C. The cells were pelleted, washed and dissolved in PBS (pH 7.0) to get an absorbance of 0.5 at 600 nm. The bacterial suspension was incubated at 30 °C and absorbance of upper suspension was measured at different time intervals (0, and 1 h). Auto aggregation percentage was expressed as: A0 - (At /A0) ×100. At represents the absorbance at different time intervals and A0 represents the absorbance at 0 h. Triplicates were maintained for all the experiments in this study unless otherwise represented.

Antibiotic susceptibility test

The susceptibility of isolate to different antibiotics was determined by placing standard antibiotic discs (Hi Media, Mumbai) on the surface of Muller Hinton agar medium seeded with a lawn of the isolate. Plates were observed for the zone of inhibition after 24 h incubation at 30ºC.

Preparation of crude cell free extracts

The crude cell free extract of each of the selected isolates were obtained by first growing them in separate sets of 50 ml of nutrient broth at 37 ± 2°C for 16 to 18 h culture, followed by centrifugation at 10,000 rpm for 15 min at 4°C. After centrifugation 2:1 ratio ethyl acetate was added to separate bioactive compounds and subsequent filtration of each supernatant through 0.2 µm membrane was performed under aseptic conditions.

Fourier trans form infra-red spectra

IR spectrum was recorded in spectrophotometer (Shimadzu), the active principle was mixed with KBr and pellet technique was adopted to record the spectra [17].

High performance liquid chromatography (HPLC)

The 500 µl of the cell free extract was injected into the loading site and was analyzed with high performance liquid chromatography. Analytical HPLC was carried out on a high performance chromatography system equipped with Clarity model CSW 32 software and alpha isocratic pump (Analytical instrumentation, India) and Gracesmart RP-18 5 µm column (250 mm x 4.6 mm). The mobile phase consisted of methanol: water in the ratio 6:4 with a flow rate of 1 ml min^{-1}. The sapphire detector monitored absorption at 254 nm. A blank solvent run was done prior to the sample run in order to remove erroneous readings.

High Performance Thin Layer Chromatography (HP-TLC)

Samples were vortexed for 1 min immediately following the addition of each solvent, and allowed to stand for about 1 h, with occasional shaking by hand. Phase separation of the biomass-solvent mixtures was achieved by adding Chloroform and Water to obtain a final ratio of Chloroform, Methanol, and Water as 1:1:0.9 by volume. The compounds extract recovered from the lower chloroform phase was dried using a rotary evaporator and finally dissolved in chloroform. Bioactive compounds, content in supernatant were first analyzed by thin layer chromatography (TLC) on precoated silica gel (20×20 cm, layer thickness 0.25 mm). The plates were developed by Hexanes-Ethyl acetate (6:1, v/v). The spots were visualized by iodine fumes. For the purification of Bioactive compounds, preparative TLC was performed on a precoated silica gel (20×20 cm, layer thickness 0.5 mm), and the plates were developed by the same solvents mentioned above. The target band from the plate was scraped, the compounds were extracted four times with Chloroform-Methanol (1:1, v/v), and the combined supernatants were brought to dryness using a rotary evaporator and finally re-dissolved in chloroform.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The bioactive compounds were extracted from the harvested bacterial culture by centrifugation at 6000 rpm

for 15 min in 4°C condition. The centrifuged cell supernatant was frozen overnight at -20 °C. Further the purification was done by chromatographic separation method and it was carried out with GC-MS-QP 2010 and the specifications of the column were: Db 30.0 column, the diameter 0.25 µm × 0.25 µm thickness. The oven temperature was programmed for the following conditions: from 70°C to 200°C with an increase of 10°C / min (isothermal for 5 minutes), in continuation the temperature 5°C / min to 280°C and ending with a 35 minute isothermal at 280°C. Mass spectra were measured at 70 eV; scan interval of 0.5 seconds, scan ranges from 40–1000 m / z. Helium was used as a carrier gas at 99.99 % and the pressure flow was maintained at 1.0 ml / min and thus the retention time, mass spectrum and the concentration of extracts were obtained.

Therapeutic screening of secondary metabolites

Antibacterial activity

The antibacterial activity of the Bioactive compounds were measured using agar disc diffusion assay against human pathogens such as *Klebsiella* (MTCC7407), *E.coli* (MTCC1303), *Serratia* (MTCC7103), *Proteus* (MTCC9493), *Vibrio cholerae* and the Fish pathogens such as *Vibrio harveyi, Vibrio parahaemolyticus* [18]

Cell lines

For the cytotoxicity studies the following cell lines were obtained from National Centre for Cell Science (NCCS), Pune. Human cervical cancer cell line (HeLa) , Breast cancer cell line (MCF-7) and Vero cell line (Normal) were cultured in Eagles Minimum Essential Medium containing 10% Fetal bovine serum (FBS). The cell lines were maintained at the following culture conditions: incubated at 37°C, supplemented with 5% CO_2 and 95% air and the relative humidity was 100%.

