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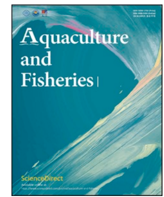

Endosymbiotic pathogen-inhibitory gut bacteria in three Indian Major Carps under polyculture system: A step toward making a probiotics consortium

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ABSTRACT

The gastrointestinal (GI) microbiome in fish plays significant roles in health and disease resistance. This investigation was accomplished to enumerate, characterize and identify the potential probiotic bacteria from three Indian Major Carps (IMCs), viz., rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) using culture dependent methods. Altogether, 105 pathogen-inhibitory bacteria (out of 1216 isolates) were detected from three IMCs by double layer assay. 16S rRNA partial gene sequence analyses and BLAST search in the NCBI GenBank unveiled that 94.29% of the pathogen inhibitory bacteria were bacilli (99 strains) and *Bacillus licheniformis* by far the most common (28%). The primarily selected 27 pathogen-inhibitory strains (cumulative inhibition score ≥13) produced extracellular enzymes, while 15 of them produced all the six exo-enzymes studied (amylase, protease, lipase, cellulase, phytase and xylanase). Gut stability of the strains became apparent by their ability to grow in fish mucus and tolerance to diluted bile-juice. Finally, 14 strains were noticed as γ-hemolytic and susceptible to the commonly used antibiotics. Further, intra-peritoneal injection with γ-hemolytic strains did not induce any pathological signs or mortalities in fish, and thus were considered as safe. These 14 γ-hemolytic isolates were represented by the genus *Bacillus* (13) and *Stenotrophomonas* (1), which might form probiotic consortia for prospective use in carp culture.

1. Introduction

Aquaculture is an important food sector for a growing global human population and has rapidly developed due to intensified culture methods (FAO, 2017). The major producer countries in farmed fish are China, India, Vietnam, Bangladesh and Egypt (FAO, 2016, p. 200). In India, Indian major carps (IMCs), i.e., rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) accounting for almost 87% of the total freshwater fish production, and these fish species represent different trophic levels and form the most important component of the carp polyculture system (ICLARM, 2001; Paul & Giri, 2015). Polyculture of carps representing different ecological niche is a traditional method for optimum utilization of trophic resources in culture ponds (Billard & Berni, 2004). However, extension, diversification and intensification of aquaculture have increased the occurrence of disease outbreaks during the past decades (Mukherjee et al., 2017), and bacteria are the most common among the pathogens in cultured fish that cause mass mortality in freshwater aquaculture (Giri et al., 2011; Swain, Behura, Dash, & Nayak, 2007). Suggested correlations between modulation in the gut microbiota with physiology and disease have received increased attention of the scientific community leading to detailed investigations on the microbial diversity in fish (Ghanbari, Kneifel, & Domig, 2015; Hoseinifar, Sun, Wang, & Zhou, 2018). A comprehensive investigation of the gut-associated microbiota of the host might shed light on the “normal” bacterial community that could help to maintain fish health under polyculture. Although several studies have condemned culture-dependent methods as they detect only a small fraction of the microbial communities (Gajardo et al., 2016; Ghanbari et al., 2015; Kim, Brunt, & Austin, 2007; Larsen, Tao, Bullard, & Arias, 2013), de Bruijn, Liu, Wiegertjes, and Raaijmakers (2018) stated in their review that

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classic culture-dependent techniques are required to validate the potential of probiotic bacteria.

The gut is one of the major infection routes in fish because they are always in intimate contact to their environment, water, and are permanently exposed to bacteria including pathogens. The gut microbiota of fish plays an important role in mediating and stimulating host gastrointestinal development, aiding digestive function, maintaining mucosal tolerance, stimulating the host immunoresponse and providing a level of protection against gastric infections (Clements, Angert, Montgomery, & Choat, 2014; Montalban-Arques et al., 2015; Rawls, Mahowald, Goodman, Trent, & Gordon, 2007; Rawls, Mahowald, Ley, & Gordon, 2006; Ringø et al., 2016). However, indiscriminate use of chemical additives and antibiotics as preventative measure towards diseases has resulted in antimicrobial resistance among pathogenic bacteria, alteration in the gut microbial community and degraded environmental conditions (Cabello, 2006; Romero, Feijoo', & Navarrete, 2012; Ringø et al., 2016). Consequently, the scientific community has searched for alternatives, for example the probiotics. At present, there is a growing interest on the application beneficial microorganisms as probiotics to reduce the incidence of fish diseases by inhibiting the growth of pathogenic microorganisms (Balcázar et al., 2006; Kesarcodi-Watson, Kaspar, Lategan, & Gibson, 2008; Mukherjee, Chandra, & Ghosh, 2019a; Munir, Hashim, Nor, & Marsh, 2018; Nandi, Banerjee, Dan, Ghosh, & Ray, 2018) and to improve the nutrient utilization (Mukherjee et al., 2019a; Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000).

In their review devoted to probiotic and prebiotics for salmonids, Merrifield et al. (2010) extended a list of criteria for potential probionts, in which some were considered as essential while others as merely favorable. Some of the essential characteristics are: (1) must not be pathogenic to the host species, (2) must be resistant to bile salts and (3) low pH. Among the favorable criteria, functionally pertinent to pursue are: (4) should be able to adhere to and/or grow well within intestinal mucus, (5) must be free of plasmid-encoded antibiotic resistance genes, (6) should exhibit antagonistic properties towards one or more key pathogens and (7) should produce relevant extracellular digestive and/or degradation enzymes (e.g. cellulase, if the diet is rich in plant ingredients). The main strategy of using probiotics is to isolate intestinal bacteria with favorable properties from mature animals and include them in the feed for immature animals of the same species (Gildberg, Mikkelsen, Sandaker, & Ringo, 1997; Hoseinifar et al., 2018; Van Doan et al., 2018). However, unlike monoculture of salmon, tilapia, rainbow trout or sea bass, the aquaculture in India is typically practiced as composite culture of carps. Consequently, application of probiotics should cross the source species barrier to ensure overall health benefit to the fish species under composite culture practice. Therefore, multi-strain and multi-species probiotics should be developed from different fish species to cover wide angel benefits under composite culture conditions.

In a recent review, Lescak and Milligan (2017) put forward the controversial statement that teleosts should be used as model organisms to understand host-microbe interactions, and that the adherent (autochthonous symbiotic) microbiota should be investigated. The aim of the present study was to investigate autochthonous endosymbiotic gut bacteria isolated from three Indian major carp species, in order to isolate potential probiotics based on; functional characterization (antibacterial activity, enzymatic production), stability within the gut micro-environment (growth in mucus, tolerance to bile juice), bio-safety (hemolytic activity, antibiotic susceptibility, in vivo validation through intra-peritoneal injection), and to identify the bacteria by 16S rRNA partial gene sequence analyses.

2. Material and methods

2.1. Sample collection and isolation of autochthonous gut bacteria

Healthy fish with no external wound or sore were collected from three different polyculture ponds in and around Burdwan (23°14′N, 87°39′E), West Bengal, India. Specimens were collected and handled following the approved guidelines of the Institutional Ethical Committee. However, approval of the committee was not required as farmed specimens were used. Three Indian major carps viz., rohu (*Labeo rohita*); catla (*Catla catla*); and mrigal (*Cirrhinus mrigala*) were used in the present study. Five fish specimens (average weight: 225 ± 10.2 g; length 29.1 ± 2.64 cm) of each species were collected from each of the three composite culture ponds. Pooled sample of each species collected from a particular pond served as a replicate, and thus the study comprised three replicates for each species. The specimens were starved for 24 h to isolate autochthonous endosymbiotic intestinal bacteria and to eliminate most of the allochthonous bacteria associated with digesta (Ghosh, Roy, Kar, & Ringø, 2010; Mukherjee et al., 2017). After starvation, fish were anaesthetized with MS-222 (tricaine methanesulfonate; Sigma-Aldrich Corp., USA) before sacrifice. The gastrointestinal (GI) tracts were divided into proximal (PI) and distal (DI) segments and the gut samples were processed for isolation of culturable autochthonous gut bacteria by the methods described previously (Mandal & Ghosh, 2013; Mukherjee et al., 2016; Mukherjee & Ghosh, 2016). Gut segments were homogenized, serially diluted and spread on soybean casein digest medium (tryptone soya agar, TSA; HiMedia). Following incubation (48 h, 30 °C), distinct colonies were randomly isolated, cultured on TSA plates and pure cultures were preserved (4 °C) for further studies.

