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Seasonal Variation of Culturable Benthic Soil Prokaryotic Microbiota as Potential Fish Pathogens and Probiotics from an Aquaculture Farm in East Kolkata Wetlands, India

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Abstract

Rising demand in the aquaculture sector tends towards finding innovative ways to promote better yield and profitability. Benthic soil microbiota can provide an insight into the potent opportunistic fish pathogens as well as probiotics present in the aquaculture system. This study reports the seasonal diversity and abundance of fifteen culturable pathogenic bacterial strains belonging to the genera of *Comamonas, Aeromonas, Providencia, Klebsiella, Escherichia, Acinetobacter, Serratia, Stenotrophomonas, Staphylococcus*, and *Enterobacter* along with nine probiotic strains native to genera of *Bacillus* and *Pseudomonas* isolated from an aquaculture farm benthic soil, located in East Kolkata Wetlands, West Bengal, India. Strains are isolated using traditional microbial culture methods and tested for their antimicrobial susceptibility against commonly available antibiotics. 16S rDNA analysis was done for the identification of the strains and the establishment of their phylogenetic relationships. Among the isolates, *B. pumilus* strain S8 in the pre-monsoon sample, *E. coli* strain M2aR1 in the monsoon sample, and *A. hydrophila* strain P6dF1 in the post-monsoon sample were the most abundant having MPN counts of 275±21 x 10^6 CFU/gram dry soil, 278±18 x 10^6 CFU/gram dry soil, and 321±28 x 10^6 CFU/gram dry soil respectively. Data on the temporal diversity, abundance, and drug-susceptibility of prokaryotic fish-pathogens and probiotics can be used to formulate measures for sustainable aquaculture practices with reduced maintenance costs.

Keywords: Aquaculture, Fish pathogens, probiotics, 16S rDNA analysis, Antibiogram

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INTRODUCTION

Fish production in India has come a long way from being dependent on natural sources (rivers, lakes, and ponds) to well-contemplated aquaculture farms and earned huge success after the implementation of composite fish culture techniques, leading to species diversification and enhanced yield[1]. The blue revolution was brought in India by the incorporation of techniques like polyculture and induced fish breeding in static ponds or tanks[2]. India stands second in terms of farmed fish production globally, with more than 5.6 million tonnes of finfish (2015-2016) from inland aquaculture farms only[3]. Among the Indian states, Andhra Pradesh, and West Bengal are major producers in aquaculture farming[4].

The immense problem to these farms is contributed by the inert fish feeds, which affect the maintenance of farm[5], harm the environment[6], escalate the production cost[3], and raises fish prices in the market[7]. One of the solutions to this problem is microbial-based aquaculture systems, which may incur better sustenance of the farm with minimal input and reduced costs[6]. Prokaryotic microbiota of the soil and water plays various functions in fish farming, including valuable roles as probiotics, prebiotics, biomarkers, sentinels, and sometimes direct food for cultured fishes[8–10] along with detrimental ones like opportunistic pathogens[11–14]. Prokaryotic pathogens cause several infectious diseases resulting in loss of revenue for the fish farm by causing fish morbidity[15]. Unfortunately, these pathogens are detected only when an outbreak occurs, and sometimes it is too late to respond. Commercially available antibiotics are used regularly, creating drug-resistant bacterial strains[16]. Benthic soil microcosm can be utilized to act as a natural feed for the aquaculture farms and hence play a part in its sustenance[17]. To implement bioaugmentation techniques, a detailed exploration of the microbial food chain, pathogenic transmission, and presence of probiotics are needed for formulating steps leading to the betterment of yield[18].

East Kolkata Wetlands, in the eastern part of the Kolkata, is designated as a Ramsar site that supplies a major fraction of the fish demand of the city[19]. These inland freshwater aquaculture farms are one of a kind and serve two-fold purposes. Firstly these farms are mainly sewage fed, and act as natural sinks for waste recycling and attenuation of floods. Secondly, these water bodies are utilised for a thriving culture of several fish species[20]. The sewage fed aquaculture ponds, locally known as bheris, are unique in their operating procedure as they utilize municipal waste products as a fish feed with the occasional addition of inert feeds[21]. However, the seasonal dynamics of the physicochemical properties of water and benthic soil play crucial roles in the microbiome of the benthic soil of aquaculture farms[22,23]. Though several references are available on the description of the microbial communities of East Kolkata Wetlands, seasonal dynamics of the benthic soil prokaryotic microbiota emphasizing on the variation of probable fish pathogens and probiotics are scarce.

MATERIAL AND METHODS

Media and Chemicals

All chemicals and media were procured from Himedia (India) and Sigma Aldrich Chemical (USA). Tryptone Soya Agar (TSA) was used as enrichment media for the isolation of strains. Routine subculture and the permanent stocks were made in Nutrient agar (NA). Antibiotic Susceptibility test was done on Mueller Hinton Agar (MHA). Culture media were subjected to sterilization at 120°C temperature and 20 psi pressure for 15 minutes before inoculation.

Sample collection

Benthic soil samples were acquired aseptically from an aquaculture farm in East Kolkata Wetlands (Lat – 22.5699° Long- 88.4394°) from the top surface of the soil below 60cm of the water column. Samples of three seasons in the year 2019 viz. Pre-monsoon sample (April), Monsoon sample (August), and Post monsoon sample (December) were chosen for prokaryotic analyses in this study.