Cytotoxicity assay

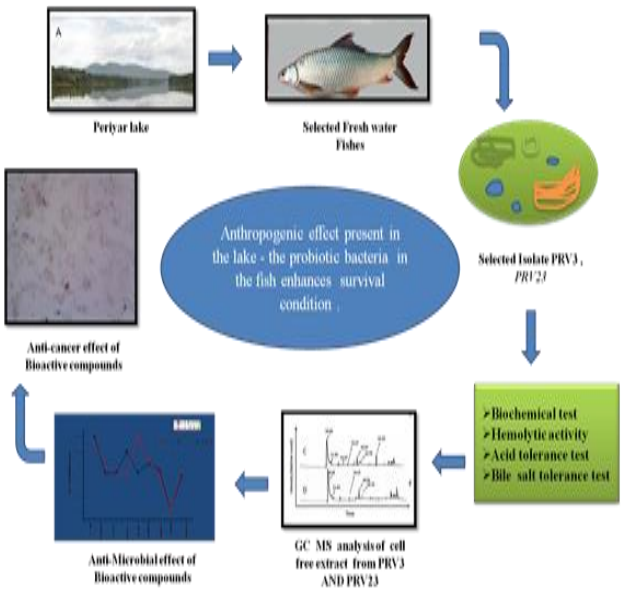
The morphological changes of the above mentioned cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) reduction assay method. The MTT added to the medium at the final concentration was 0.5 mg/ml and further incubated for 4h in a humidified atmosphere at 37 °C with 5% CO_2 The growth media was removed from the wells leaving formazone crystals at the bottom, and the crystals were further dissolved in 200 µl with dimethyl sulfaxide. The resulted absorbance was recorded at 570 nm immediately. Optical density (OD) values of each well were normalized against the control wells without treatment.

RESULTS AND DISCUSSION

Isolation and characterization of probiotic bacteria from fish gut

The water probiotics contain multiple bacterial strains like *Bacillus subtilis*, *B.licheniformis*, *Nitrobacter sp*., *Aerobacter sp*., and *Saccharomyces cerevisiae* while feed probiotics contain *Bacillus sp*., *Lactobacillus sp*. or *Saccharomyces cerevisiae*. These are reported to give better survival and growth and improve the protective response especially in the larval stages. In the present study, fresh water fish species namely *Hypselobarbuskolus* (Kooralfish), *Oreochromis mossambicus* (Tilapia), *Punitus melanampyx* (Kudukonda), *Channamurulius* (Cherumeen), *Nemacheilusmenoni* (Ayira), were collected 8 species are *Bacillus sp* one sp *Pseudomonas* sp sp and finally *Enterobacter.* In the present study, fresh water fish species namely *Hypselobarbuskolus* (Kooralfish), *Oreochromis mossambicus* (Tilapia), *Punitus melanampyx* (Kudukonda), *Channamurulius* (Cherumeen), *Nemacheilus menoni* (Ayira), were collected 6 species are *Bacillus sp* one sp *Pseudomonas* sp and finally *Enterobacter* sp. (Fig.1:Table S1).

Fig.1



Supplementary table S1: Morphological, biochemical and physiological characteristics of the isolates from Periyar lake

Among them, Eight bacterial strains were considered as probiotic based on their hemolytic, acid and bile tolerance properties. Survival in extremely low pH is one of the major selection criteria for probiotic strains.

Our results were well accordance with the results of [19] who reported that the *lactobacillus casei* exhibited higher survival rate at higher pH value (2.0). This is due to fact that the percentage of viability increases while increasing the pH value [20]. The acid tolerance test suggest that freshwater ecosystem play an vital role for the bacterial cells to be able to adabt the stress condition. However, *Lac rhamnosus* strains isolated from Parmigiano Reggiano cheese were able to survive at bile salt concentration of 10,000, 15,000 and 20,000 ppm after 48 hour of incubation at 37°C [21].

Parameters	*Periyar lake*							
Isolates	*Bacillus sp*	*Bacillus sp*	*Bacillus sp*	*Pseudomonas fluorescence*	*Bacillus sp*	*Bacillus sp*	*Bacillus sp*	*Enterobacter sp*
Fish species	Cherumeen	Cherumeen	kudukonda	Kudukonda	Kooral	Ayira	Tilapia	Tilapia
Gram stain	G+	G+	G+	G–	G+	G+	G+	G–
Shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Motility	+	+	+	+	+	+	+	+
Catalase	–	–	–	–	–	–	–	–
MR test	+	+	+	+	+	+	+	+
Citrate utilization	+	–	–	+	+	+	–	–
Indole test	–	–	–	–	–	–	–	–
Mannitol	–	–	+	–	+	+	–	+
25°C	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Arabinose	–	–	–	–	–	–	–	–

Twenty two bacterial strains were isolated from fish gut. Initial studies revealed that all the isolated strains were gram positive and rod shaped (Fig.1A).

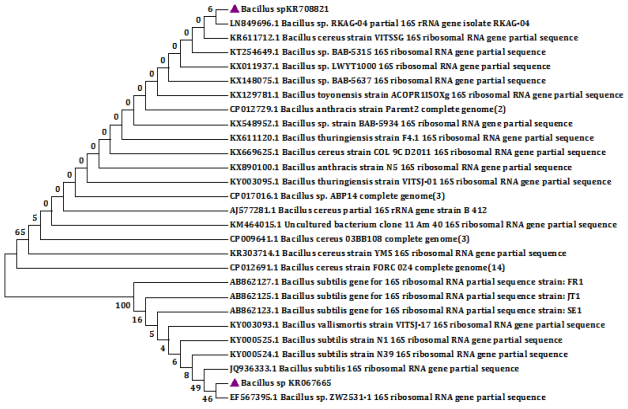


Figure 1: Graphical represention data of whole research work. A is indicating that construction of phlogentic tree in two *Bacillus* sp

The acid tolerance test suggests that freshwater ecosystem play an vital role for the bacterial cells to be able to adabt the stress condition. Bacterial isolates, while increasing the pH slightly up to 2.5, there was a slight increases in the optical density was noticed for most of the bacterial isolates. This disclosed that the growth of bacteria has slightly increased in accordance with slight increase in the pH further enhancement in the pH values from 2.5 to 3.5 and 4.5 resulted in significant increment in the optical density values irrespective of the all bacterial isolates which procured that bacterial growth was superior at higher pH values (3.5 and 4.5) over lower pH values (1.5 and 2.5).