2.2. Antimicrobial activity assay

Antibacterial activity of the isolated gut bacteria was tested towards seven fish pathogenic strains by the 'double-layer' method of Dopazo et al. (1988). The pathogenic strains, *Aeromonas hydrophila* MTCC-1739 (AH), *Aeromonas salmonicida* MTCC-1945 (AS), *Aeromonas sobria* MTCC-3613 (ASo), *Pseudomonas fluorescens* MTCC-103 (PF), *Pseudomonas putida* MTCC-1072 (PP) and *Bacillus mycoides* MTCC-7538 (BM) were obtained from the Microbial Type Culture Collection, Chandigarh, India, while, *Aeromonas veronii* KT737240 (AV) was isolated from a diseased catla (Mukherjee & Ghosh, 2016). Growth inhibition of the pathogenic strains was determined as halo zones and presented as inhibition scores: 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–25 mm), and 4 (very high, ≥ 26 mm). The most promising antagonistic bacteria were primarily selected based on cumulative inhibition scores ≥13.

2.3. Molecular identification and phylogenetic analysis

All antagonistic bacteria were analysed by 16S rRNA partial gene sequences as described elsewhere (Mukherjee & Ghosh, 2016; Ringø, Sperstad, Myklebust, Mayhew, & Olsen, 2006). Universal primers, 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5-GGTTACCTTGT TACGACTT-3′) were employed to amplify the gene encoding 16S rRNA. Amplified products were sent to the commercial house for Sanger sequencing using automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA). Sequenced data were edited (BioEdit Sequence Alignment Editor; Version 7.2.5), the closest known (type strain) alignment identities were retrieved from National Centre for Biotechnology Information (NCBI) GenBank, and deposited to the NCBI GenBank to obtain accession numbers.

2.4. Enzymatic activity assay

The selected antagonistic strains were further screened for production of extracellular digestive (amylase, protease, lipase) and antinutritional degrading (cellulase, xylanase, phytase) enzymes. The bacteria strains grown in selective broth media were analysed for production of the enzymes. Quantitative determination of amylase (Bernfeld, 1955), protease (Walter, 1984, pp. 270–277), lipase (Bier, 1955), cellulase (Denison & Koehn, 1977), xylanase (Bailey, Biely, & Poutanen,

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1992) and phytase (Yanke, Selinger, & Cheng, 1999) activities were carried out following standard methodologies and expressed as unit activity (U).

2.5. Stability in gut micro-environment: growth in mucus and tolerance to bile juice

A description relating to growth potential of bacteria in fish mucus and bile tolerance has been depicted elsewhere (Balcázar et al., 2008; Mukherjee et al., 2017; Mukherjee & Ghosh, 2016; Nikoskelainen, Salminen, Bylund, & Ouwehand, 2001). Mucus from intestine and skin of live carp specimens (average weight 145.45 ± 8.7g; length 16.8 ± 1.27 cm) was collected and processed separately following the methods described by Mukherjee and Ghosh (2016) and Ross, Firth, Wang, Burka, and Johnson (2000), respectively. Protein concentration of the mucus was determined (Lowry, Rosenbrough, Fair, & Randall, 1951) and adjusted to 1 mg mL^{-1}. Mucus samples were filter sterilized (0.8 and 0.22 μm porosity; HiMedia, Mumbai, India) and inoculated with the selected strains (30 °C, 24 h) to confirm their growth potential in fish mucus. Crude bile juice (pH 5.7) was collected by puncturing gall bladder taken out from live specimens (IMCs), filter sterilized and stored at −20 °C for further use. Sterile PBS supplemented with 20% (v/v) fish bile juice was inoculated with the selected bacteria, incubated (30 °C, 1.5 h) and viable counts were determined by spreading on TSA media plates.

2.6. Bio-safety assay

2.6.1. Hemolytic activity

Selected strains were investigated for hemolytic activity to determine their pathogenic potential (Nurhidayu, Ina-Salwany, Mohd-Daud, & Harmin, 2012). The assay was performed by streaking the bacteria cultures onto plates containing Columbia blood agar base (HiMedia, India) supplemented with goat blood (5%) and incubated at 30 °C for 24 h. Appearance of hemolytic zones around the colonies were noticed and classified as: α (greenish halo), β (clear halo) or γ (no halo) hemolysis based on lysis of the red blood cells in the media around and under the colonies.

2.6.2. Determination of antibiotic susceptibility

Antibiotic susceptibility of the selected strains was determined on TSA plates with susceptibility test discs (HiMedia, India) following disc-diffusion methodand zones around discs were measured. The studied antibiotics (Ampicillin, Amoxicillin, Azithromycin, Chloramphenicol, Clindamycin, Erythromycin, Gentamicin, Kanamycin, Neomycin, Novobiocin, PenicillinG, Streptomycin, Tetracycline, Vancomycin) were used at prescribed doses and sensitivity was determined following the recommendation of National Committee for Clinical Laboratory Standards (NCCLS, 2012).

2.6.3. Small-scale in vivo validation

In vivo bio-safety evaluation for each of the γ-hemolytic bacteria was carried out separately as described by Mukherjee and Ghosh (2016) and Mukherjee et al. (2017). Briefly, experimental fish (rohu, 15.6 ± 1.2 g) were given intra-peritoneal (IP) injection (1.0 mL) of a selected bacterium (10^9 cells/mL, in sterile 0.9% saline) and observed for 4 weeks for development of any external pathological symptoms (loss of scale or mucus, hemorrhage, lesion). Control fish were injected with sterile 0.9% saline (Mesalhy, Abd-El-Rahman, John, & Mohamed, 2008).

2.7. Statistical analysis

Results on exo-enzyme producing ability, growth potential in fish mucus and bile tolerance were presented as mean ± standard error (SE). Data on exo-enzyme producing ability was subjected to analysis of variance following Zar (1999) using SPSS version 17 (Kinnear & Gray, 2009), and differences between means were tested by Tukey's range test ($P \leq 0.05$).

3. Results

3.1. Fish species, bacterial isolates and antimicrobial activity

Totally, 1216 strains were randomly isolated from the three IMCs, of which, 545 strains were isolated from PI and 671 strains from DI. Amongst them, 47 strains from PI (8.62%) and 58 strains from DI (8.64%) exhibited antagonistic activity against at least one of the pathogens evaluated. Total number of isolates from PI and DI regions and antagonistic isolates from respective portions with reference to each fish species are presented in Fig. 1. While demonstrating pathogen-inhibition by the isolated strains, 53 strains that revealed antagonism against ≥4 pathogenic strains and acquired cumulate inhibition score of

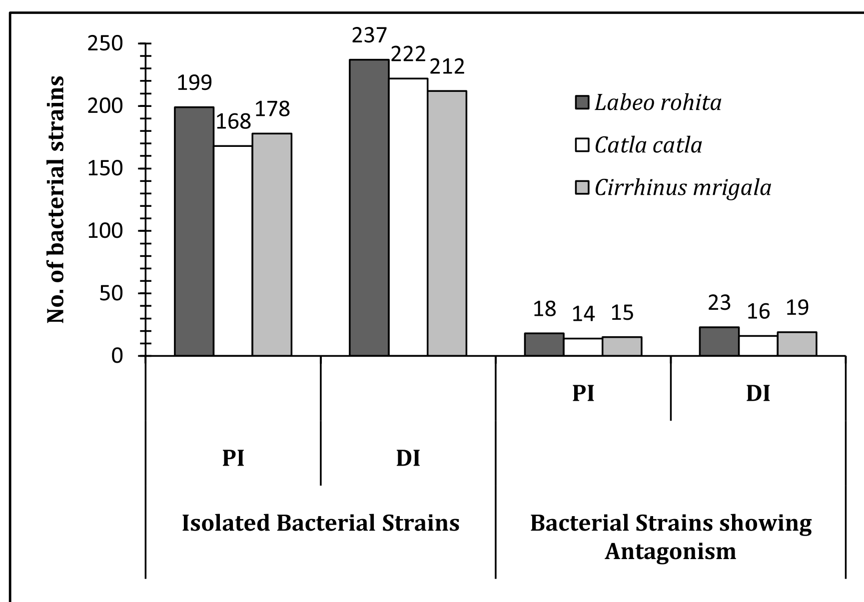

Fig. 1. Bacteria strains isolated from the proximal (PI) and distal (DI) regions of the gut in Indian major carps.