Isolation of bacterial strains

Isolation of culturable bacterial strains was done according to the method given by Vieira and Nahas 2005[24], with some minor alterations. 5gms (wet weight) of each soil sample was first diluted and homogenized in sterile water (50 ml) with intermittent cooling in an ice bath for 30 minutes. The homogenate was then passed through a sterile 2mm mesh. Filtrates were serially diluted up to 10^8 fold, and 100 µl from the last four

dilutions (10^5 to 10^8) of each filtrate were spread on TSA plates (in triplicates), which were then incubated at 37°C for 48 hours. A dry weight of soil was calculated by incubating 50 grams of benthic soil in 105°C for a period of 8 hours, and the final weight was used to calculate the wet weight to dry weight conversion factor[25].

Most Probable Number Count

Colonies that appeared on Each TSA plate after 48 hours of incubation, were marked based on colony morphology (shape, size, colour, transparency, margin contour, and surface topology) and were analysed for the Most Probable Number (MPN) count was done according to the method given by Janssen et al. 2002[26] with some minor alterations. Conversion factors of wet weight to dry weight of each soil sample and dilution factor of the inoculum were considered in the MPN count. The count was taken as a mean along with standard deviation for each dilution (from the triplicate count) and results were expressed in CFU x 10^6/gram dry soil. For the process of purification, colonies were transferred to NA plates and repeated subcultures were done until the strains were free from conglomeration. Each purified strain was stored permanently in NA stab cultures at 4°C and 80% glycerol stock at -20°C.

Biochemical characterization

A total of fourteen biochemical tests, including determination of Gram Character, Methyl Red (MR), tests for gelatinase, Triple Sugar Iron (TSI), degradation of starch, Voges- Proskauer (VP), indole production, citrate, oxidase, motility tests, etc. were performed with each purified strains and they were characterized following the methods of Bergey's Manual of Systematic Bacteriology[27].

Antibiogram

Antibiogram was prepared by testing all the isolated strains with a multitude of antibiotics by disk diffusion method[28] using commercially available disks with a total of thirteen antibiotics viz. A, Ampicillin (10 µg); Cf, Ciprofloxacin (5 µg); Co, Cotrimoxazole (25 µg); E, Erythromycin (15 µg), Fr, Furazolidone (100 µg); C, Chloramphenicol (30 µg); G, Gentamycin (30 µg); K, Kanamycin (30 µg), Of, Ofloxacin (5 µg), Na, Nalidixic acid (30 µg). S, Streptomycin (10 µg); T, Tetracycline (30 µg); VA, Vancomycin (30 µg) on MHA. Cell concentrations from overnight grown culture (in MHB) of each isolate were measured spectrophotometrically at 660 nm (Systronics 2202 Double Beam Spectrophotometer, Systronics, India) and inoculums were spread on sterile MHA ($1x10^9$ CFU/ml). Antibiotic discs were placed 15 cm apart on the spread plates followed by incubation at 37°C for 24 hours[29]. Strains were marked as susceptible, intermediate, or resistant by measurement of the diameter of the inhibitory zones to a particular antibiotic that was matched with the manufacturer's interpretive table as per the recommendations given by the National Committee for Clinical Laboratory Standards[30]. An ATCC strain (*Escherichia coli* ATCC 25922) was used as a quality control.

Molecular Characterization

Isolation of Genomic DNA and 16S rRNA gene amplification

Isolation of Genomic DNA from the cultured bacterial strains was done using a bacterial genomic DNA isolation kit (GCC Biotech, India). Genomic DNA was electrophoresed through 0.8% agarose gel and was measured spectrophotometrically at 260 nm wavelength to estimate purity. The samples were subjected to 16S rRNA gene amplification using a Gradient Thermal Cycler (Biorad Laboratories, USA). Universal primer 27f (5'- AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') encompassing V1-V9 hypervariable region of 16S rDNA were used for PCR amplification in conjunction with 2X PCR MasterMix (Thermo Fisher Scientific, India). The PCR amplification was carried out for 30 cycles and the amplicons (1.4 kbp approx.) were tested in 1.5% agarose gel using Ethidium Bromide stain. Purification of the amplicons was done using the Agarose Gel purification kit (NEB, USA) and sequencing was done from Xcelris Labs Ltd. (Ahmedabad, India).

In – silico analysis

Sequences were analysed in-silico using BLASTn (NCBI database), the hits were recorded for finding the nearest neighbour with the highest Max score. The aligned sequences were obtained in the FASTA format for downstream analyses in MEGA 7[31]. CLUSTALW algorithm was used for alignment analyses and the data thus obtained were considered for phylogenetic analyses using Neighbor-Joining method[32]. The evolutionary distance was calculated by Maximum Likelihood