The result of the acid tolerance test for the bacterial isolates fish sp collected from Periyar lake Although in the stomach, the pH level is as low as 1.5, in most *in vitro* assays pH 4.5 has been preferred. For selection, the strains resistant to low pH used medium buffered with PBS to corresponding pH. The probiotic bacterial strains were cultured on medium with varying pH (1.5, 2.5, 3.5 and 4.5) for three hours (median log phase) to study the

acid tolerance effect on their growth (Table -2A). Bacterial isolates, while increasing the pH slightly up to 2.5, there was a slight increases in the optical density was noticed for most of the bacterial isolates. This disclosed that the growth of bacteria has slightly increased in accordance with slight increase in the pH further enhancement in the pH values from 2.5 to 3.5 and 4.5 resulted in significant increment in the optical density values irrespective of the all bacterial isolates which procured that bacterial growth was superior at higher pH values (3.5 and 4.5) over lower pH values (1.5 and 2.5). The result of the acid tolerance test for the bacterial isolates fish sp collected from Periyar lake Bile salts are toxic for living cells because they disrupt the structure of cell membranes. Tolerance to bile is considered one of the essential properties required for probiotic bacteria to survive in the small intestine [22]. According to this study, Based on our observation the bacterial strains were subjected bile salt test to elucidate their growth tendency at different bile salt concentrations of 0.3%, 0.6%,1% and the observed results are furnished. At high bile salt concentration of 0.6%, 1% all the bacterial isolates showed lower optical density values because of high resistance induced by the higher concentration of bile salt for the bacterial growth.

Nevertheless the optical density values of bacterial isolates was considerably improved at lower bile salt concentration of 0.3% which could be inferred that lower concentration of bile salt favour the bacterial growth of all bacterial isolates. The result of bacterial isolates belongs to Periyar Lake. Bile salt concentration is important for growth of the intestinal bacteria. The suitable bile salt concentration is 0.3 w/v. In our study, the probiotic bacteria were made to grow at various concentrations (0.3%, 0.6% and 1%). And the bacterial growth was observed by using UV-Visible spectrophotometer at 600 NM.

The isolates were more in 0.3 % of bile salt, whereas growth at 0.6 % and 1.0 % bile salts were less (Table -2B). According to this study, Based on our observation the bacterial strains were subjected bile salt test to elucidate their growth tendency at different bile salt concentrations of 0.3%, 0.6%, 1% and the observed results are furnished. At high bile salt concentration of 0.6%, 1% all the bacterial isolates showed lower optical density values because of high resistance induced by the higher concentration of bile salt for the bacterial growth. Nevertheless the optical density values of bacterial isolates was considerably improved at lower bile salt concentration of 0.3% which could be inferred that lower concentration of bile salt favour the bacterial growth of all bacterial isolates. The results of bacterial isolates belong to Periyar lake.

The antibacterial activity induced by bacteria can be attributated to any of the following factors, either individually is combined such as antibiotics production, bacteriocin, siderophores, Lysozyme and proteases and pH alteration as a consequence of organic acid production. Nevertheless works carried out in the plant could not elucidate the prime factors responsible for the antibacterial activity of intestinal bacteria [23].

Among the probiotic bacterial strains the highest antibacterial activity was noticed for the bacillus prv4 against Serratia with zone of inhibition 30±2 mm. The next highest antibacterial activity was exhibited by prv 15±2 against the same pathogenic bacteria Serratia with zone of inhibition value of ± mm which was followed by spk1, prv4, prv20, prv21, prv23, mbs against various human pathogenic strains such as E.coli, Klebsiella, bacillus sp, proteus, Serratia, staphylococcus zone of inhibition of approximately 10mm the probiotic bacterial strains such as spk1, prv3, prv5, prv20, prv21 showed lowest antibacterial activity against few pathogenic bacterial strains with zone of inhibition approximately 5mm. When the antibacterial activity was conducted against fish pathogenic bacterial starains the highest antibacterial activity was observed for probiotic bacterial isolates V.harveii and V.parahemolytics against prv3 and prv23 with zone of inhibition 18±1 mm. The probiotic bacterial strains PRV20 Exhibited lowest antibacterial activity against *V.parahemolytics* with zone of inhibition of 06±1 mm respectively.

Overall results of the antibacterial study revealed that the antibacterial substance bacteriocins present in the freshwater fishes *Hypselobarbuskolus* (Kooralfish), *Oreochromis mossambicus* (Tilapia), *Punitus melanampyx* (Kudukonda), *Channamurulius* (Cherumeen), *Nemacheilus menoni* (Ayira), *Oreochromis mossambicus* (Tilapia) and *Labeo rohita* (Rogu) of Periyar lake and Vaigai river can be employed as biological agents towards controlling the bacterial population of both human and fish orgin.However it is necessary to purified and characterize the antibacterial substances in the future for exploring the substances responsible for antibacterial activity. In this respect of,8 probiotic bacterial isolates were collected from periyarlake. The probiotic bacterial strains are used against selected human and fish pathogenic strains. All the probiotic bacterial strains possessed zone of inhibition antagonistic effect against human pathogenic bacteria. (Fig. 2E&2F).

