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Screening of extracellular enzyme-producing and pathogen inhibitory gut bacteria as putative probiotics in mrigal, *Cirrhinus mrigala* (Hamilton, 1822)

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Abstract
The present study was intended to screen autochthonous bacteria as novel probionts in mrigal, *Cirrhinus mrigala.* Altogether 117 extracellular enzyme-producing bacteria were isolated from the proximal (PI) and distal (DI) regions of the gut, out of which 30 strains were primarily selected through qualitative and quantitative assay of extracellular enzyme activities (viz., amylase, protease, lipase, cellulase, phytase, xylanase). Further, study of antagonism against 7 potential fish pathogens revealed 13 strains to be antagonistic against ≥1 pathogen(s) by cross-streaking and double-layer method. The strains CMH1X and CM2H3L, isolated from the DI region were capable of producing six studied extracellular enzymes and antagonistic to 5 tested fish pathogens. Both the strains were competent to grow in fish gut mucus and could resist fish bile juice (8%). Fingerlings of *C. mrigala* were intraperitoneally injected with these strains separately and no mortality or external disease symptoms were noticed. Based on the results, the strains CMH1X and CM2H3L were selected as putative probiotics and identified as *Bacillus amyloliquefaciens* (KF623290) and *Bacillus sonorensis* (KF623291), respectively, through 16S rRNA partial gene sequence analysis. Although, the presently reported study depicts enzyme-producing capacity and antimicrobial potential of the gut bacteria in carps, *in vivo* studies are essential prior to their application in commercial aquaculture.

Keywords: Antagonism, autochthonous bacteria, *Bacillus*, extracellular enzymes, Indian major carp

1. Introduction
The use of beneficial microbes as probiotics has a long tradition in animal husbandry [1]. Since the last two decades probiotics are also being more frequently used in aquaculture. However, the use of commercial probiotics in fish is somewhat futile as most of the commercial formulations contain strains isolated from non-fish sources and might not remain viable at high cell density in the intestinal micro-environment of fish [2]. Hence, screening and selection of putative probiotics from the host species seems to be reasonable. Extensive research on the gut microbiota in marine and freshwater fish has confirmed that the gastrointestinal (GI) tract of fish is an abode of dense microbial population [3, 4, 5] and wide variety of enzymes produced by GI bacteria could be a contributing source of enzymes in fish [6]. However, screening and characterization of efficient probiotic isolates from tropical freshwater species is less studied and merits further exploration.

Preceding studies have suggested varied bacterial species from the GI tract of Indian major carps, exotic carps and other cultivable teleosts, and apparent beneficial functions of the gut microbiota pertaining to nutrition of the host fish have been emphasized [6]. On the other hand, some of the previous studies have attempted selection of probiotic bacteria based on *in vitro* antagonism against pathogens [7], or the adhesion and growth in fish intestinal mucus [8, 9]. To the author's knowledge, reports on the antibacterial efficiency of the extracellular enzyme producing gut bacteria isolated from the Indian major carps are scarce [10, 11]. Apart from the functional role that a putative probiotic bacterium might play, viability within the host gut is often believed to be one of the main selection criteria for prospective probiotics [12, 13]. Therefore, an appraisal of functional attributes together with reports on growth in fish mucus, resistance to fish bile juice and safety evaluation for the target fish species might be considered as an ideal scheme to screen novel probionts from the fish. The presently reported study primarily aimed as screening and validation of efficient putative probiotics from an Indian major carp, mrigal, *Cirrhinus mrigala*.

2. Materials and methods

2.1. Collection and processing of sample

Specimens of mrigal, *Cirrhinus mrigala* (Hamilton) were collected from three composite carp culture ponds located around Burdwan (23°24′N, 87°86′E), West Bengal, India. Along with natural feeding, the fish were fed a mixture of fish meal, rice bran and different oil cakes as supplementary feed. The pond was free from sewage discharge and other anthropogenic activities. The ranges of water quality parameters during the collection period were: temperature 26.1–27.5 °C, pH 7.1–7.5 and dissolved oxygen 5.8–6.9 mg L^{-1}. Three specimens were obtained from each collection pond, and thus altogether nine fish (average live weight 350±11.32 g) were collected, brought to the laboratory with oxygen packing and distributed separately over 3 aquaria of 75 L each on the basis of their source.

Prior to sacrifice, the fish were starved for 48 h in order to clear their GI tracts and anaesthetized by applying 0.03% tricaine methanesulfonate (MS-222). Ventral surfaces were sterilized using 70% ethanol and fish were dissected aseptically to remove the intestine [14]. The GI tract was divided into proximal (PI) and distal (DI) parts and processed according to [15] for isolation of autochthonous microorganisms. Gut segments from three specimens of *C. mrigala* were pooled together region-wise for each replicate, and thus there were three replicates for the study. Pooled samples of 3 fish were used for each replicate to avoid erroneous conclusions due to individual disparity in gut microbiota as described elsewhere [16].