≥10 (with respect to halo zone) are presented in Table 1. Of these, 27 promising pathogen inhibitory strains were primarily selected from rohu (16), catla (4) and mrigal (7) on the basis of a cumulative inhibition score of ≥13 and were further characterized to validate their potential probiotic attributes.

3.2. Molecular identification and phylogenetic analysis

Identity of the pathogen-inhibitory bacteria as evidenced through nucleotide homology and 16S rRNA partial gene sequence analyses are depicted in Table 2. Out of the 105 pathogen-inhibitory gut isolates, 99 strains (94.29%) belonged to the genus *Bacillus* (similarity between 94 and 100%), while the other isolates were represented by *Pseudomonas fluorescens* (similarity = 98%)*, Micrococcus aloeverae* (similarity = 99%), *Micrococcus yunnanensis* (similarity = 89%)*, Stenotrophomonas pavanii* (similarity = 99%)*, Lactococcus lactis* (similarity = 97%) and *Staphylococcus capitis* (similarity = 99%). Bacteria identified as *Bacillus licheniformis* were most common (28%) among the pathogen-inhibitory bacteria, followed by *B. safensis* (17%) and *B. aerius* (12%). Diversity of the pathogen-inhibitory bacteria at species level as appeared through molecular identification of the isolated autochthonous pathogen-inhibitory bacteria is presented in Fig. 2.

3.3. Enzymatic activity

Results of the quantitative determination of exo-enzyme producing ability with respect to both, digestive (amylase, protease and lipase) and degradation (cellulase, phytase and xylanase) enzymes, revealed

Table 1
Determination of antagonism (double layer method) by the isolated gut bacteria against fish pathogens. Zones of inhibition (halo diameter) were presented as scores[¶].

Fish	Strains	AH	AV	AS	ASo	PF	PP	BM	Total Score
Labeo rohita	LR1HG9	4	0	0	1	2	2	3	12
	LR2FG18	0	3	3	2	3	2	2	15
	LR2FG27	3	3	0	4	2	2	3	17
	LR2FG31	2	2	0	3	1	2	2	12
	LR2FG32	2	3	1	1	2	2	3	14
	LR2FG33	2	2	0	4	0	2	4	14
	LR2HG4	4	3	0	3	2	2	3	17
	LR2HG12	0	3	1	3	0	3	4	14
	LR2HG14	2	3	0	2	0	0	4	11
	LR2HG15	2	2	0	3	0	2	3	12
	LR2HG16	2	3	0	2	1	2	3	13
	LR2HG21	3	2	2	0	0	2	3	12
	LR2HG22	2	3	0	4	1	1	3	14
	LR3FG19	4	2	0	3	2	3	4	18
	LR3FG25	3	3	0	4	0	3	4	17
	LR3HG13	0	3	3	1	0	1	4	12
	LR1D	3	2	0	2	2	2	3	14
	LR2F	0	4	2	3	2	2	2	15
	LR1C	3	2	0	2	0	0	3	10
	LRF2X	2	2	0	4	0	0	2	10
	LRF3X	0	2	3	3	0	0	2	10
	LRF2C	3	2	0	3	0	2	3	13
	LRF1Ch	2	0	2	3	2	2	2	13
	LRH1C	0	2	2	2	0	2	2	10
	LRH3C	3	1	2	2	3	2	2	15
	LRH2X	0	2	2	0	2	2	2	10
	LRH5X	3	0	0	2	2	2	2	11
	LRH8X	2	2	3	0	2	2	2	13
	LRH6Ch	3	3	2	0	0	2	1	11
Catla catla	CC1HG6	1	2	4	3	2	0	2	14
	CC1HG7	0	4	0	2	2	4	0	12
	CC3HG13	3	3	0	0	3	3	0	12
	CC2F3L	2	0	1	1	2	2	2	10
	CC2F1Ph	0	2	0	3	0	2	3	10
	CC2H8L	0	1	2	3	0	2	2	10
	CCH3L	3	2	2	3	2	2	2	16
	CCH2P	3	3	3	2	2	2	2	17
	CC1C	2	2	3	3	2	2	1	15
	CCF1X	2	1	2	3	2	0	0	10
Cirrhinus mrigala	CM2FG16	0	0	0	4	3	2	3	12
	CM2HG2	2	2	0	3	3	0	2	12
	CM2HG6	2	0	4	4	0	2	0	12
	CM3FG14	3	2	2	4	2	0	0	13
	CM3HG11	0	0	2	3	2	3	2	12
	CM2H2L	0	0	2	3	0	2	3	10
	CMH1P	2	1	2	2	2	3	3	15
	CMH4X	3	2	2	2	3	3	2	17
	CMH1L	0	2	0	2	2	2	3	11
	CMF2A	2	2	1	0	1	2	2	10
	CMF1Ph	3	2	3	1	3	0	2	14
	CMF5C	0	2	2	4	0	3	2	13
	CMF X3	3	2	0	2	2	2	3	14
	CMH C2	2	2	1	3	1	2	2	13

[¶]1, low (6–10 mm); 2, moderate (11–20 mm); 3, high (21–25 mm); 4, very high (≥26 mm). Data represents mean value of three observations.
AH, *A. hydrophila*; **AV**, *A. veronii*; **AS**, *A. salmonicida*; **ASo**, *A. sobria*; **PF**, *Psudomonas fluorescence*; **PP**, *Pseudomonas putida*; **BM**, *Bacillus mycoides*.

Table 2
Identification of gut bacteria isolated from three Indian Major Carps, viz., *L. rohita*, *C. catla* and *C. mrigala* with their closest type strains retrieved from NCBI GenBank.