The present study revealed that the isolation of probiotic bacteria from fish samples of Periyar Lake and to assess their antibacterial activity against human and fish pathogenic bacteria. According to the morphological characterization of 8 isolates, 6 species were identified as *Bacillus* sp, one was identified as *Enterobacter* sp and the another being *Pseudomonas sp.* In this context, nine pathogenic bacteria (seven human pathogens, two fish pathogens) were tested and the results explained that probiotic bacteria inhibited the pathogens.

The ability of strains to auto aggregation seems to be an essential prerequisite for the adhesion of bacterial cells to intestinal epithelium, whereas their co aggregation abilities with pathogens enable forming the effective barrier that prevents colonization of epithelium by

harmful bacteria [24]. The culture PRV23 *Bacillus* sp showed the highest auto-aggregation activity (94.5%) and the culture Prv3 Bacillus sp had the lowest (76.6 %). These antibiotic resistant strains could create a risk to human health as there is a chance of horizontal gene transfer [25]. The auto aggregation test was performed to found out the growth of bacterial isolates in the absence of growth medium using pbs solution. The results of the aggregation tests for the fish 8 bacterial isolates from periyarlake. The higher result activity for auto aggregation test for Periyar lake isolates (PRV3 and PRV23) This could be attributed to the fact that the bacterial isolates of current concern highly dependent on nutrient supplementation. Collado et al. (2007) stated that aggregation is useful for preliminary screening to identify the potent probiotic strains suitable for food, human, or animal use. Thus, the higher aggregation property of the culture *Enterobacter* MBS1 represents its characteristic feature for interaction with pathogens, which is of importance from the point of view of both food preservation and the therapeutic impact of food on intestinal microbiota. The auto aggregation test was performed to found out the growth of bacterial isolates in the absence of growth medium using pbs solution. The results of the aggregation tests for the fish eight bacterial isolates from periyarlake . The higher result activity for auto aggregation test for Periyar Lake isolates (PRV3, PRV23,) this could be attributed to the fact that the bacterial isolates of current concern highly dependent on nutrient supplementation.

On the other hand, antibiotics inhibit or kill beneficial microorganisms and thus, disturb the microbiological balance of gut flora. Also, use of antibiotics has led to the immune suppression in animals and harmful effects on the environment and concerns on food safety. It is reported that fish ingest only 20–30% antibiotics applied in the farm and rest reach out to the environment. Besides, the antibiotics ingested by aquatic animals may be excreted as metabolites which may also harmful to the animal and human consumers [26]. In this respect of, 14 probiotic bacterial isolates were used against selected antibiotic susceptibility test. Among the probiotic bacterial strains the highest antibiotic activity was noticed for the bacillus (prv5,mbs1,k1,prv4 and prv 20) is the highest antibiotic susceptibility test against different type types of antibiotic namely penicillin, Cephalothin, tetracycline, Lincomycin and Amoxcillin with zone of inhibition.In this respect of, eight probiotic bacterial isolates were used against selected antibiotic susceptibility test. Among the probiotic bacterial strains the highest antibiotic activity was noticed for the bacillus (prv5, mbs1, k1, prv4 and prv 20) is the highest antibiotic susceptibility test against different type types of antibiotic namely penicillin, Cephalothin, tetracycline, Lincomycin and Amoxcillin with zone of inhibition. In the current study, identification of resistance and susceptibility properties in probiotic bacteria are used for further studies (Fig .2D).

Bioactive compounds were extracted from the two probiotic bacterial isolates that showed higher activity and are used for therapeutic applications.