2.2. Isolation of bacterial strains

Homogenates of the pooled intestinal segments of the two regions were serially (1:10) diluted [17] and each diluted sample (0.1 mL) was poured aseptically onto sterilized Soyabean Casein Digest Agar (Tryptone Soya Agar, TSA; HiMedia, Mumbai, India) media plates to determine the autochthonous culturable heterotrophic aerobic/facultative anaerobic bacterial population. For isolation and enumeration of different extracellular enzyme-producing bacteria (e.g., amylase, protease, lipase, cellulase, xylanase and phytase), the diluted gut homogenates were spread onto starch (ST), peptone-gelatin (PG), tributyrin (TB), carboxymethylcellulose (CMC), xylan (XY) and modified phytase screening (MPS) media plates, respectively, following enrichment culture technique. ST, PG, TB and CMC media were prepared following [18]. XY and MPS media were prepared following [19] and [20], respectively. The culture plates were aerobically incubated at 30 °C for 48h to count bacterial colonies following dilution plate count method and expressed as log viable counts g^{-1} GI tract (LVC) [15]. Number of colonies reported in the present study was an average of three replicates. The well-separated colonies were randomly collected, streaked individually on respective media plates and re-streaked repeatedly to acquire pure cultures. Pure cultures were maintained on slants in a refrigerator (4 °C) for further study.

2.3. Fish pathogens and culture maintenance

Four fish pathogenic strains *Aeromonas salmonicida* MTCC-1945 (AS), *Pseudomonas fluorescens* MTCC-103 (PF), *Pseudomonas putida* MTCC-1072 (PP) and *Bacillus mycoides* MTCC-7538 (BM) were acquired from the Microbial Type Culture Collection, Chandigarh, India. In addition, *Aeromonas hydrophila* (AH), *Aeromonas veronii* (AV) and *Pseudomonas* sp. (P) were isolated from diseased fish. The fishes were suffering from pale gills, bloated appearance, skin ulcerations and hemorrhage. Pathogenicity of the isolated strains was checked experimentally by intravenous injection to *C. mrigala* and by observing the onset of disease in the fish. The pathogenic strains were maintained in the laboratory on TSA (HiMedia, Mumbai, India) slants at 4 °C. Stock cultures in tryptone soya broth (TSB) were stored at -20 °C in 0.9% NaCl with 20% glycerol to provide stable inoculums throughout the study [21].

2.4. Evaluation of probiotic properties

2.4.1. Extracellular enzyme production: Qualitative and quantitative assay

Following growth on respective media plates, appearance of halo zone by flooding the plates with 1% Lugol's iodine or 15% $HgCl_2$ indicated amylase and protease activities, respectively [22]. Clear zone around colonies grown on TB and MPS media plates represented lipase [23] and phytase [20] activities, respectively. Cellulolytic and xylanolytic activities were determined on CMC [24] and [19] plates flooded with Congo red dye. There were three replicates for each experimental set. Qualitative extracellular enzyme activity observed by the appearance of halo (diameter in mm) around the colony was presented as scores [25] as follows; 0, nil (no halo); 1, low (6-10 mm halo); 2, moderate (11-20 mm halo); 3, good (21-30 mm halo); 4, high (31-39 mm halo); 5, very high (≥ 40 mm halo).

On the basis of the cumulative scores, efficient extracellular enzyme producing isolates were selected for quantitative assay. Respective selective broth media were used as production media for the enzymes. Quantitative assay for the production of amylase, cellulase, protease and lipase were performed following the methods described by [26, 27, 28, 29], respectively. A comprehensive description for measurement of these extracellular enzymes and quantitative enzyme assay has been mentioned elsewhere [18]. Quantitative assay of xylanase and phytase activities were measured after [30, 31], respectively. Protein content of the enzyme source was measured after [32] and specific activity of the respective enzymes was expressed as units (U).

2.4.2. Assay for pathogen inhibitory activity

The pathogen inhibitory activity of the promising enzyme-producing isolates were primarily tested against seven fish pathogens by 'co-culture' or 'cross streaking' method [33, 34] and the strains that showed antagonism against ≥1 studied fish pathogens were further evaluated by the 'double-layer' method [35] with minor modification. There were three replicates for each experimental set. A clear zone of inhibition (halo) around growth of the selected gut bacteria indicated antibacterial activity and the halo (diameter in mm) around the colony was presented as follows; +, low (6-10 mm); ++, moderate (11-20 mm); +++, high (21-25 mm); ++++, very high (≥ 26 mm).

2.4.3. Growth on fish mucus

Fish gut mucus was collected from live *C. mrigala* and thereafter processed following [36]. Growth on mucus was determined at 30 °C by counting the number of bacterial cells with a Petroff-Hausser counting chamber at 24 h, 48 h and 72 h intervals. To determine viable bacterial cells in mucus, diluted (upto 10^{-5}) 24 h, 48 h and 72 h cultures were inoculated (0.1 mL) onto TSA plates, incubated at 30 °C for 24 h and colony-forming units (CFU mL^{-1}) were counted. Sterilized un-inoculated mucus was served as the control.