Fish species	Code of Strains	Identified as	Accession No.	Query cover	Accession No. of the Closest type strains
Labeo rohita	LR1HG4	*Bacillus altitudinis*	KU664835	95%	NR_042337.1
	LR1HG9	*Bacillus amyloliquefaciens*	KU664836	96%	NR_117946.1
	LR2FG15	*Bacillus subtilis*	KU664837	95%	NR_113265.1
	LR2FG18	*Bacillus tequilensis*	KU664839	95%	NR_104919.1
	LR2FG19	*Bacillus subtilis*	KU664841	98%	NR_113265.1
	LR2FG27	*Bacillus licheniformis*	KU664843	97%	NR_074923.1
	LR2FG31	*Bacillus safensis*	KU664844	98%	NR_113945.1
	LR2FG32	*Pseudomonas fluorescens*	KU588182	98%	NR_113647.1
	LR2FG33	*Bacillus safensis*	KU664846	98%	NR_113945.1
	LR2HG4	*Bacillus licheniformis*	KU664845	98%	NR_118996.1
	LR2HG12	*Bacillus pumilus*	KU588181	99%	NR_112637.1
	LR2HG14	*Bacillus pumilus*	KU664847	98%	NR_112637.1
	LR2HG15	*Bacillus safensis*	KU664838	98%	NR_113945.1
	LR2HG16	*Bacillus safensis*	KU664840	98%	NR_113945.1
	LR2HG21	*Bacillus safensis*	KU664842	98%	NR_113945.1
	LR2HG22	*Bacillus safensis*	KU588180	99%	NR_113945.1
	LR3FG19	*Bacillus licheniformis*	KU664848	98%	NR_118996.1
	LR3FG25	*Bacillus licheniformis*	KU588179	98%	NR_118996.1
	LR3HG13	*Bacillus safensis*	KU664849	98%	NR_113945.1
	LR1D	*Bacillus altitudinis*	KX273991	98%	AJ831842
	LR2F	*Bacillus aerius*	KX273995	99%	JX009139
	LR1C	*Bacillus licheniformis*	KX377645	98%	NR_118996.1
	LR1G	*Bacillus aerius*	KX273992	99%	JX009139
	LR2H	*Bacillus aerius*	KX364920	99%	JX009139
	LR2D	*Bacillus aerius*	KX364921	98%	JX009139
	LRF2X	*Bacillus licheniformis*	KX364925	99%	NR_118996.1
	LRF3X	*Bacillus safensis*	KX364926	99%	NR_113945.1
	LRF4X	*Micrococcus aloeverae*	KX364928	99%	NR_134088.1
	LRF1C	*Bacillus licheniformes*	KX364930	98%	NR_118996.1
	LRF2C	*Bacillus amyloliquefaciens*	KX364929	98%	NR_117946.1
	LRF1Ch	*Bacillus licheniformes*	KX364931	98%	NR_118996.1
	LRH1C	*Bacillus stratosphericus*	KX388229	98%	NR_042336.1
	LRH3C	*Bacillus licheniformis*	KX377640	99%	NR_118996.1
	LRH5C	*Bacillus altitudinis*	KX388230	98%	NR_042337.1
	LRH2X	*Bacillus aerius*	KX377644	98%	NR_118439.1
	LRH4X	*Bacillus aerius*	KX377643	99%	NR_118439.1
	LRH5X	*Bacillus safensis*	KX377642	99%	NR_041794.1
	LRH8X	*Bacillus aerius*	KX377641	99%	NR_118439.1
	LRH4Ch	*Bacillus aerius*	KX388226	99%	NR_118439.1
	LRH6Ch	*Bacillus aerius*	KX388227	98%	NR_118439.1
	LRH7Ch	*Bacillus aerius*	KX388228	98%	NR_118439.1
Catla catla	CC1HG6	*Bacillus methylotrophicus*	KU601350	98%	NR_116240.1
	CC1HG7	*Bacillus amyloliquefaciens*	KU564242	98%	NR_117946.1
	CC2FG1	*Bacillus subtilis*	KU564241	98%	NR_113265.1
	CC2FG2	*Bacillus tequilensis*	KU601351	98%	NR_104919.1
	CC2FG4	*Bacillus safensis*	KU601352	99%	NR_113945.1
	CC2FG16	*Bacillus aerius*	KU564244	96%	NR_118439.1
	CC2HG6	*Bacillus subtilis*	KU601353	94%	NR_102783.1
	CC3FG9	*Bacillus subtilis* subsp. *spizizenii*	KU601354	97%	NR_112686.1
	CC3HG6	*Bacillus subtilis* subsp. *spizizenii*	KU564243	99%	NR_112686.1
	CC3HG10	*Bacillus cereus*	KU601355	97%	NR_074540.1
	CC3HG13	*Bacillus licheniformis*	KU601356	97%	NR_118996.1
	CC3HG16	*Bacillus cereus*	KU601357	98%	NR_074540.1
	CC2F3L	*Bacillus aerius*	KX273993	99%	JX009139
	CC2F1Ph	*Bacillus licheniformis*	KX273994	99%	NR_118996.1
	CC2H8L	*Bacillus safensis*	KX364922	98%	AB681259
	CCH3L	*Bacillus stratosphericus*	KX377649	98%	AJ831841
	CCH2P	*Bacillus cereus*	KX424371	98%	AE016877
	CC1C	*Bacillus subtilis* subsp. *spizizenii*	KX377646	99%	AB325584
	CC2F16P	*Bacillus licheniformis*	KX377647	95%	NR_118996.1
	CC2F1A	*Bacillus licheniformis*	KX377648	99%	NR_118996.1
	CC2H1A	*Bacillus aerophilus*	KP940381	98%	AJ831844
	CC2H2L	*Bacillus cereus*	KP940382	98%	AE016877
	CC2H2Ph	*Bacillus licheniformis*	KX424372	98%	NR_118996.1
	CC2H6L	*Bacillus licheniformis*	KX424374	98%	NR_118996.1
	CCH4X	*Bacillus subtilis* subsp. *spizizenii*	KP940380	98%	AB325584
	CCH3C	*Bacillus licheniformis*	KP940379	97%	NR_118996.1
	CCF1Ch	*Bacillus licheniformes*	KX398848	99%	NR_118996.1
	CCF1X	*Bacillus licheniformes*	KX398849	99%	NR_118996.1
	CCF2X	*Bacillus licheniformes*	KX398851	99%	NR_118996.1
	CCF4X	*Bacillus licheniformes*	KX398850	98%	NR_118996.1
Cirrhinus mrigala	CM1FG1	*Bacillus flexus*	KP006751	98%	NR_113800.1
	CM1FG4	*Bacillus licheniformis*	KP006752	99%	NR_118996.1

(*continued on next page*)

Table 2 (*continued*)

Fish species	Code of Strains	Identified as	Accession No.	Query cover	Accession No. of the Closest type strains
	CM1FG12	*Bacillus flexus*	KP006753	98%	NR_113800.1
	CM1HG1	*Bacillus flexus*	KP006754	98%	NR_113800.1
	CM1HG8	*Micrococcus yunnanensis*	KP006755	89%	NR_116578.1
	CM2FG3	*Bacillus subtilis*	KU601346	97%	NR_113265.1
	CM2FG5	*Bacillus firmus*	KU601347	97%	NR_112635.1
	CM2FG9	*Bacillus altitudinis*	KU601348	98%	NR_042337.1
	CM2FG16	*Bacillus licheniformis*	KU601349	99%	NR_118996.1
	CM2HG2	*Bacillus flexus*	KU664826	97%	NR_113800.1
	CM2HG3	*Bacillus flexus*	KU664827	98%	NR_113800.1
	CM2HG4	*Bacillus aerius*	KU664828	98%	NR_118439.1
	CM2HG6	*Bacillus stratosphericus*	KU664829	97%	NR_042336.1
	CM2HG7	*Bacillus licheniformis*	KU664830	98%	NR_118996.1
	CM3FG12	*Bacillus licheniformis*	KU664831	98%	NR_118996.1
	CM3FG14	*Stenotrophomonas pavanii*	KU664832	99%	NR_118008.1
	CM3FG15	*Lactococcus lactis*	KU664833	97%	NR_113958.1
	CM3HG11	*Bacillus licheniformis*	KU664834	97%	NR_118996.1
	CM2H2L	*Bacillus stratosphericus*	KX269834	100%	AJ831841
	CMH1P	*Bacillus safensis*	KX269835	99%	AB681259
	CMH4X	*Bacillus subtilis*	KX269836	99%	AB598736
	CMH1L	*Bacillus subtilis*	KX269838	99%	AB598736
	CMF2A	*Bacillus safensis*	KX364927	99%	AB681259
	CMF1Ph	*Bacillus licheniformis*	KX424373	99%	NR_118996.1
	CMH5X	*Bacillus safensis*	KX269837	99%	AB681259
	CMF2Ph	*Bacillus licheniformis*	KX364932	95%	NR_118996.1
	CMH1Ph	*Bacillus licheniformis*	KX424374	96%	NR_118996.1
	CMH2L	*Bacillus safensis*	KX432181	97%	AB681259
	CMF5C	*Bacillus safensis*	KX273999	94%	NR_113945.1
	CMF X3	*Bacillus stratosphericus*	KX364923	95%	NR_042336.1
	CMH C2	*Bacillus subtilis*	KX273998	98%	NR_113265.1
	CMH X2	*Bacillus stratosphericus*	KX364924	98%	NR_042336.1
	CMH3X	*Staphylococcus capitis*	KX273996	99%	NR_113348.1
	CMH1Ch	*Bacillus safensis*	KX273997	96%	NR_113945.1