Table 1. Biochemical Test results of the Isolates

Strain	TSI				Oxidase	Catalase	Gram Staining	KOH String Test	MR	VP	Gelatinase	Indole	Motility	DNase	MacKonkey	Urease	Citrate	Amylase
	Slant	Butt	H_2S	Gas														
S1a	R	R	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+
S2	Y	Y	-	-	-	+	-	-	+	-	+	+	-	+	W	-	+	+
S8	R	R	-	-	+	-	+	+	-	+	+	-	+	-	-	-	-	-
S8a1	Y	Y	-	-	+	-	+	+	+	+	-	-	-	-	-	-	+	-
SAC1	R	R	-	-	-	-	-	-	-	-	-	-	+	-	R	+	-	-
SWA6a	R	Y	-	-	+	-	+	+	-	+	+	-	+	-	-	+	-	-
MI0	R	R	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-
MI1	Y	Y	-	-	+	-	-	-	+	+	+	+	-	-	W	-	+	-
M2aR1	Y	Y	-	-	-	+	-	-	+	-	-	+	+	-	W	-	-	+
M2F1	Y	R	-	-	+	+	-	-	+	+	-	+	+	-	W	-	-	+
M3	Y	Y	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+
M5fR1	Y	Y	-	-	+	+	-	-	-	-	-	-	-	-	W	-	-	+
M5hR1	Y	Y	-	-	-	+	-	-	-	-	-	-	+	-	W	-	-	+
M6aR1	R	R	-	-	-	+	-	-	-	-	-	-	+	-	R	+	+	-
P1bR1	Y	Y	-	-	-	+	-	-	+	-	-	+	+	-	W	-	-	+
P2fR1	R	R	-	-	-	+	-	-	-	-	-	-	+	-	R	-	-	-
P4cR1	Y	Y	-	-	-	+	-	-	+	+	-	-	+	-	W	-	-	+
P5a	Y	R	-	-	+	-	+	+	-	+	+	+	+	+	-	+	-	-
P5aR1	Y	Y	-	+	-	+	-	-	-	+	-	-	-	-	W	+	+	+
P5bR1	Y	Y	-	-	-	+	-	-	-	-	-	-	+	-	W	-	-	+
P6b1	Y	Y	-	-	+	-	+	+	+	+	+	-	+	-	-	-	-	+
P6dF1	Y	Y	-	+	+	+	-	-	+	+	+	+	+	-	W	-	+	+
P7	R	R	-	-	-	-	-	-	-	-	-	-	+	-	R	+	-	-
P8d3a1	Y	Y	-	-	+	-	+	+	+	+	-	-	-	+	-	-	-	+

[+ = Positive result, - = Negative result, Y = Sugar Fermenters (Slant – Anaerobic/ Butt – Aerobic) R = Negative result (TSI and Mackonkey media)]

Composite method[33] and the branch lengths were calculated based on base substitution per site. Bootstrap tests of 500 replicates were done to construct the consensus tree to represent the evolutionary history of the taxa analysed[34]. Nearest four neighbours of each isolate from the BLASTn hits were used to create the phylogenetic tree. The partial 16S rDNA sequences of all strains were deposited into the GENBANK database.

Statistical analysis

Experiments on the enumeration of bacterial MPN were performed in triplicate and mean values were indicated along with standard deviation (SD). SPSS 17.0 (SPSS Inc., Chicago, USA) was used for the Statistical analyses.

RESULTS

Isolation of bacterial strains

In total, two hundred and forty-eight culturable bacterial strains were isolated and purified from the aquaculture benthic soil samples collected from three seasons. Molecular phylogenetic analyses revealed that several bacterial strains were common in samples from more than one season. Amongst the isolates, only twenty-four strains were revealed to have either presumptive pathogenic or probiotic values to fish and are reported in this communication. In the pre-monsoon season sample, a total of six strains (*Bacillus flexus* strain S1a, *Aeromonas punctata* strain S2, *Bacillus pumilus* strain S8, *Bacillus subtilis* strain S8a1, Comamonas aquatica strain SAC1 and *Bacillus cereus* strain SWA6a) were isolated. Cumulatively, eight strains were found in the monsoon season sample (*Bacillus thuringiensis* strain M10, *Aeromonas enteropelogenes* strain M11, *Escherichia coli* strain M2aR1, *Pseudomonas aeruginosa* strain M2F1, *Bacillus flexus* strain M3, *Acinetobacter junii* strain M5fR1, *Serratia marcescens* strain M5hR1 and *Stenotrophomonas maltophilia* strain M6aR1). Finally, from the post-monsoon season sample, another ten strains were isolated (*Escherichia coli* strain P1bR1, *Providencia vermicola* strain P2fR1, *Enterobacter cloacae* strain P4cR1, *Bacillus flexus* strain P5a, *Klebsiella pneumoniae* strain P5aR1, *Serratia marcescens* strain P5bR1, *Bacillus cereus* strain P6b1, *Aeromonas hydrophila* strain P6dF1, *Comamonas aquatica* strain P7, and *Staphylococcus aureus* strain P8d3a1). Isolate *B. flexus* strain S1a, *A. punctata* strain S2, *B. pumilus* strain S8, *B. subtilis* strain S8a1 were found in the samples from both the pre-monsoon and monsoon season whereas, *E. coli* strain M2aR1, *B. flexus* strain M3 and *S. marcescens* strain M5hR1 strains were found to occur in the monsoon and post-monsoon samples.