2.4.4. Bile tolerance
Bile tolerance of the selected gut bacteria was evaluated through determination of minimum inhibitory concentration (MIC). Crude bile juice (pH 5.6) was collected from dissected gall bladder in aseptic condition, filter sterilized through 0.8 µm and 0.22 µm pore size filter papers (HiMedia, Mumbai, India) and stored at -20 °C until use. Different concentrations of bile (5 - 100%, v/v) were used in the agar-wells to determine the lowest concentration (MIC) that completely inhibited macroscopic growth of the organisms.

2.4.5. Safety evaluation
Bio-safety evaluation of the two putative probionts was carried out through *in vivo* studies conducted in 75L glass aquaria using 45 healthy *C. mrigala* fingerlings (16 ± 1.27 g). The fish were acclimatized in the laboratory condition for 2 weeks and divided into three equal groups (two experimental and one control), each with three replicates. The candidate probionts were grown in TSB at 30 °C for 24 h, centrifuged (2800 × g for 15 min, at 4 °C) and cell pellets were suspended in sterile 0.9% saline. Each experimental fish received intra-peritoneal (IP) injection of 1.0 mL containing 10^9 cells mL^{-1} of a candidate probiotic bacterium. The fish in control group were injected with sterile 0.9% saline [37]. Fish were fed *Ad libitum* with a diet containing approximately 35% crude protein having fish meal as the chief protein source. All groups were kept under observation for 21 days and health status was checked every day for development of any disease symptom.

2.5. Identification of isolates by 16S rRNA gene sequence analysis
The most promising two putative probiotics were identified through 16S rRNA partial gene sequence analysis after isolation and PCR amplification following the methods described in [25]. The gene encoding 16S rRNA was amplified from the isolates by polymerase chain reaction (PCR) using universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). Sequenced data were edited using BioEdit Sequence Alignment Editor (Version 7.2.0), aligned and analyzed for finding the closest homolog using National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases. Sequences were deposited to the NCBI GenBank and accession numbers were obtained. Phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using MEGA 5.1Beta4 software following the Maximum Likelihood Method.

2.6. Statistical Analysis
Statistical analysis of the quantitative enzyme activity data was performed by the one-way analysis of variance (ANOVA), followed by Tukey's test according to [38] using SPSS Version 10 software [39].

3. Results
Enumeration of gut-bacteria revealed that autochthonous heterotrophic as well as protease, amylase, lipase, cellulase, xylanase and phytase producing bacterial populations were present in the proximal (PI) and distal (DI) segments of the GI tract in *C. mrigala* (Figure 1). Heterotrophic and diverse extracellular enzyme-producing bacterial populations were found to be predominantly high in the DI region. While considering extracellular enzyme-producing bacteria, occurrence of amylolytic bacteria was found to be the highest (LVC = 5.63) followed by cellulolytic bacteria (LVC = 5.34). While, xylan-degrading population was noticed to be the lowest (LVC = 3.87).