significant differences in the enzyme activities among the 27 primarily selected bacteria (Table 3). Amylase, protease, lipase and cellulase were produced by all of the studied bacteria, although at varying levels. Fifteen bacteria produced all six enzymes studied. *Bacillus subtilis* CMHC2 exhibited maximum amylase activity (271.39 ± 2.14 U). Maximum protease (80.08 ± 4.15 U) and lipase (5.68 ± 0.27 U) activities were recorded with *Bacillus pumilus* LR2HG12 and *Bacillus safensis* CMF5C, respectively. Maximum cellulase (69.55 ± 2.58 U) and phytase (265.42 ± 5.22 U) activities were noticed with the strain *Bacillus subtilis* subsp. *spizizenii* CC1C. Phytase-producing ability by the strains *B. licheniformis* LR2FG27, *Pseudomonas fluorescens* LR2FG32, *B. licheniformis* LR2HG4, *B. licheniformis* LR3FG25, *B. aerius* LR2F, *B. stratosphericus* CCH3L, *B. cereus* CCH2P, *Stenotrophomonas pavanii* CM3FG14, *B. safensis* CMH1P and *B. subtilis* CMH4X were not recorded. *Bacillus aerius* LRH8X demonstrated the highest xylanase activity (13.28 ± 1.27 U), while xylanase activity was not detected with the strains *B. altitudinis* LR1D, *B. licheniformis* LR3FG19 and *B. licheniformis* LR3FG25.

3.4. Stability in gut micro-environment

3.4.1. Growth in mucus

Log viable cell counts (Log CFU/mL) revealed that the 27 primarily selected strains were competent to grow in both, intestinal mucus as well as skin mucus (Table 4). In general, the strains were more potent to grow in intestinal mucus than the skin mucus. The lowest growth (6.38 ± 0.01 Log CFU/mL) was revealed by *B. licheniformis* LRF1Ch in skin mucus, while the highest growth (7.32 ± 0.10 Log CFU/mL) was recorded for *B. licheniformis* LR2HG4 grown in mucus collected from intestine.

3.4.2. Tolerance to bile juice

All of the primarily selected strains exhibited tolerance towards diluted (20%) bile juice (pH 5.5–7), even after exposure for a period of 24h. Growth detected on TSA plates inoculated with bacteria suspension treated with 20% bile has been presented in Table 5. The highest and lowest growth potential in terms of viable counts (Log CFU/mL) have been recorded for the strains *Bacillus pumilus* LR2HG12 (7.23 ± 0.01) and *Bacillus subtilis* subsp. *spizizenii* CC1C (6.12 ± 0.01), respectively.

3.5. Bio-safety assays

3.5.1. Hemolytic assay

Hemolytic activities of the primarily selected strains are shown in Table 6. When grown on blood agar media plates, 11 strains produced greenish halo under or around the colonies and thus were categorized as α-hemolytic. The strains *B. licheniformis* CMF1Ph and *P. fluorescens* LR2FG32 were considered as β-hemolytic as they produced clear halo around colonies. Another 14 strains, *B. tequilensis* LR2FG18, *B. licheniformis* LR2HG4, *B. pumilus* LR2HG12, *B. safensis* LR2HG22, *B. altitudinis* LR1D, *B. licheniformis* LRH3C, *B. aerius* LRH8X, *B. methylotrophicus* CC1HG6, *B. stratosphericus* CCH3L, *Bacillus cereus* CCH2P, *S. pavanii* CM3FG14, *B. safensis* CMH1P, *B. stratosphericus* CMFX3, and *B. subtilis* CMHC2 did not produce halo zone around the colonies indicating that these strains were γ-hemolytic. Only bacteria with γ-hemolytic property were selected for the antibiotic susceptibility assay.

3.5.2. Determination of antibiotic susceptibility

The finally selected 14 γ-hemolytic bacteria were evaluated for susceptibility against 14 antibiotics, and were noticed as susceptible to 13 commonly used antibiotics (Table 7). All of the studied bacteria were only intermediately susceptible to the amoxycillin (Am) (10mcg), while no resistance was revealed against the other tested antibiotics.

3.5.3. Small-scale in vivo validation

At the end of the 4 weeks trial following intra-peritoneal injection, no external anomalies, disease symptoms or mortality was revealed in the control group, as well as in the experimental groups (results not shown). Consequently, the selected bacteria appeared as harmless to the fingerlings of Indian major carps.

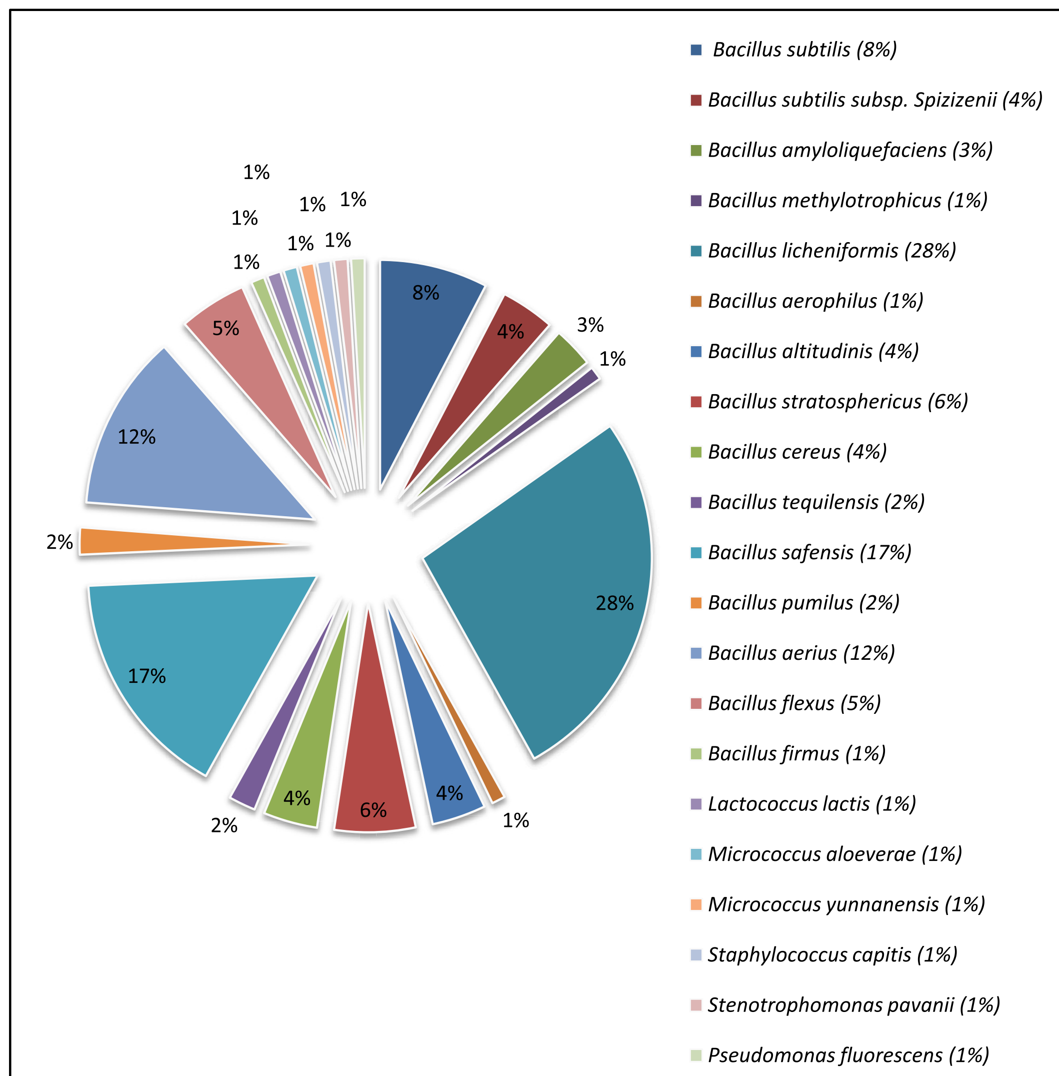

Fig. 2. Pathogen-inhibitory endosymbiotic bacteria detected in the gut of the three Indian major carps. Out of the 105 pathogen-inhibitory gut isolates, 99 strains (94.29%) belonged to the genus *Bacillus*.