MPN count

The MPN counts of bacterial strains isolated from all three seasons are depicted in Figure 1. Isolate *B. pumilus* strain S8 in the pre-monsoon sample, isolate *E. coli* strain M2aR1 in the monsoon sample, and *A. hydrophila* strain P6dF1 in the post-monsoon sample were the most abundant having MPN counts of 275±21 x 10^6 CFU/gram dry soil, 278±18 x 10^6 CFU/gram dry soil, and 321±28 x 10^6 CFU/gram dry soil respectively. Strains were found to vary drastically in abundance in different seasons revealed by the difference in seasonal abundances of *B. flexus* strain S1a, *A. punctata* strain S2, *B. pumilus* strain S8, *B. subtilis* S8a1, *E. coli* strain M2aR1, *B. flexus* strain M3, and *S. marcescens* strain M5hR1. Coliform bacteria belonging to genus *Escherichia, Klebsiella, Serratia,* and *Enterobacter,* were found as the most abundant groups during the monsoon (25.87%), and the post-monsoon (45.92%) season samples. The values of the MPN count of all bacterial isolates collected from all seasons ranged from 113 to 321 x 10^6 CFU/gram dry soil.

Biochemical Characterization

The biochemical characteristics of all the isolates are compiled in Table 1. Among the isolates, nine were Gram positives and rest were Gram-negative bacteria. Fermentation of sugar as analysed by TSI tests revealed a varying degree of fermentation potentiality amongst the isolates, in both aerobic and anaerobic conditions. All the strains tested were DNase negative and were unable to produce H_2S in TSI media.

Antibiogram

The results of the antibiogram of the bacterial strains are given in Table 2 (in a color-coded format). Isolate *S. marcescens* strain M5hR1 was most resistant against six antibiotics followed by *P. vermicola* strain P2fR1, *B. subtilis* S8a1, *E. coli* strain M2aR1, which were resistant against five different antibiotics each. Thirteen strains showed suppressed growths in Erythromycin, Furazolidone, Kanamycin, Ofloxacin,

and Vancomycin of which four strains showed intermediate results in Erythromycin. Nearly all of the isolates were susceptible to Cotrimoxazole and Gentamycin except *B. pumilus* strain S8 and *B. subtilis* S8a1 respectively.

Molecular Characterization and phylogenetic analyses

BLASTn results revealed the identity of the isolates and taxonomic names were assigned based on the nearest neighbour of the NCBI database. Table 3 depicts the BLASTn analysis of the bacterial strains. Phylogenetic analysis of the fifteen pathogenic strains is given in Figure 2 and the optimal sum of the branch length was found to be 3.522. The phylogenetic relationship of the nine probiotic isolates is shown in Figure 3 with an optimal sum of branch length 1.478. Both the trees are drawn to scale and are shown as legend in the figure.

Probiotic and pathogen analyses

From several strains isolated from the enrichment cultures, post identification it was found that fifteen of the isolates (S2, SAC1, M5fR1, M11, M2aR1, M5hR1, M6aR1, P6dF1, P7, P1bR1, P5aR1, P8d3a1, P5bR1, P4cR1, and P2fR1) are opportunistic pathogens of Indian major carps, exotic carps, pangasiid catfishes and cichlid fishes cultivated in the farm., whereas nine isolates (S8, S8a1, S1a, SWA6a, M2F1, M10, M3, P6b1, and P5a) have possible probiotic values related to aquaculture.

With the help of online literature databases, a review was done highlighting the pathogenic effect of the isolates on fish species cultivated in the farm and also to ascertain the efficacy of the species isolated as probiotics. Data obtained from the literature survey is summarised for the pathogens and probiotics in Table 4 and

Table 2. Antibiogram results of twenty-four isolates from the soil sample against thirteen different Antibiotics. The table is colour coded according to the legend given below

Isolates	Antibiotics												
	A	C	Cf	Co	E	Fr	G	K	Of	Na	S	T	VA
M10	R	S	S	I	S	I	S	S	S	R	S	R	S
M11	R	S	S	S	I	S	S	R	S	S	S	S	S
M2aR1	R	S	S	R	S	S	S	S	R	S	R	S	R
M2F1	R	S	S	S	S	S	S	S	R	S	S	R	R
M3	S	S	S	S	S	S	S	S	S	R	R	S	S
M5fR1	R	S	R	R	S	R	S	S	S	S	S	R	R
M5hR1	R	S	S	S	R	R	S	S	S	S	S	S	R
M6aR1	R	S	S	S	R	S	S	I	S	S	I	R	R
P1bR1	R	S	S	S	I	R	S	S	S	S	S	S	R
P2fR1	R	S	R	R	S	S	S	S	S	S	S	R	R
P4cR1	R	S	S	R	S	S	S	S	S	S	S	R	R
P5a	R	S	S	R	S	S	S	S	I	S	S	R	R
P5aR1	R	S	R	S	R	S	S	S	S	R	S	S	S
P5bR1	R	S	S	S	R	S	S	S	I	S	S	S	R
P6b1	S	S	R	S	S	S	S	S	R	S	S	R	S
P6dF1	R	S	S	I	R	S	S	S	S	S	S	R	R
P7	R	S	S	S	I	S	S	R	S	S	S	S	S
P8d3a1	R	S	S	R	S	S	S	S	S	R	S	R	I
S1a	S	S	S	R	S	S	S	S	S	R	S	R	S
S2	R	S	S	S	R	S	S	I	S	S	S	R	R
S8	S	S	S	S	S	S	R	S	S	R	S	S	S
S8a1	R	R	S	R	S	S	S	S	I	R	S	R	S
SAC1	R	S	S	S	I	S	S	S	S	S	S	S	S
SWA6a	R	S	S	S	S	S	S	R	S	S	I	R	S

R – Resistant, I – Intermediate, S – Susceptible

[A – Ampicillin, C – Chloramphenicol, Cf – Ciprofloxacin, Co – Cotrimoxazole, E - Erythromycin, Fr – Furazolidone, G – Gentamycin, K – Kanamycin, Of - Ofloxacin, Na - Nalidixic acid, S – Streptomycin, T – Tetracycline, VA - Vancomycin.]