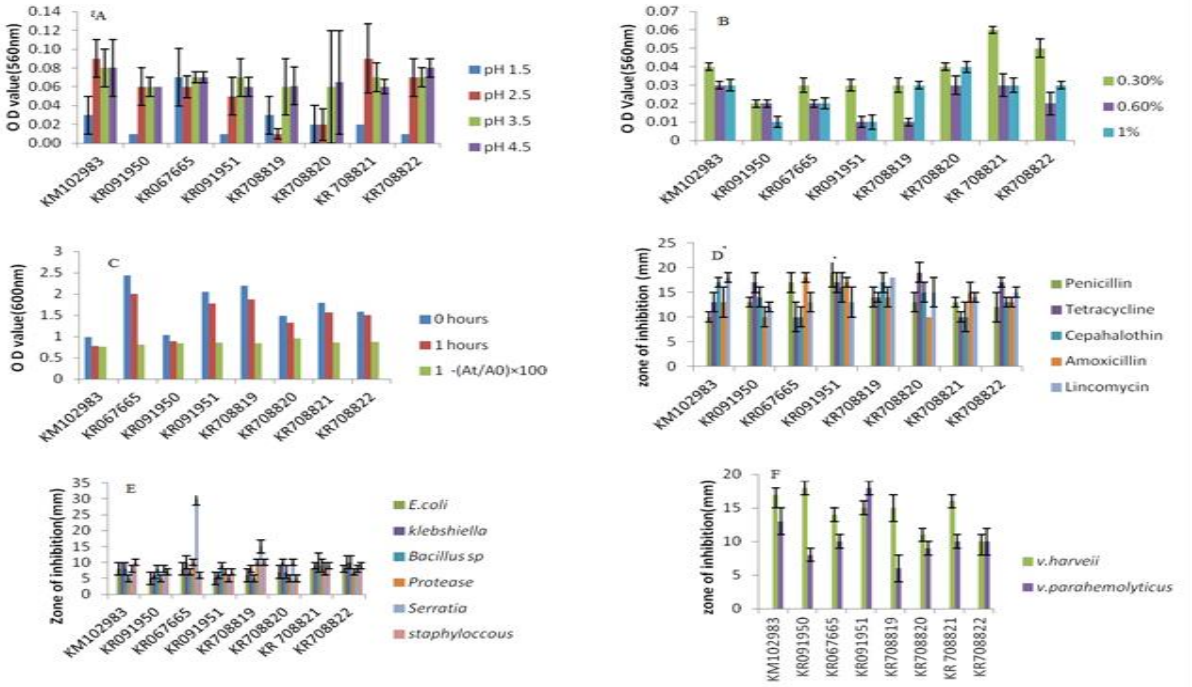


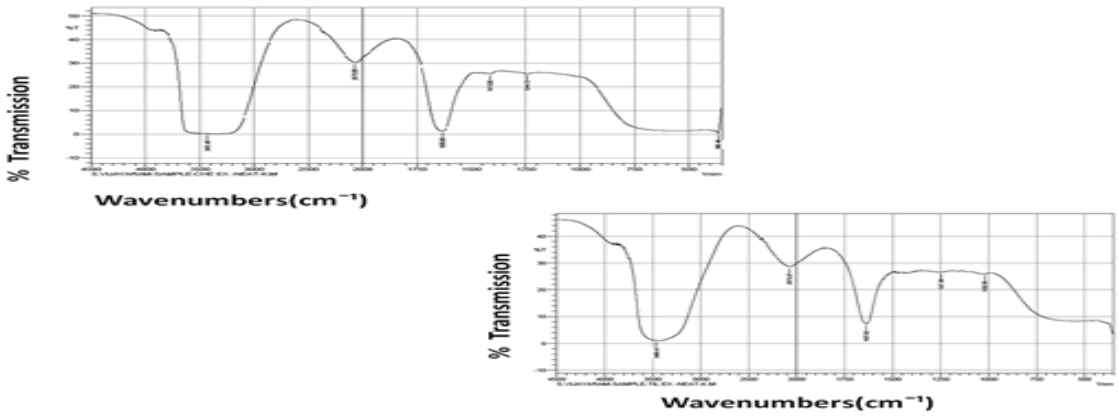
Figure 2: Probiotic characterization analysis of eight isolates from fish gut.A is acid tolerance test for probiotic bacteria. B is bile salt concentration tests for probiotic bacteria C is autoaggregation test for probiotic bacteria D is antibiotic susceptibility tests against probiotic bacteria E is antibacterial activity tests for clinical pathogens F is antibacterial activity tests for fish pathogens

Characterization of bioactive compounds from cell free extract

The FTIR spectral analysis showed the characteristic features of aliphatic compound with one or more C=C groups. The major peaks are at 1020.38 (C-H) stretch, 1247.99 (C=N) stretch, 1637.62 (C=C), 2075.47 (C=O) stretch and 3460.41 cm^{-1} that can be attributed to O-H stretch. Several earlier studies reported that some *Bacillus* strains could produce bacteriocins or bacteriocin-like substances to kill bacterial pathogens [27]. The results of FTIR analysis confirmed the presence of phenol, alkanes, aldehyde, Secondary alcohol, amino acid, aromatic amines and halogen compound. It is believed that crude extracts from bacteria are more biologically active than isolated compounds due to their synergistic effects.The FTIR spectral analysis showed the characteristic features of aliphatic compound with one or more C=C groups. The major peaks are at 1020.38 (C-H) stretch, 1247.99 (C=N) stretch, 1637.62 (C=C), 2075.47 (C=O) stretch and 3460.41 cm^{-1} that can be attributed to O-H stretch. (Table 2; Fig .S1).

Table 2: IR spectrum analysis from crude cell free extract Prv3 and Prv23.*

peak value	Assignment and Intensity	Functional groups	peak value	Assignment and Intensity	Functional groups
366.49	(C=O)Stretch	Carboxylic acid	1020.38	(C-H)stretch	Aliphatic amines
1244.13	(C=O)Stretch	Alkenes	1247.99	(C=N)stretch	Aliphatic amines
1415.8	(CH2 -CH3)	Alkyl halides	1637.62	(C=C) stretch	Amines
1635.9	(N-H)stretch	Amines	2075.47	(C=O)stretch	Alkenes
2073.55	(C=C)stretch	Nitriles	3460.41	(O-H)stretch	Alchol,phenols
3431.48	(O-H) stretch	Alchol,phenols			



Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity. HPLC results for PRV3, PRV23, SVSK2 and SVSK5 strains bioactive compounds. Totally ten peaks were observed in each bacterial cell free extracts and represents the Rt value of each peaks.Fig.3 shows HPLC results for PRV3 and PRV23 strains bioactive compounds. Totally ten peaks were observed in each bacterial cell free extracts and table 2 represents the Rt value of each peaks.

Natural products are a rich source of valuable medicinal agents. More than half of the currently available drugs are natural or related compounds [29]. 3,5-Dihydroxy-4-isopropylstilbene (DHPS) (also named 2-isopropyl-5-(2-phenylethenyl)-benzene-1,3-diol) belongs to the stilbene family and was first identified as a bacterial metabolite of the antimicrobial compound [30]. In addition HPTLC was used for the assessment of the effectiveness of the fractionation step shows Rf values and color of all fractions of PRV3 and PRV23 samples. Nevertheless, it is evident that the secondary metabolites of PRV3 and PRV23 may include some potent chemotherapeutic substance notably antibiotics mediated by free radical

scavenging effect, antioxidant effect and some potent anticancer principles that includes bioactive compounds (Hptlc Table 3).