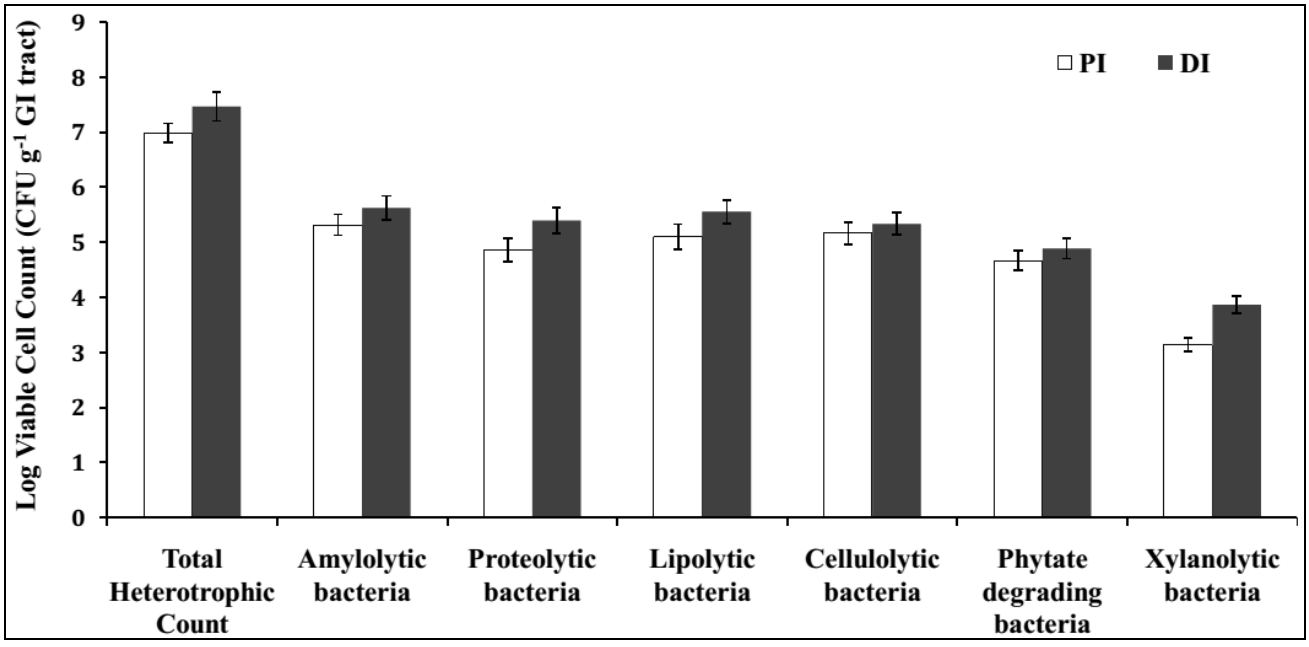

Fig 1: Log viable counts (LVC g^{-1} GI tract) of autochthonous bacteria isolated from the proximal intestine (PI) and distal intestine (DI) of *Cirrhinus mrigala.* Each column with error bar represents Mean ± Standard error (n=3).

Altogether 117 bacteria were isolated, out of which 30 extracellular enzyme producing strains (14 from PI and 16 from DI) were primarily selected through qualitative enzyme assay, maximum and minimum scores being 27 and 10, respectively (data not shown). Results of the quantitative enzyme assay revealed significant differences in the enzyme

Table 1: Specific activity of the enzymes (U) produced by the primarily selected bacteria isolated from the gut of *Cirrhinus mrigala.* Data are Means ± Standard error (n=3)

Strains	Amylase(U)[1]	Protease(U)[2]	Lipase(U)[3]	Cellulase(U)[4]	Phytase (U)[5]	Xylanase(U)[6]
Proximal Intestine						
CMF1A	161.32±4.31^{hi}	ND	4.21±0.23^{ab}	ND	201.37±5.62^{g}	4.91±0.22^{bc}
CMF2A	241.14±5.27^{lm}	31.84±1.14^{b}	5.38±0.26^{i}	58.89±2.15^{b}	ND	9.96±0.21^{l}
CMF1L	138.83±3.14^{g}	55.83±2.39^{f}	5.08±0.24^{h}	70.16±2.13^{d}	83.64±2.58^{c}	9.07±0.20^{k}
CMF1Ph	157.84±4.29^{h}	56.81±2.33^{f}	4.30±0.21^{bc}	73.52±2.64^{de}	89.73±2.59^{d}	7.39±0.21^{h}
CMF2Ph	163.51±4.84^{hi}	58.84±2.49^{fg}	4.29±0.23^{b}	71.41±2.73^{d}	309.81±6.03^{k}	7.36±0.28^{h}
CMF3Ph	131.06±3.64^{f}	35.67±1.54^{cd}	4.27±0.26^{b}	60.06±2.91^{b}	208.71±5.53^{gh}	7.35±0.25^{h}
CM2F1L	168.49±4.23^{ij}	71.88±2.19^{i}	4.47±0.27^{d}	55.26±2.17^{ab}	212.29±5.41^{h}	6.86±0.23^{de}
CM2F2L	160.37±4.47^{hi}	60.37±2.67^{g}	4.30±0.24^{bc}	58.36±2.57^{b}	95.67±2.59^{ef}	7.03±0.25^{ef}
CM2F1Ph	119.76±3.06^{d}	77.53±2.58^{j}	4.18±0.26^{a}	58.71±2.04^{b}	86.63±2.65^{cd}	6.66±0.26^{d}
CM3F3C	98.07±2.33^{c}	38.85±1.63^{e}	4.29±0.24^{b}	64.16±2.44^{c}	69.73±2.49^{b}	7.41±0.22^{hi}
CM3F2A	163.52±4.19^{hi}	29.88±1.41^{b}	4.29±0.21^{b}	61.27±2.27^{bc}	96.67±2.65^{f}	6.89±0.21^{de}
CM3F2X	89.94±2.31^{b}	18.03±1.06^{a}	4.27±0.22^{b}	51.17±2.46^{a}	93.26±2.09^{e}	10.65±0.74^{n}
CM3F2Ph	143.68±3.27^{h}	ND	ND	59.96±2.67^{b}	288.75±5.38^{i}	10.09±0.87^{lm}
CM3F3Ph	233.67±5.33^{l}	ND	ND	63.37±2.44^{bc}	206.53±5.48^{gh}	4.74±0.23^{ab}
Distal Intestine						
CMH1L	155.46±4.26^{h}	70.43±2.34^{i}	4.51±0.25^{e}	71.85±2.87^{d}	81.57±2.43^{c}	7.34±0.25^{h}
CMH2L	ND	54.85±2.56^{f}	4.19±0.26^{a}	64.96±2.56^{c}	ND	11.16±0.23^{op}
CMH1P	258.47±5.32^{mn}	33.68±1.41^{c}	4.32±0.22^{c}	57.43±2.41^{ab}	ND	11.53±0.23^{q}
CMH3P	165.05±4.14^{i}	71.99±2.38^{i}	4.21±0.21^{ab}	63.16±2.70^{bc}	66.53±2.61^{ab}	6.88±0.22^{de}
CMH1Ph	134.21±3.69^{fg}	34.59±1.64^{c}	4.30±0.24^{bc}	70.97±2.39^{d}	199.83±4.59^{g}	7.29±0.23^{h}
CMH1X	262.14±5.34^{o}	82.06±2.67^{jk}	5.60±0.26^{j}	72.28±2.67^{de}	362.41±6.76^{l}	10.89±0.46^{o}
CMH4X	123.63±3.11^{de}	56.83±2.64^{f}	5.07±0.22^{h}	72.09±2.51^{de}	93.17±2.26^{e}	10.03±0.47^{lm}
CMH5X	ND	31.70±1.02^{b}	4.35±0.26^{c}	71.14±2.49^{d}	91.41±2.23^{de}	7.36±0.24^{h}
CM2H2L	135.52±3.64^{fg}	57.46±2.69^{f}	4.31±0.29^{bc}	60.19±2.41^{b}	ND	4.97±0.28^{bc}
CM2H3L	320.39±6.83^{p}	72.82±2.35^{i}	4.58±0.26^{f}	66.34±2.28^{c}	370.56±6.94^{lm}	17.82±1.13^{r}
CM2H1P	176.53±4.41^{k}	80.24±2.39^{jk}	4.81±0.24^{g}	73.64±2.26^{de}	ND	6.85±0.24^{de}
CM2H2P	134.76±3.43^{fg}	54.86±2.47^{f}	4.28±0.28^{b}	56.69±2.37^{ab}	ND	7.11±0.25^{fg}
CM3H1A	250.37±5.37^{m}	19.75±1.53^{a}	4.23±0.20^{ab}	53.37±2.36^{a}	207.16±5.63^{gh}	7.08±0.24^{ef}
CM3H4C	100.37±3.03^{c}	65.53±2.06^{h}	4.25±0.25^{ab}	61.13±2.65^{bc}	64.49±2.36^{a}	4.88±0.23^{bc}
CM3H1X	81.19±2.64^{a}	16.59±1.11^{a}	4.29±0.22^{b}	ND	292.14±5.34^{ij}	4.56±0.22^{a}
CM3H2P	ND	73.35±2.37^{i}	ND	59.61±2.37^{b}	92.52±2.43^{de}	7.69±0.31^{j}