Table 5 respectively. Notably, among the isolates, three species are belonging to genus *Aeromonas* were found that cause Aeromoniasis, which is by far the worst affecting disease, causing high fish morbidity in aquaculture fish cultivation scenario and is advised by several citations[35–39]. However, it is also appealing that among the probiotics isolated, several of them are reported to impart resistance against the pathogens isolated from the same source as well as boosting the immunity of the cultured fish specimens[40–43].

DISCUSSION

This study reveals several pathogens and probiotic species present in the benthic soil of the aquaculture farm under study. The farm cultivates Indian and exotic major carps along with some catfishes and cichlid fishes and there is ample chance that any of these fish species may get affected by the pathogens revealed in this study. The presence of pathogens in a fish farm is never evaluated until or unless a disease breakout occurs with high fish morbidity[44]. Mass fish morbidity is reported caused by pathogens like *A. hydrophila*[10,35,45], *A punctata*[36], *K. pneumonia*[46,47] causing a huge loss in revenue. Bacterial isolates identified in this study revealed that the benthic soil sample collected from post-monsoon season harbors a huge number of opportunistic pathogens with *A. hydrophila* being the most abundant. It is also noteworthy that, in comparison with the other two seasons, diversity and abundance of the opportunistic pathogens are much higher in the post-monsoon season sample as the occurrence of new genera of pathogens viz. *Klebsiella, Enterobacter* and *Providencia* were noticed along with strains of *Escherichia* and *Serratia*. This high abundance of diverse opportunistic pathogens could be attributed to the mixture of urban sewage and rainwater runoff from adjacent areas during monsoon and post-monsoon seasons.

Table 3. The assigned taxonomic name and Max Score of the twenty-four bacterial strains along with GenBank accession numbers

Isolate	Assigned Taxonomic Name	Max Score	GenBank Accession Number
M10	*Bacillus thuringiensis*	1565	MT254902
M11	*Aeromonas enteropelogenes*	1790	MT254904
M2aR1	*Escherichia coli*	1724	MT279647
M2F1	*Pseudomonas aeruginosa*	1502	MT254901
M3	*Bacillus flexus*	1714	MT279648
M5fR1	*Acinetobacter junii*	1729	MT279649
M5hR1	*Serratia marcescens*	1744	MT279650
M6aR1	*Stenotrophomonas maltophilia*	1716	MT279675
P1bR1	*Escherichia coli*	1766	MT254905
P2fR1	*Providencia vermicola*	1775	MT256362
P4cR1	*Enterobacter cloacae*	1729	MT279676
P5a	*Bacillus flexus*	1838	MT279679
P5aR1	*Klebsiella pneumoniae*	1772	MT254946
P5bR1	*Serratia marcescens*	1759	MT279682
P6b1	*Bacillus cereus*	1692	MT279685
P6dF1	*Aeromonas hydrophila*	1186	MT254947
P7	*Comamonas aquatica*	1434	MT254973
P8d3a1	*Staphylococcus aureus*	933	MT279691
S1a	*Bacillus flexus*	1725	MT279688
S2	*Aeromonas punctata*	1555	MT254982
S8	*Bacillus pumilus*	1620	MT254983
S8a1	*Bacillus subtilis*	1482	MT254984
SAC1	*Comamonas aquatica*	1236	MK849930
SWA6a	*Bacillus cereus*	1555	MF554643

Table 4. List of bacterial strains isolated in this study that are earlier reported to cause pathogenesis among common fish species cultivated in freshwater aquaculture farms

Sl. No.	Isolate	Identified as	Pathogen of	Pathogenesis	Reference
1	M11	*Aeromonas enteropelogenes*	*L. rohita*	• Motile Aeromonad septicemia • Ulceration in gastrointestinal mucosa • Produce putative toxins that enhances the chance of other opportunistic infections	37,39
2	M2aR1, P1bR1	*Escherichia coli*	All major carps	• Found in intestinal tracts, gills muscle, and skin. • Indicator of water pollution • Transfers antibiotic resistance to several other fish pathogens in the intestinal milieu.	44,59
3	P2fR1	*Providencia vermicola*	*L. rohita*	• Ulceration and reddish colouration in abdominal surfaces, the base of the pectoral fins and also on the cephalic region	60
4	P5aR1	*Klebsiella pneumoniae*	*C. carpio, L. rohita*	• Haemorrhagic subcutaneous ulcer and red spots in the body	46,47
5	P6dF1	*Aeromonas hydrophila*	Wide variety of freshwater fishes including all Indian major carps and exotic carps.	• Motile Aeromonad septicemia • Tail/ fin rot • Epizootic Ulcerative Syndrome	35,38,39
6	P7, SAC1	*Comamonas aquatica*	*L. rohita*	• Tail fin rot.	61
7	S2	*Aeromonas punctata*	*C. carpio* *Labeo rohita*	• Haemorrhagic septicemia • Epizootic Ulcerative Syndrome	36
8	M5fR1	*Acinetobacter junii*	*L. rohita, Hypophthalmichthys molitrix*	• Emerging fish pathogen • Causes septicemia leading to death	62
9	M5hR1, P5br1	*Serratia marcescens*	Variety of fishes	• A probable pathogen of fishes causes high morbidity. • Opportunistic pathogen derived from highly polluted environment	63
10	M6aR1	*Stenotrophomonas maltophilia*	Catfishes	• Surface discoloration with hemorrhages. • Tail fin rot, inflammation of internal organs	64
11	P8d3a1	*Staphylococcus aureus*	*Hypophthalmichthys molitrix*	• Causes high morbidity due to severe eye infection and ultimately damaging the brain	65
12	P4cR1	*Enterobacter cloacae*	*Mugil cephalus*	• Causes high fish morbidity. Pathogenicity caused by unspecified cationic factors.	45,66