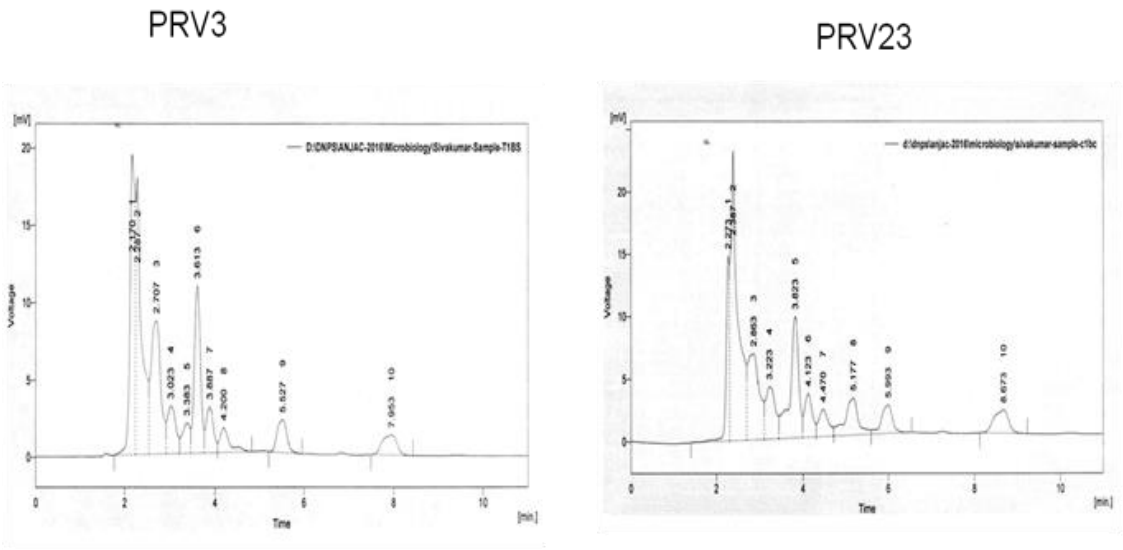


Figure 3: The selected probiotic bacterial isolates are used to carried out HPLC analysis. Two bacterial sp(PRV3 and PRV23) crude cell free extracts are using to identification of peaks, mass spectrum, retention time.

This compound is commonly used as a food additive due to its antioxidant activity [28]; however, it is reported to also have antimicrobial activity [29]. The mechanism of toxicity to microorganisms, of this and other hydrocarbons, was discussed in the comprehensive review by [28]. It is obvious that the active compounds extracted are phenolic compounds. However, coumarin, caffeic acid and some unknown compounds were also found in the samples of PRV3 and PRV23 Table 2.

Hptlc **Table 3:** Identification of bioactive compounds from crude cell free extract PRV3 and Prv23

PRV3	Bacillus sp KR067665
0.72	L-methionine hydrazide derivative
0.83	L-alanine derivatives
0.69	N-4chlorobenzylidine derivatives
0.44	Benzoic group
0.66	Gallic acid
0.79	Coumarin
0.81	Coumarin
PRV23	Bacillus sp KR708821
0.65	N-4\-chloro-benzylidine-glycine-hydrazonederivative
0.58	N-benzylidine-glycine derivative
0.81	thiazolidine-4-carbonyl L-alanine hydrazide derivative
0.79	caffeic acid
0.85	Coumarin
0.69	Gallic acid
0.86	Coumarin

The microbial extracts have served as a valuable source of diverse molecules in drug discovery efforts and let to the isolation of several important drugs[31]. Bioactive compounds overproduction was attributed to stress conditions such as alkaline pH, oxidative stress and cell wall stresses[32]. The biochemical structure of Bioactive compounds (Figure 2H). Because of a double bonded structure of six CH3 compounds the isoprenoid may have

strong antioxidant effect and thus they can act like a natural antibiotic [33]. It is to be noted that bioactive compounds have been proposed as a chemo preventive substance that protects from cancer [34] The identification of bioactive compounds was carried out by using GC MS analysis. The crude cell free extracts of selected strains from Periyar lake (PRV3, PRV23) sample some of the bioactive compounds are similarly found in the both field isolates (Neopentyl Glycol, Phenol, 2,4-Bis(1,1-Dimethylethyl, Methoxyacetic Acid, Hentriacontane, Phthalic Acid). (Table 4).

Table 4: GC MS analysis of crude cell free extract PRV3 and PRV 23.