4. Discussion

The presently reported study demonstrated diversity of the autochthonous pathogen-inhibitory gut bacteria in three IMCs reared under polyculture system and portrayed their likely probiotic potential. The study used culture based-techniques for isolation of autochthonous gut microbiota, however, universal primers guided amplification and analyses of the 16S rRNA partial gene sequences were employed to identify the pathogen-inhibitory gut microbiota. Commonly, the use of conventional culture-based techniques is argued because of lacking accurateness, requiring more time, and being incapable to represent a correct picture of the bacterial diversity as majority of the microorganisms are unculturable (Asfie, Yoshijima, & Sugita, 2003; Egerton, Culloty, Whooley, Stanton, & Ross, 2018; Gajardo et al., 2016; Li et al., 2015; Ray, Roy, Mondal, & Ringø, 2010). Thus, culture dependent methods based on 16S rRNA gene sequences using universal primers may not reproduce the core diversity of a given environment (Gajardo et al., 2016; Marchesi et al., 1998; Suzuki & Giovannoni, 1996), including the gut microenvironment. On the contrary, it may be apprehended that the presence of any bacterium would not suggest its functional role (Ray, Ghosh, & Ringø, 2012), e.g. antagonistic or enzymatic potential, within the gut. Therefore, as the major aim of the present study was to decipher pathogen-inhibitory gut bacteria in the IMCs, the use of a culture-based technique seemed to be logical.

The presently reported study revealed that pathogen-inhibitory bacterial community in the three IMCs were almost similar being dominated by *Bacillus* spp., which were in accordance with previous reports on gut bacterial community in freshwater teleosts (Ghosh et al., 2010; Ray et al., 2010; Mondal, Roy, & Ray, 2010). Occurrence of *Bacillus* spp. in the GI tract of finfish and shellfish, and their probiotic potential in aquaculture has been widely investigated (for review, see Soltani et al., 2019; Kuebutornye, Abarike, & Lu, 2019). Although, pathogen inhibition by gut bacteria in fish has been less studied, likely antagonism against different fish pathogens has been suggested to be considered as one of the desired criteria in the probiotic screening process during recent times (Dutta, Banerjee, Mukherjee, & Ghosh, 2018; Mohapatra, Chakraborty, Kumar, de Boeck, & Mohanta, 2013; Mukherjee et al., 2017, 2019b; Nandi et al., 2018). In accordance to the present study, *B. subtilis* SG4 isolated from *C. mrigala* showed antagonistic activity against fish pathogenic *P. fluorescens*, *A. hydrophila* and *E. tarda* (Ghosh, Sinha, & Sahu, 2007). Pathogen inhibition by bacilli isolated from gastrointestinal (GI) tract of rohu, *L. rohita* (Giri et al., 2011) has been reported. Probiotic *B. subtilis* BT23 and *Bacillus* spp. could inhibit growth of pathogenic *Vibrio harveyi*, both *in vitro* and in vivo (Janarthanam, George, John, & Jeyaseelan, 2012; Vaseeharan & Ramasamy, 2003). The antagonistic activity of *Lactobacillus casei* and

Table 3
Spectrum of extracellular enzyme production by the selected bacteria. Data are means ± SE (n = 3). Values with the same superscripts in the same vertical column are not significantly different (P < 0.05).

Strains	Amylase§	Protease‡	Lipase†	Cellulase*	Phytase¶	Xylanaseϕ
LR2FG18	209.37 ± 5.34^{v}	40.02 ± 2.73^{b}	$4.11 \pm 0.28^{d,e,f,g,h,i}$	$51.40 \pm 2.28^{f,g,h}$	$95.40 \pm 5.73^{e,f}$	$6.53 \pm 1.01^{a,b,c}$
LR2FG27	$136.48 \pm 2.73^{f,g}$	$64.56 \pm 3.52^{\circ,p,q,r,s,t}$	$4.05 \pm 0.21^{d,e,f,g}$	$58.56 \pm 2.26^{m,n,o}$	ND	$6.14 \pm 0.55^{a,b}$
LR2FG32	102.30 ± 5.70^{a}	$40.33 \pm 3.27^{b,c}$	$3.95 \pm 0.30^{b,c,d,e,f}$	$61.26 \pm 2.35^{m,n,o,p,q,r}$	ND	$7.73 \pm 0.28^{c,d,e,f,g,h,i}$
LR2FG33	$109.55 \pm 3.81^{a,b}$	71.66 ± 2.62^{u}	$4.15 \pm 0.28^{d,e,f,g,h,i,j}$	$59.01 \pm 2.17^{m,n,o,p,q}$	71.66 ± 2.26^{a}	$7.47 \pm 0.33^{c,d,e,f,g}$
LR2HG4	$129.37 \pm 3.72^{d,e}$	$62.59 \pm 1.29^{\circ,p}$	$4.55 \pm 0.29^{g,h,i,j,k,l,m,n}$	$58.53 \pm 2.10^{l,m,n}$	ND	$7.03 \pm 0.21^{b,c}$
LR2HG12	$199.30 \pm 4.26^{t,u}$	80.08 ± 4.15^{v}	$4.83 \pm 0.21^{l,m,n,o,p,q,r}$	$62.34 \pm 2.04^{n,o,p,q,r,s}$	78.51 ± 2.22^{c}	$8.45 \pm 1.29^{c,d,e,f,g,h,i,j,k}$
LR2HG16	$137.77 \pm 3.86^{f,g,h}$	$53.66 \pm 2.36^{g.h.i.j}$	$3.90 \pm 0.21^{b,c,d}$	44.48 ± 1.18^{b}	$105.54 \pm 2.41^{g,h,i,j}$	$6.06 \pm 0.96^{a,b}$
LR2HG22	165.26 ± 3.74^{k}	$43.65 \pm 2.35^{b,c,d}$	$3.90 \pm 0.28^{b,c,d,e}$	$48.56 \pm 2.46^{e,f}$	$99.51 \pm 4.24^{f,g}$	$6.44 \pm 0.55^{a,b}$
LR3FG19	$211.23 \pm 4.62^{v,w}$	49.34 ± 1.18^{f}	$4.11 \pm 0.24^{d,e,f,g,h,i}$	$52.64 \pm 2.01^{f,g,h,i,j}$	$102.52 \pm 1.45^{g,h,i}$	ND
LR3FG25	153.47 ± 5.70^{j}	$55.51 \pm 2.42^{i,j,k,l,m,n}$	$4.06 \pm 0.32^{b,c,d,e,f,g,h}$	$54.68 \pm 1.19^{h,i,j,k,l}$	ND	ND
LR1D	$115.52 \pm 3.61^{b,c}$	35.21 ± 1.58^{a}	$4.15 \pm 0.28^{d,e,f,g,h,i,j}$	$51.16 \pm 2.51^{f,g}$	$73.51 \pm 2.74^{a,b}$	ND
LR2F	$177.68 \pm 3.21^{\circ,p,q}$	$45.36 \pm 1.31^{d,e}$	$4.69 \pm 0.30^{j,k,i,m,n,o,p,q}$	$63.55 \pm 2.41^{q,r,s,t}$	ND	$6.13 \pm 0.28^{a,b}$
LRF2C	195.32 ± 2.45^{t}	$51.55 \pm 1.08^{f,g}$	2.87 ± 0.28^{a}	38.37 ± 1.11^{a}	$102.33 \pm 1.43^{g,h}$	$8.25 \pm 0.25^{i,j}$
LRF1Ch	$168.65 \pm 2.05^{k,l,m}$	40.92 ± 1.03^{b}	$4.02 \pm 0.29^{b,c,d,e,f,g}$	$57.83 \pm 1.41^{l,m}$	89.51 ± 1.24^{e}	$7.45 \pm 0.28^{c,d,e}$
LRH3C	$173.28 \pm 1.86^{n.o}$	$45.46 \pm 1.11^{d,e}$	$3.92 \pm 0.22^{b,c,d,e}$	$44.55 \pm 1.12^{b,c}$	112.56 ± 1.41^{l}	$7.07 \pm 0.26^{b,c}$
LRH8X	125.43 ± 1.18^{d}	$51.56 \pm 1.15^{f,g,h}$	$4.19 \pm 0.28^{d,e,f,g,h,i,j,k}$	$68.46 \pm 2.19^{u,v}$	97.35 ± 0.83^{f}	$13.28 \pm 1.27^{m,n}$
CC1HG6	$167.07 \pm 4.82^{k,l,m,n}$	$63.76 \pm 3.36^{\circ,p,q,r}$	$3.48 \pm 0.33^{a,b,c}$	$54.08 \pm 2.19^{h,i,j,k}$	$101.14 \pm 2.53^{f,g}$	5.16 ± 1.02^{a}
CCH3L	$227.14 \pm 5.21^{x,y}$	$54.16 \pm 2.58^{g,h,i,j,k,l}$	$4.55 \pm 0.31^{f,g,h,i,j,k,l,m,n,o}$	$51.47 \pm 2.44^{f,g,h,i}$	ND	$7.31 \pm 0.38^{b,c,d}$
CCH2P	$231.41 \pm 5.16^{y,z}$	$53.86 \pm 2.82^{g,h,i,j,k}$	$4.68 \pm 0.24^{k,l,m,n,o,p}$	$68.43 \pm 2.63^{t,u}$	ND	$7.46 \pm 0.31^{c,d,e,f}$
CC1C	$141.38 \pm 4.51^{g,h,i}$	$63.58 \pm 2.14^{\circ,p,q}$	$4.95 \pm 0.21^{n,o,p,q,r,s}$	$69.55 \pm 2.58^{u,v,w}$	265.42 ± 5.22^{n}	$7.23 \pm 0.29^{b,c}$
CM3FG14	$166.58 \pm 3.31^{k,l}$	$54.56 \pm 2.71^{g,h,i,j,k,l,m}$	$4.06 \pm 0.28^{c,d,e,f,g,h}$	$58.32 \pm 2.40^{k,l,m,n}$	ND	$6.54 \pm 0.46^{a,b,c}$
CMH1P	$132.56 \pm 3.67^{e,f}$	$64.13 \pm 2.56^{\circ,p,q,r,s}$	$3.88 \pm 0.24^{b,c,d}$	$45.59 \pm 2.56^{b,c,d,e}$	ND	$6.54 \pm 0.34^{b,c}$
CMH4X	$187.69 \pm 4.26^{r,s}$	$61.77 \pm 2.34^{\circ}$	$4.05 \pm 0.26^{c,d,e,f,g}$	$54.22 \pm 2.36^{h,i,j,k,l}$	ND	$6.87 \pm 0.36^{b,c}$
CMF1Ph	$176.38 \pm 4.31^{\circ,p}$	$44.54 \pm 2.51^{c,d,e}$	$4.36 \pm 0.26^{d,e,f,g,h,i,j,k,l}$	$58.66 \pm 2.47^{m,n,o,p}$	154.36 ± 4.68^{m}	$7.51 \pm 0.37^{c,d,e,f,g}$
CMF5C	189.48 ± 2.32^{r}	$44.55 \pm 0.36^{d,e}$	5.68 ± 0.27^{t}	$45.27 \pm 0.67^{b,c,d}$	86.34 ± 0.91^{d}	$11.43 \pm 1.06^{l,m}$
CMFX3	221.48 ± 1.18^{x}	53.42 ± 0.12^{i}	$3.44 \pm 0.32^{a,b}$	$51.28 \pm 0.57^{f,g}$	$107.24 \pm 1.04^{j,k}$	$7.55 \pm 0.55^{c,d,e,f,g,h,i}$
CMH C2	$271.39 \pm 2.14^{z``}$	41.62 ± 0.11^{b}	$4.41 \pm 0.31^{d,e,f,g,h,i,j,k,l,m}$	46.73 ± 0.75^{e}	95.68 ± 0.97^{f}	10.58 ± 0.49^{l}