Table 5. List of bacterial strains isolated in this study that are earlier reported to act as probiotics among common fish species cultivated in freshwater aquaculture farms

Sl. No.	Isolate	Identified as	Pathogenesis	Reference
1	M10	*Bacillus thuringiensis*	• Maintenance of water quality. • Better conversion of organic matters by progressing the biogeochemical cycles. • Shows antimicrobial activity against several fish pathogens. • Possess immunostimulant activity against other pathogens • Immunity boosting activity when part of fish diet	9,51,54,67–69
2	M2F1	*Pseudomonas aeruginosa*	• Provide disease resistance and immunity against several pathogens • Can be used in conjunction with other bacterial pathogens with enhanced effect	43,70,71
3	S8	*Bacillus pumilus*	• Imparts resistance to *A. hydrophila* infections • Have a modulatory effect on the gut microbiota on dietary supplementation • It improves the growth rate and health of several fish species.	42,58,72,73
4	S8a1	*Bacillus subtilis*	• Enhances growth rate, boost immunity and impart disease resistant when used as a dietary supplement • Disease resistance against Vibriosis. • Produces a wide range of antibacterial substances and imparts resistance against Aeromoniasis.	10,74–76
5	S1a, M3, P5a	*Bacillus flexus*	• Imparts disease resistance against pathogens • Produce cellulolytic and lipolytic enzymes and contributes to biogeochemical cycles	41,77,78
6	SWA6a, P6b1	*Bacillus cereus*	• Imparts resistance to *A. hydrophila* infections in L. rohita. • Can be used as a dietary supplement is several types of aquaculture	10,79

Several antibiotic-resistant strains have been reported by scientists due to the indiscriminate antibiotic use in aquaculture farms[48,49] and also pathogens like *E. coli* can transmit the resistance to other pathogens possibly through horizontal gene transfer[44]. Since the study reveals the antibiotic susceptibility of the strains, indiscriminate use of antibiotics could be avoided and specific treatment measures can be implemented.

The prokaryotic probiotics present in soil and water also boost the immunity of culturable fishes[8,50–53] against several pathogens, and thus, steps could be formulated for the aid of the probiotics. These probiotic bacteria can also act as a dietary supplement for the fish specimens, mitigating the problem raised by inert fish feeds[43,54–56]. Probiotic species also engage in the continuance of biogeochemical cycles by several enzymes that help in the biodegradation