Values with the same superscripts in the same vertical column are not significantly different ($P < 0.05$).
[1] µg of maltose liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
[2] µg of tyrosine liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
[3] µg of free fatty acid liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
[4] µg of glucose liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
[5] µg of inorganic phosphate liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
[6] mg of D-xylose liberated ml^{-1}of enzyme extract mg^{-1} protein min^{-1}
ND= Not detected

activities between different bacterial isolates (Table 1). The highest amylolytic was noticed with the isolate CM2H3L (320.39±6.83U). The strain CMH1X exhibited maximum protease activity (82.06±2.67U). Both these strains were isolated from the DI region. Maximum lipase activity was recorded with the strain CMH1X (5.60±0.26U) isolated from the DI region. Maximum cellulase activity was documented with the strain CM2H1P (73.64±2.26U) isolated from the DI region. Maximum phytase (370.56±6.94U) and xylanase (17.82±1.13U) activities were noticed with the strain CM2H3L. Overall examination of the six different extracellular enzyme activities revealed that the strains CMH1X and CM2H3L (qualitative activity score being 26 and 27, respectively) were the most efficient among the 30 primarily selected bacterial strains.

To verify pathogen inhibitory activity, the primarily selected 30 extracellular enzyme-producing bacterial isolates were further screened against fish pathogens. Out of the 30 isolates, 13 strains (4 from PI and 9 from DI regions) were found to inhibit at least one of the tested fish pathogens through cross-streaking method. Pathogen inhibitory activity of these 14 isolates were further assessed by double layer method and the

zone of inhibition (halo) produced by the gut isolates were depicted in Table 2. In consequence of the maximum extracellular enzyme-producing capacities, the strains CMH1X and CM2H3L were also noticed to be antagonistic against 5 out of the 7 tested fish pathogens. Therefore, these promising strains (CMH1X and CM2H3L) were analyzed further for evaluation of other probiotic properties. Both the strains grew well in gut mucus of *C. mrigala* (Figure 2). As evident from the log total microscopic count (cells mL^{-1}) following incubation in the fish gut mucus, the putative probiotic strains exhibited maximum growth at 48h, after which growth started to decline. Log viable cell count (CFU mL^{-1}) also reached maximum when TSA plates were inoculated with the 48h growth in mucus. Both the candidate probiotic bacteria, CMH1X and CM2H3L, showed tolerance against diluted bile juice and were capable to grow at or below 8% fish bile juice (MIC=8% bile juice). After 21 days of small-scale *in vivo* experiment, it was revealed that along with the control set intra-peritoneal injection of the candidate probiotics did not induce any pathological signs/disease symptoms or mortalities in both treatment groups.