§µg maltose liberated per mL of enzyme extract per min
‡µg tyrosine liberated per mL of enzyme extract per min
†µmole free fatty acid liberated per mL of enzyme extract per min
*µg glucose liberated per mL of enzyme extract per min
¶g inorganic phosphate liberated per mL of enzyme extract per min
ϕ mg D-xylose liberated per mL of enzyme extract per min
ND= not detected

Table 4
Log values of viable count (Log CFU mL^{-1}) of the selected gut bacteria (Initial count: 6 Log CFU mL^{-1} mucus) grown in skin and intestinal mucus of carps. Viable count was done on TSA plates inoculated with respective bacteria cultures of 24h in fish mucus. Data are mean ± SE (n = 3). No growth detected on plates inoculated with sterilized mucus.

Strains	Intestinal mucus	Skin mucus
LR2FG18	7.01 ± 0.10	6.89 ± 0.08
LR2FG27	6.96 ± 0.06	6.78 ± 0.10
LR2FG32	7.11 ± 0.08	6.59 ± 0.09
LR2FG33	7.06 ± 0.11	6.91 ± 0.01
LR2HG4	7.32 ± 0.10	7.01 ± 0.06
LR2HG12	7.10 ± 0.10	6.98 ± 0.01
LR2HG16	6.69 ± 0.10	6.51 ± 0.07
LR2HG22	7.23 ± 0.09	6.95 ± 0.10
LR3FG19	7.32 ± 0.06	7.09 ± 0.04
LR3FG25	6.99 ± 0.10	6.86 ± 0.10
LR1D	7.30 ± 0.01	7.04 ± 0.01
LR2F	6.81 ± 0.03	6.43 ± 0.01
LRF2C	6.76 ± 0.01	6.51 ± 0.01
LRF1Ch	6.59 ± 0.02	6.38 ± 0.01
LRH3C	7.24 ± 0.01	7.03 ± 0.01
LRH8X	7.18 ± 0.01	6.98 ± 0.01
CC1HG6	7.18 ± 0.10	6.96 ± 0.10
CCH3L	7.03 ± 0.04	6.82 ± 0.01
CCH2P	7.24 ± 0.01	7.02 ± 0.02
CC1C	6.68 ± 0.03	6.49 ± 0.02
CM3FG14	7.28 ± 0.10	6.88 ± 0.10
CMH1P	7.11 ± 0.01	6.94 ± 0.02
CMH4X	6.66 ± 0.01	6.83 ± 0.01
CMF1Ph	6.91 ± 0.02	6.75 ± 0.01
CMF5C	6.45 ± 0.03	6.74 ± 0.02
CMF X3	7.32 ± 0.02	7.16 ± 0.02
CMH C2	7.27 ± 0.01	7.08 ± 0.01

Table 5
Tolerance of the selected gut bacteria at different concentrations of fish bile juice for 1.5 h at 30 °C. Viable count was determined on TSA plates inoculated with bile exposed bacterial suspension. Data are mean ± SE (*n* = 3).

Strains	Log values of viable count (CFU/ml) on TSA plates inoculated with 20% bile-juice exposed (1.5 h at 30 °C) bacterial suspension
LR2FG18	7.11 ± 0.01
LR2FG27	7.16 ± 0.01
LR2FG32	6.91 ± 0.01
LR2FG33	6.84 ± 0.01
LR2HG4	7.04 ± 0.01
LR2HG12	7.23 ± 0.01
LR2HG16	7.08 ± 0.01
LR2HG22	6.99 ± 0.01
LR3FG19	6.88 ± 0.01
LR3FG25	6.74 ± 0.01
LR1D	6.76 ± 0.01
LR2F	6.18 ± 0.01
LRF2C	6.25 ± 0.01
LRF1Ch	6.13 ± 0.01
LRH3C	6.74 ± 0.01
LRH8X	6.62 ± 0.01
CC1HG6	6.64 ± 0.01
CCH3L	6.59 ± 0.02
CCH2P	6.46 ± 0.01
CC1C	6.12 ± 0.01
CM3FG14	6.90 ± 0.01
CMH1P	6.73 ± 0.01
CMH4X	6.28 ± 0.01
CMF1Ph	6.44 ± 0.01
CMF5C	6.38 ± 0.01
CMFX3	6.85 ± 0.01
CMHC2	6.71 ± 0.01

Table 6
Hemolysis assay of the selected bacteria performed on Columbia blood agar base supplemented with goat blood. Appearance of hemolytic zones were classified as: α (greenish halo), β (clear halo) or γ (no halo).