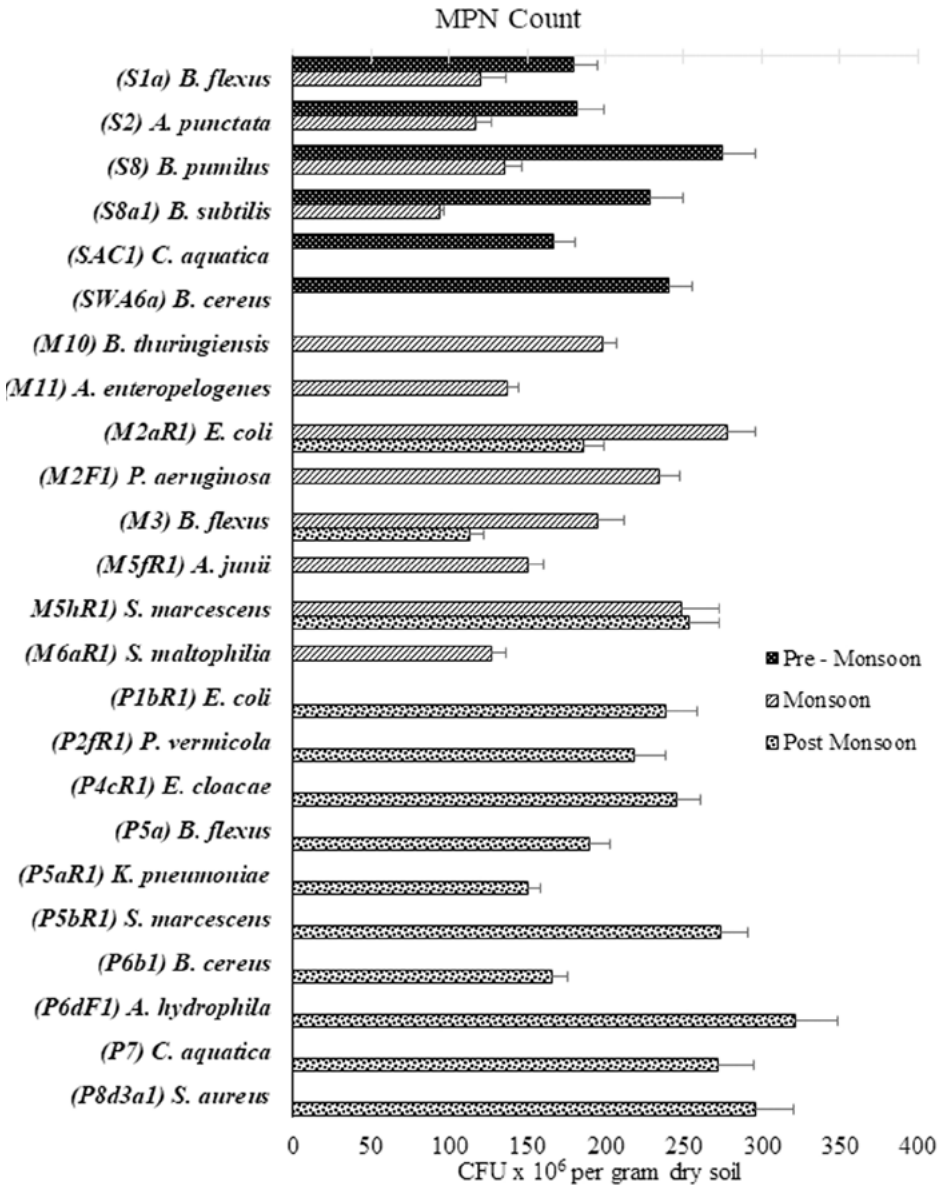

Fig. 1. Most Probable Number count of all the isolates in all three seasons

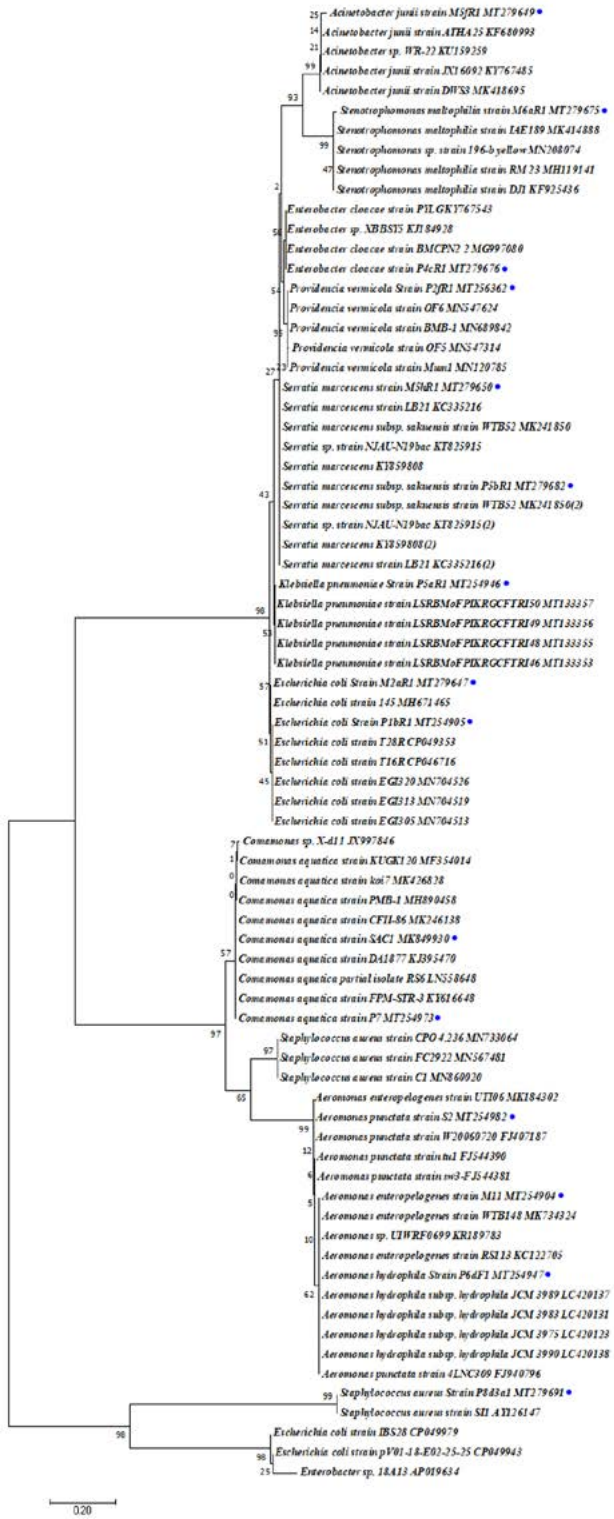

Fig. 2. Tree showing the phylogenetic relationship amongst the fish pathogenic strains. A total of 75 nucleotide sequences were used involving 180 positions in the final dataset

of detritus materials in the benthos[18]. Probable probiotic species isolated in this study produces enzymes like cellulase[57], lipase (unpublished data), and hence could be forerunners in the maintenance of the aquatic ecosystem. These probiotics are often consumed from the soil by