Table 2: Inhibition zone* produced by the selected gut bacteria in double layer method against the tested fish pathogens[#]

Strains	AH	PP	AS	BM	AV	P	PF
CMF1L	+	-	-	-	-	-	+
CM2F1Ph	-	-	++	-	-	+	-
CM3F2X	-	+	-	-	-	++	-
CM3F2A	-	++	-	-	-	+	-
CMH1X	++	+++	-	++++	+	-	++
CMH4X	+	-	-	-	-	-	-
CMH1L	-	+	-	-	-	+	-
CM2H3L	-	+++	++++	++++	-	+++	+
CM2H2P	-	-	-	-	+	-	-
CM2H2L	++	-	-	-	-	-	+
CM2H1P	-	++	-	+	+	-	-
CM3H2P	-	++++	-	-	-	-	-
CM3H4C	-	-	-	+	-	-	+

[#]AH=*Aeromonas hydrophila*; PP=*Pseudomonas putida*; AS= *Aeromonas salmonicida*; BM=*Bacillus mycoides*; AV=*Aeromonas veronii*; P=*Pseudomonas sp.*; PF=*Pseudomonas fluorescence*.

* +, low (6-10 mm halo diameter); ++, moderate (11-20mm halo diameter); +++, high (21-25 mm halo diameter); ++++, very high (≥ 26 mm halo diameter)

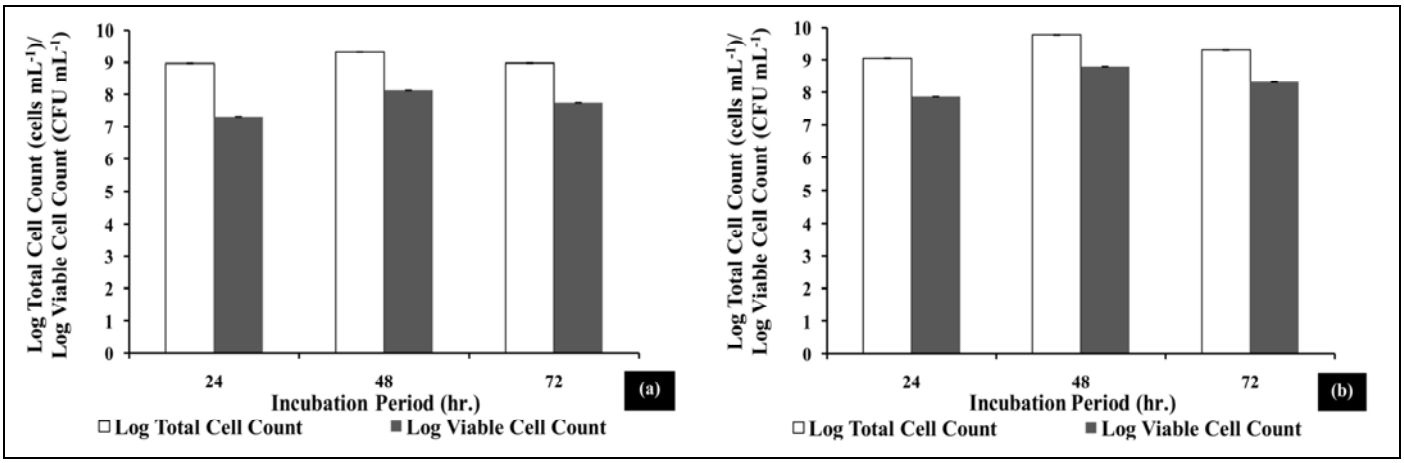

Fig 2: Log values of total microscopic count (cells mL^{-1}) and viable count (CFU mL^{-1}) of the selected gut bacteria (a) CMH1X (*Bacillus amyloliquefaciens*) and (b) CM2H3L (*Bacillus sonorensis*) grown in gut mucus of *Cirrhinus mrigala.* Viable count was done on TSA plates inoculated with respective bacteria cultures of 24 h, 48 h and 72 h duration in fish gut mucus. Each column with error bar represents Mean ± Standard error (n=3)

Based on the nucleotide homology and phylogenetic analysis of the 16S rRNA partial gene sequences by nucleotide blast in the NCBI GenBank and RDP databases, the putative probiotic strain CMH1X was identified as *Bacillus amyloliquefaciens* (GenBank Accession no. KF623290), which was closest to the type strain *Bacillus amyloliquefaciens* (AB255669.1). The other isolate, CM2H3L was identified as *Bacillus sonorensis* (GenBank Accession no. KF623291) that showed maximum similarity with the type strain *Bacillus sonorensis* (AF302118.1). Phylogenetic relation of the two identified bacterial isolates with other closely related type strains retrieved from the RDP database were presented in the dendrogram (Figure 3).