S.No	Cds	M.Formula	Act	Ref	S.No	Cds	M.Formula	T.App	Ref
1	Propanoic Acid, Ethyl Ester	$C_3H_6O_2$	Anti Diabetic Activity Anti Oxidant Antitubercular Activity Antimalarial Activity	Vytautas Mickevičius et al. 2013	1	N-Propyl Acetate	$C_5H_{10}O_2$	Probiotic fermentation	Aristide Guillaume Silapeux Kamda et al 2015
2	Neopentyl Glycol	$C_5H_{12}O_2$	Anti Microbial	S j jiang 2013	2	Neopentyl Glycol	$C_5H_{12}O_2$	Anti Microbial	S j jiang 2013
3	Hentriacontane	$C_{31}H_{64}$	Anti Cancer Activity Anti Inflammatory Activity Anti Oxidant Antimicrobial	Jeffery et al. 1983	3	Hexanoic Acid, 2-Methyl	$C_7H_{14}O_2$	Anti Oxidant Anti Microbial	Salah Ali Idan et al. 2015
4	Phenol, 2,4-Bis(1,1-Dimethylethyl	$C_{14}H_{22}O$	Anti Fungal Anti Microbial Anti Oxidant	Rangel-Sánchez 2014	4	Pentanoic Acid, 4-Methyl-	$C_6H_{12}O_2$	Anti Cancer Anti Microbial	Ming-Xia Songa et al *2015*
5	Methoxyacetic Acid, 3-Pentadecyl	$C_{18}H_{36}O_3$	Anti Bacterial	Vaithiyanathan et al 2015	5	Hexadecane	$C_{16}H_{34}$	Anti Alarial Anti Oxidant Anti Acterial	Zakaria et al. 2011
6	Sulfurous Acid, Hexyl Tetradecyl	H_2SO_3	Anti-Diabetics Anthelmintic Antibacterial Antifungal	Vadivel and Gopalakrishnan 2011	6	Phenol, 2,4-Bis(1,1-Dimethylethyl)	$C_{14}H_{22}O$	Anti Acterial Anti Oxidant Anti labetic	Rangel-Sánchez 2014
7	Heptacosane	$C_{27}H_{56}$	Anti Oxidant Anti Cancer Anti Microbial	Elshiekh et al. *2015*	7	Sulfurous Acid, 2-Propyl Tetrade	$C_{18}H_{38}O_3S$	Anti-Diabetics Anthelmintic Antibacterial Antifungal	Vadivel and Gopalakrishnan 2011
8	Methyl 3-(1-Pyrrolo)Thiophene -2-.	$C_{10}H_9NO_2S$	Anti Oxidant Anti Malarial Anti Tumour	Jarak et al. 2006	8	Heptadecane	$C_{17}H_{36}$	Antimicrobial Anti Oxidant	Varsha Jadhav et al. 2014

Nevertheless, it is evident that the secondary metabolites of PRV3 (KR067665) and PRV23 (KR708821) may include some potent chemotherapeutic substance notably antibiotics mediated by free radical scavenging effect, antioxidant effect and some potent anticancer principles that includes bioactive compounds . In order to identify their therapeutic potential we screened these metabolites for antimicrobial and anticancer effects in vitro. The results of the antimicrobial effect of the isolates against select human and fish pathogens show that majority of the isolates have inhibitory activity against

human pathogens such as *Escherichia coli*, *Klebshiell*a, *Proteus mirabilis*, *Serratiamarcescens*, *Staphylococcus aureus*, *Vibrio parahaemolyticus, Vibrio chlorae.* However, the two lead strains PRV3 (KR067665) and PRV23 (KR708821) that are under investigation showed significant inhibitory activity against human pathogen *Serratia marcescens* (Fig.4).

This study helps to identify the environmental stress on fish by accessing the probiotic metabolic alteration as an effective investigative strategy. Adaptation and resilience of probiotic communities to anthropogenic stresses have increased the bioactive compounds production. Further, our study showed promising results to exploit the isolated strains not only as commercial probiotics as supplements and food in aquaculture but also as a biochemical machinery to synthesize novel therapeutic compounds such as antibiotics and cancer therapeutic agents. We

used Vero, MCF7 and HeLa cell line (normal, breast cancer and cervical cancer cell lines respectively) to study the *in vitro* anticancer effect of bioactive compounds were isolated from PRV3 and PRV23 MTT cytotoxicity assay using secondary metabolites of *B. cereus* (KR067665) showed no effect on normal cell line (Vero), indicating safer for normal cells, however, MCF7 cells were significantly inhibited in dose dependent manner. HeLa cells were inhibited.

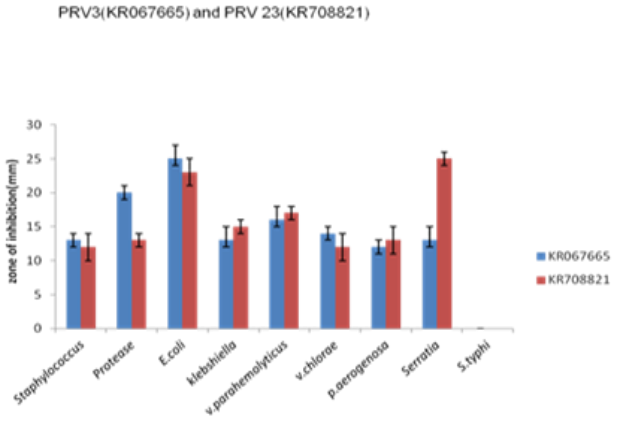
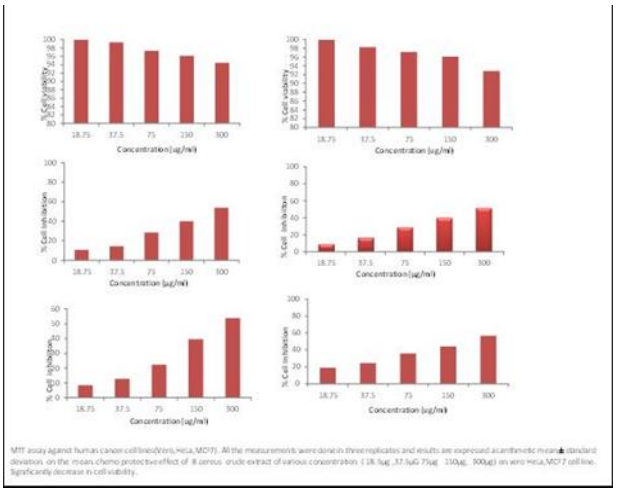
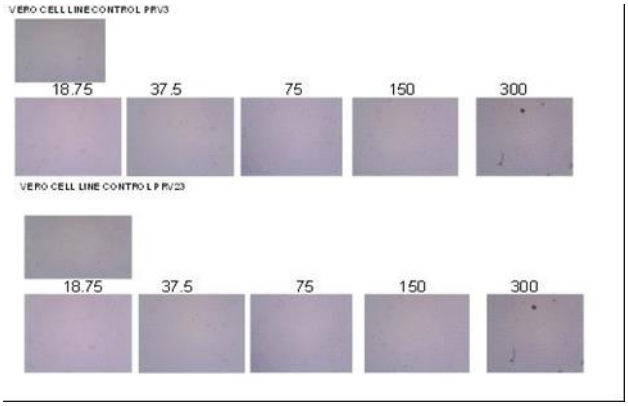