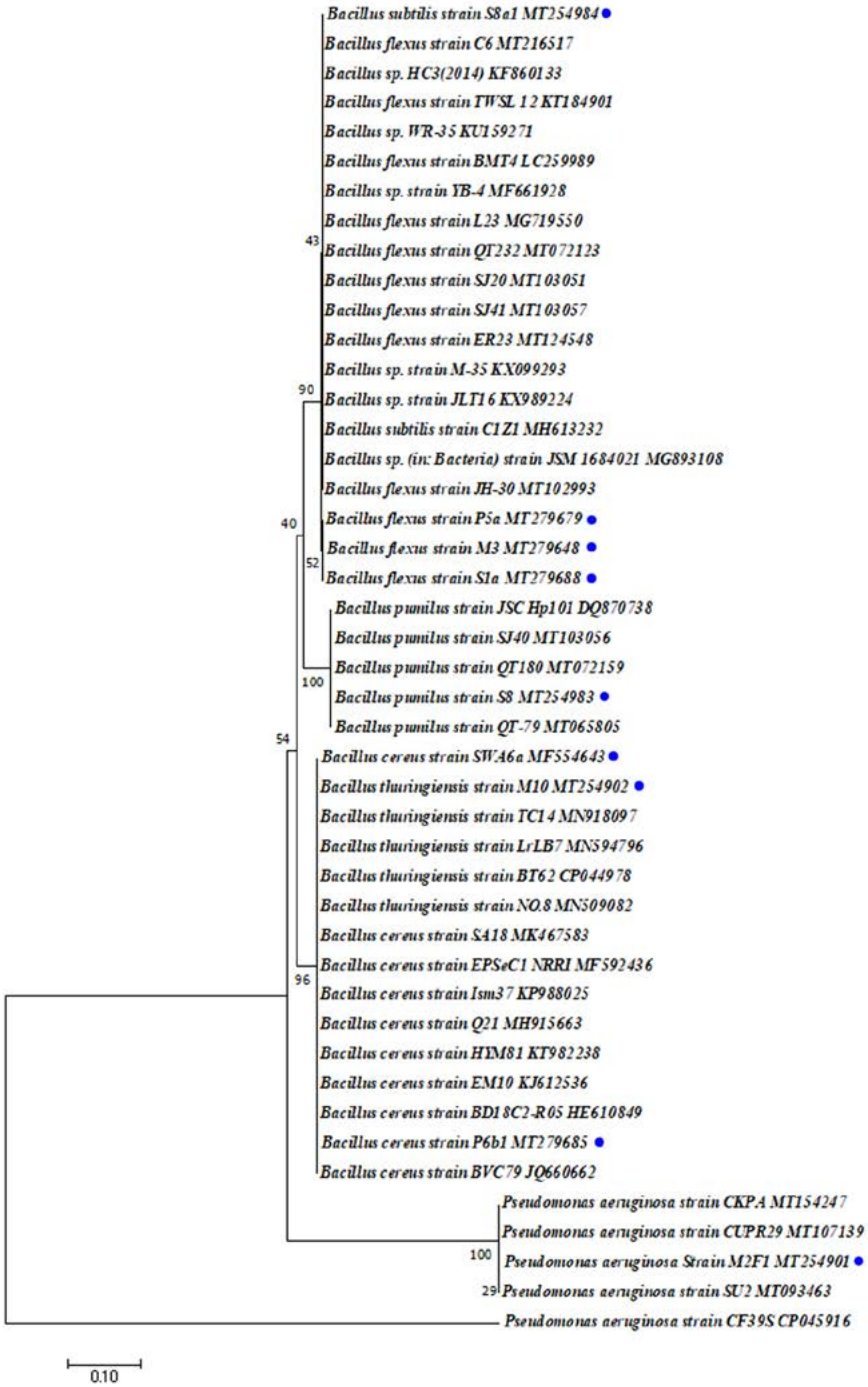

Fig. 3. Tree showing the phylogenetic relationship amongst the probiotic strains. A total of 45 nucleotide sequences were used involving 709 positions in the final dataset

bottom feeders thus circulating them along the food chain and could alter the gut microcosm of fish beneficially[58]. Further, it was revealed that the presence of probiotics is maximum is pre-monsoon season and declines rapidly from the monsoon season and thereafter. This is because the farm practices liming and the addition of organic fertilizers once annually in the pre-monsoon season leading to the amplification of probiotics. The monsoon rains bring a lot of agricultural and domestic runoffs as well as sewage overloads reducing the quality of water and soil, thus diminishing the number of probiotics and leading to the growth of pathogens. Coliforms are also found abundant in the soil during monsoon and post-monsoon season. This is probably the first report that provides a more in-depth insight into the culturable microcosm from an aquaculture farm of East Kolkata Wetlands, emphasizing their roles as fish pathogens and probiotics. This project could lead to early detection of pathogens and formulation of remedial measures even before the onset of the fish pathogenesis. This could be done by formulating a suitable bioaugmentation program to reinforce the growth of probiotics and eradication of the pathogens ensuring sustainable and profitable aquaculture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

The contribution of the authors are as follows: AD: Conceptualization, Methodology, Investigation, and Writing – Original Draft, SM: Conceptualization, Writing - Review & Editing, GCS: Supervision, NCS: Supervision and Project administration.

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DATA AVAILABILITY

The datasets generated are included in this manuscript and 16S rRNA gene sequences of the bacterial strains are available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers MT254902, MT254904, MT279647, MT254901, MT279648, MT279649, MT279650, MT279675, MT254905, MT256362, MT279676, MT279685, MT254946, MT279682, MT279679, MT254947, MT254973, MT279691, MT279688, MT254982, MT254983, MT254984, MK849930, MF554643.

ETHICS STATEMENT

Not applicable

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