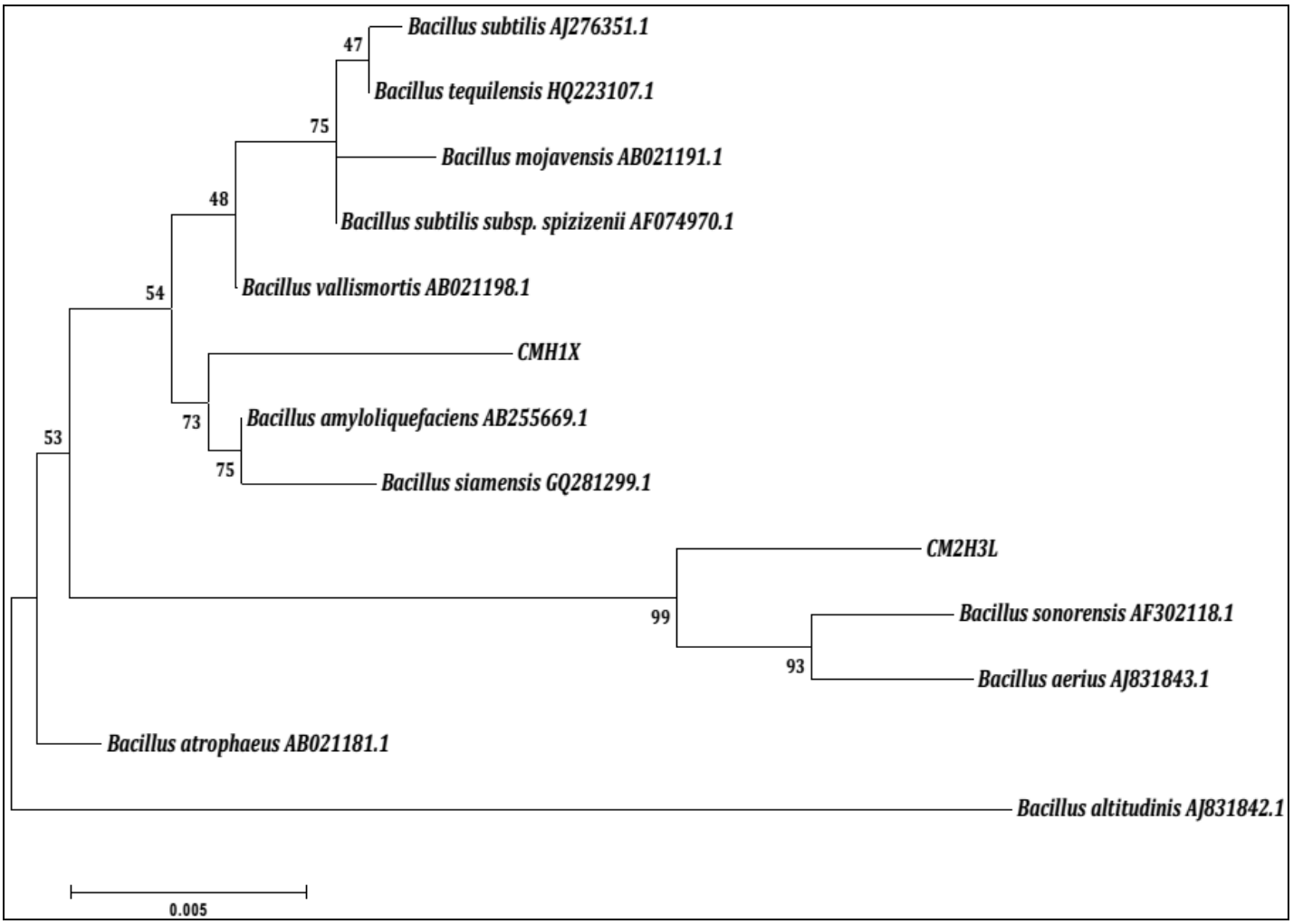

Fig 3: Dendrogram showing phylogenetic relations of the two potential probiotic bacterial strains, *Bacillus amyloliquefaciens* CMH1X (KF623290) and *Bacillus sonorensis* CM2H3L (KF623291), with other closely related strains retrieved from NCBI *GenBank* and RDP. The GenBank accession numbers of the reference strains are shown besides the names. Horizontal bars in the dendrogram represent the branch length. Similarity and homology of the neighbouring sequences have been shown by bootstrap values. Distance matrix was calculated by Hasegawa-Kishino-Yano model. The scale bar indicates 0.005 substitutions per nucleotide position. *Bacillus altitudinis* AJ831842.1 served as an out group.

4. Discussion

In the present study emphasis has been given on the autochthonous microorganisms, as the native flora are supposed to be well adapted to the intended ecological niche. Prior to isolation of microorganisms, the fish were starved for 48 hours and their GI tracts were thoroughly washed with sterile 0.9% saline. Therefore, it may be assumed that the isolated microorganisms described in the present study belonged to the autochthonous microbiota as suggested elsewhere [14, 15]. Screening through *in vitro* tests are commonly being used to eliminate the less potential microorganisms from available large pool to a more acceptable number for further testing [40]. Selection of strong extracellular enzyme producers was considered as the primary criteria for the candidate probionts in view of inducing improved nutrient utilization to support growth. This preliminary screening has resulted in an elimination of 74.4% (87 strains out of 117) of the total isolates from current study. Results of the present study depicted that heterotrophic community within the GI tract of *C. mrigala* were represented by diverse bacteria capable of producing digestive (amylase, protease, lipase) and anti-nutritional factor degrading (cellulase, phytase, xylanase) enzymes, which were compliant with the previous observations recorded from the GI tracts of the Indian major carps [6]. The Indian major carp, *C. mrigala* has been described as either omnivore, or feeding on detritus arising out from the plant feedstuffs [41]. Therefore, in accordance with the hypothesis that gut bacteria might contribute to the digestion in fish [6]; amylase, cellulase and xylanase activities by the gut bacteria noticed in the present study might indicate their ability to aid in digestion of plant feedstuffs in *C. mrigala*.