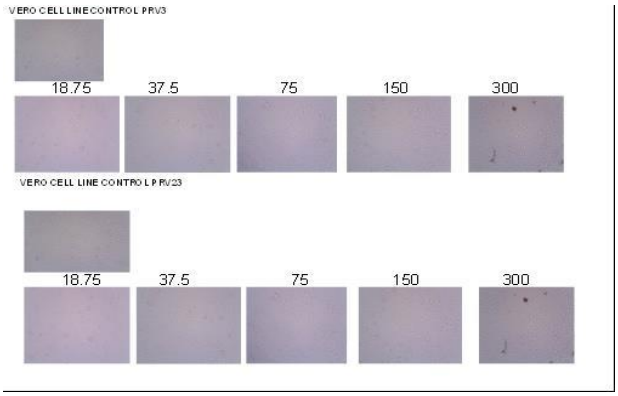
Figure 4: The selected bacterial crude cell free extracts are using against antibacterial activity.

The HeLa cells are highly inhibited to the bioactive compounds various concentrations are used to inhibit the HeLa (18.75 to 300µg/ml) and MCF7 cell line (18.75 to 300µg/ml). HeLa cell lines are the highly inhibited to the probiotic crude cell free extracts. In order to identify the anticancer activity the bacterial cell free extract was used against Vero, MCF7 and Hela cell line (normal, breast cancer and cervical cancer cell lines [35]The PRV3 and PRV23 strains cell free extracts were used for the identification of anticancer activity against Vero, MCF7 and Hela cell lines (general, breast cancer and cervical cancer cell lines). The cytotoxicity assay of bioactive compounds of PRV3 and PRV23 showed no harmful effects on normal cell line (Vero), thus indicating these compounds can be used for therapeutic purpose (Fig.5;S2). And the activity was analyzed by dose dependent manner. The IC_{50} value of PRV3 and PRV23 cell free extracts for MCF7 300 µg/ml and HeLa 200 µg/ml cells were respectively (Fig.5;S2). The cellular morphology of normal cells remain eloquent while the MCF7 300 µg/ml and HeLa 200 µg/ml cells showed reduced growth and disrupted cell wall indicating apoptotic like behavior for both PRV3 and PRV23 bioactive compounds. Thus the result of the present study reveals that the bacterial metabolites namely PRV3 and PRV23 act as potential compounds for bio-therapeutic treatment.

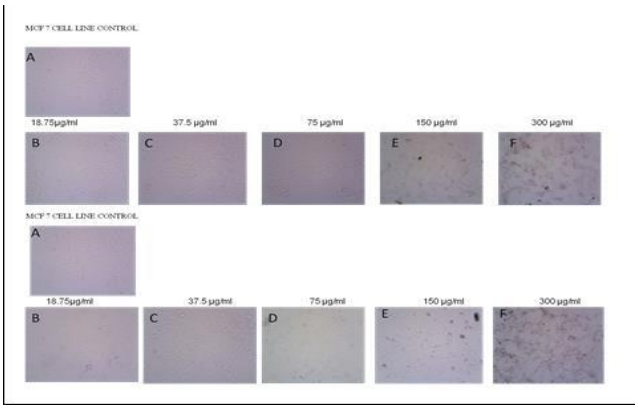




A



B



C

Figure 5: Two bacterial sp (PRV3 and PRV23) crude cell free extracts are using to treat anticancer activity.normal cell line no harmful effects of bioactive compounds.5A is the HeLa cell line IC_{50} values are measured concentration level is (18.75µg/ml to 300 µg/ml).5B is indicate that MCF 7 cell line IC_{50} values are measured concentration level is (18.75µg/ml to 300 µg/ml).The bioactive compounds are best activity for HeLa cell line

CONCLUSION

The use of probiotics in enhancing the gut microflora of human, animals and in fishes shows signs of becoming a success which infers that the isolated strains can be used as probiotics both in human and fishes after proper certification. The probiotic bacteria followed by characterization test such as Acid tolerance, Bile salt tolerance, Auto aggregation, Antibiotic susceptibility and Antimicrobial tests. The bioactive compounds of isolated probiotic organisms were used as the relative scale to correlate the stress experienced by the fishes through their environmental habitat and food chain. Our study showed promising results to exploit the isolated strains not only as commercial probiotics as supplements and food in aquaculture, but also as a source biochemical substances to synthesize novel therapeutic compounds such as antibiotics and cancer therapeutic agents. For therapeutic purposes, our study lays a rudimentary foundation and further characterization of metabolites and extensive *in vivo* studies may yield interesting results.

Ethical approval

In this study we have conducted only *in vitro* analysis and not involving to *in-vivo* study. Hence we are not getting the ethical statement.

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