The ability of a bacterial strain to inhibit the growth of pathogenic bacteria has been proposed as the major criteria for the selection of probiotics in many studies [40]. A general consensus developed that probiotic treatment might lead to improved protection in fish against several diseases [36]. Therefore, demonstration of antagonistic properties against some fish pathogens was considered as the second criteria for the selection of candidate probionts in the present study. Besides *Aeromonas* spp. as the well-known fish pathogens, both *Pseudomonas* spp. [42] and *Bacillus mycoides* [43] are described as opportunistic pathogens in fish and therefore, included in the presently reported study to evaluate antibacterial efficiency of the putative probiotics. Precisely, two isolates (CMH1X and CM2H3L) with antagonistic activity against five of the seven pathogenic strains tested were

categorized as strong antagonists. The inhibition zones exhibited by *Bacillus amyloliquefaciens* CMH1X (GenBank Accession no. KF623290) and *Bacillus sonorensis* CM2H3L (GenBank Accession no. KF623291) in the cross-streaking method were similar to those reported by [44] for *Alteromonas* sp. P7 (4.0±0.25 mm) and [45] for *Bacillus* strain BY-9 (3-6 mm) against the pathogenic *Vibriosis harveyi*. Gut microbiota in the freshwater teleosts were fairly dominated by *Bacillus* spp. [14, 46, 47, 48], which is in agreement with the present study. *Bacillus* spp. have been shown to possess adhesion abilities, provide immunostimulation and produce bacteriocins [49, 50, 51]. Furthermore, *Bacillus* spp. are favoured as probiotics as they can be kept in the spore form and stored indefinitely [52]. Probiotic effects of *B. amyloliquefaciens* have been addressed in few of the recent observations. Application of probiotic *B. amyloliquefaciens* led to improvement of the growth performance, feed conversion ratio, and immunological parameters in Nile tilapia, *Oreochromis niloticus* [53]. In another study, *B. amyloliquefaciens* FPTB16 has been reported as putative probiotic for *Catla catla* on the basis of systemic and cutaneous mucosal immune responses and disease resistance [54]. Besides, pathogen inhibitory potential of several bacilli has been indicated elsewhere [10, 55, 56, 57]. Nevertheless, enzyme producing capacity together with anti-pathogenic potential has not been addressed widely. In this context, extracellular digestive enzyme-producing *Bacillus thuringiensis* isolated from the GI tract of the Atlantic salmon (*Salmo salar* L.) was shown to inhibit the growth of 4 pathogenic bacteria (*in vitro*) *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio (Listonella) anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum* [58]. In consequence of the results observed in the present study, *B. amyloliquefaciens* and *B. sonorensis* may be suggested as putative probiotics for mrigal. In addition, it could be hypothesized from the present investigation that the extracellular enzyme-producing bacteria colonizing within the GI tract of *C. mrigala* might offer protection against some of the fish pathogens.

A probiotic bacterium should have the capacity to tolerate fish GI conditions. Therefore, growth in fish gut mucus and tolerance to diluted fish bile juice were considered as additional criteria for verification of probiotic potential. Both the selected gut bacteria grew well in fish mucus, although, minor differences were noted in bacterial growth rate which might be due to specific nutritional requirements of the bacteria [57]. Furthermore, results of the MIC of bile juice indicated that the selected probiotic bacteria could tolerate diluted fish bile juice up to 8%. The physiological concentration of bile was estimated to be approximately 0.4 – 1.3% within the fish GI tract [59]. Hence, the bile tolerance by the gut isolates shown in the present study was comparatively high. Similar observations were recorded by several authors to establish bile tolerance of the putative probotic bacteria against fish bile [60, 61] or commercial bile salts [57, 62]. Safety of the host is another prerequisite for any probiotic bacterium as suggested elsewhere [7]. In this study, the selected isolates were evaluated for safety measurement through small scale *in vivo* study and experimental results revealed that the isolates did not induce any pathological signs or mortalities in *C. mrigala*.

5. Conclusions

The present study encompasses isolation of the gut bacteria by culture dependant methods, which has been generally criticized as time consuming, lack accuracy and do not represent an exact description of the bacterial diversity in fish gut [63, 48]. However, as the major aim of the present study was to investigate likely function and identify autochthonous gut bacteria in *C. mrigala* as putative probiotics, the application of culture dependent process seems to be rationale. In this study, we isolated putative probionts from fish intestine and screened their potentiality through *in vitro* experiments. Nevertheless, assumptions based on *in vitro* experiments might not comply exactly with *in vivo* conditions. Therefore, *in vivo* studies are warranted with these autochthonous strains to determine their effects on growth and wellbeing of *C. mrigala*.

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