Strains	α-hemolytic	β-hemolytic	γ-hemolytic
LR2FG18	–	–	+
LR2FG27	+	–	–
LR2FG32	–	+	–
LR2FG33	+	–	–
LR2HG4	–	–	+
LR2HG12	–	–	+
LR2HG16	+	–	–
LR2HG22	–	–	+
LR3FG19	+	–	–
LR3FG25	+	–	–
LR1D	–	–	+
LR2F	+	–	–
LRF2C	+	–	–
LRF1Ch	+	–	–
LRH3C	–	–	+
LRH8X	–	–	+
CC1HG6	–	–	+
CCH3L	–	–	+
CCH2P	–	–	+
CC1C	+	–	–
CM3FG14	–	–	+
CMH1P	–	–	+
CMH4X	+	–	–
CMF1Ph	–	+	–
CMF5C	+	–	–
CMF X3	–	–	+
CMH C2	–	–	+

'+' Positive; '-'Negative.

Lactobacillus plantarum, isolated from common carp (*Cyprinus carpio*) intestines was studied using *in vitro* double agar layer method against *Yersinia ruckeri* (Andani, Tukmechi, Meshkini, & Sheikhzadeh, 2012). The presently reported study also noticed the presence of pathogen-inhibitory lactic acid bacteria (*Lactococcus lactis* CM3FG15) in the gut of mrigal. However, due to low cumulative inhibition score (<10), *L. lactis* CM3FG15 was not included in further characterization of probiotic properties. Furthermore, strains of *B. aerius*, *B. sonorensis* (Dutta, Banerjee, Mukherjee, & Ghosh, 2015) and *B. methylotrophicus* (Mukherjee & Ghosh, 2016) isolated from catla and *B. cereus* and *B. circulans* isolated from the GI tract of some other fish species (Lalloo, Moonsamy, Ramchuran, Görgens, & Gardiner, 2010; Geraylou et al., 2014) were established as antagonistic against diverse strains of pathogenic *A. hydrophila*. Strains of *B. methylotrophicus* isolated from channel catfish (*Ictalurus punctatus*) intestine inhibited fish pathogens causing enteric septicaemia (*Edwardsiella ictaluri*) and motile aeromonad septicaemia (*Aeromonas hydrophila*) (Ran et al., 2012). In another study, *B. subtilis* isolated from the GI tract of channel catfish inhibit *in vitro* growth of *A. hydrophila*, *A. sobria*, and *A. caviae*, *in vitro* (Luo, Bai, & Chen, 2014); while a strain of *B. sonorensis* CM2H3L isolated from the gut of *C. mrigala* was reported to inhibit pathogenic *A. salmonicida* (Dutta & Ghosh, 2015). Mukherjee et al. (2016) documented strains of *B. stratosphericus*, *B. aerophilus*, *B. licheniformis* and *Solibacillus silvestris* isolated from the GI tract of mrigal inhibited *in vitro* growth of some *Aeromonas, Psudomonas* and *Bacillus* fish pathogens. Inhibition of pathogenic aeromonads by diverse strains of bacilli (*B. methylotrophicus*, *B. amyloliquefaciens*, *B. licheniformis*) isolated from rohu were also revealed by Mukherjee et al. (2017) and Dutta et al. (2018) (*B. subtilis* subsp. *spizizenii*, *B. tequilensis*).

Although the present study detected antagonism of gut-associated bacteria against some fish pathogens, the mechanism of inhibition was not addressed. Preceding reports indicated that inhibitory activity of bacteria could be due to single or combined production of anti-microbial compounds, e.g., antibiotics (Williams & Vickers, 1986), bacteriocins (Desriac et al., 2010; Pisano et al., 2014), siderophores, lysozymes, proteases and hydrogen peroxide (Sugita, Fujie, Sagesaka, & Itoi, 2009). The double agar layer method used in the present study to evaluate the antagonistic effect of the putative probiotics detects the influence of diffusing antimicrobial substances on the growth of pathogenic bacteria (Kesarcodi-Watson et al., 2008). Thus, the present study confirmed the production of one or more antibacterial substances by fish gut bacteria isolates inhibiting *in vitro* growth of fish pathogens. In the present investigation, 105 out of 1216 bacterial isolates depicted inhibitory activity against *A. hydrophila*, *A. salmonicida* and *A. sobria*, which are most common among diseases causing by bacteria of freshwater fish (Mukherjee et al., 2017). Another pathogen used in the present study, *P. fluorescens*, has been described as an opportunistic pathogen of various fish species (Bøgwald & Dalmo, 2014). Thus, the presently reported study might support the hypothesis that antagonism between endogenous fish gut bacteria (i.e. "normal" or protective microbiota) and pathogens constitute a major component of '*defensive barrier function*' in fish (Cain & Swan, 2010, pp. 111–134; Pérez-Sánchez et al., 2011; Sahoo, Jena, Patel, & Seshadri, 2016; Hoseinifar et al., 2018; Mukherjee et al., 2019b).

Another beneficial property for the selection of probiotics is; enzyme-producing abilities (Dutta et al., 2015; Kesarcodi-Watson et al., 2008; Merrifield et al., 2010), as extracellular enzyme producing bacteria in fish gut exert positive effects to the digestive processes of the host (Ray et al., 2012). The present study demonstrated extracellular

Table 7
Antibiotic sensitivity of the selected bacteria.

Selected strains	Antibiotic concentration (mcg)													
	Am (10)	T (30)	Co(25)	Cf(5)	G (10)	E (15)	C (30)	Cp(30)	Ak (10)	B (10)	Ch(30)	Nv(30)	O (30)	Va (30)
LR2FG18	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LR2HG4	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LR2HG12	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LR2HG22	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LR1D	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LRH3C	±	–	–	–	–	–	–	–	–	–	–	–	–	–
LRH8X	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CC1HG6	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CCH3L	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CCH2P	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CM3FG14	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CMH1P	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CMFX3	±	–	–	–	–	–	–	–	–	–	–	–	–	–
CMHC2	±	–	–	–	–	–	–	–	–	–	–	–	–	–

Am: Amoxycillin; T: Tetracycline; Co: Co-Trimoxazole; Cf: Ciprofloxacin; G: Gentamicin; E: Erythromycin; C: Chloramphenicol; Cp: Cephalexin; Ak: Amikacin; B: Bacitracin; Ch: Cephalothin; Nv: Novobiocin; O: Oxytetracycline; Va: Vancomycin.
'+' Resistant; '±' Intermediately sensitive; '-'Sensitive.

amylase, protease and lipase producing abilities, and anti-nutritional factors degrading-enzymes; cellulase, phytase and xylanase of selected bacterial strains. Presence of amylolytic, proteolytic, cellulolytic and lipolytic bacteria in the GI tracts of tropical freshwater fish has been widely studied (Banerjee, Dora, & Chowdhury, 2013; Mondal et al., 2010; Ray et al., 2012). Ghosh, Sen, and Ray (2002) isolated *Bacillus* spp. strains from in the gut of rohu which were good protease and cellulase producers. Likewise, Mondal et al. (2010) isolated amylolytic, cellulolytic and proteolytic *B. licheniformis* and *B. subtilis* from the digestive tract of bata (*Labeo bata*). Ray et al. (2010) detected a large population of amylase, cellulase and protease producing bacteria in the gastrointestinal (GI) tract of three Indian major carps, catla, mrigal and rohu. However, none of these studies considered the antimicrobial potential of the enzyme producers. Previously, few reports dealt with both enzymatic and pathogen-inhibitory potential of the gut bacteria in tropical freshwater fishes (Dutta et al., 2015, 2018; Kavitha, Raja, & Perumal, 2018; Midhun et al., 2017; Mukherjee et al., 2017; Mukherjee & Ghosh, 2016), which were in accordance with the present report. Based on the present findings, we put forward the hypothesis, that the exoenzyme-producing bacteria colonizing within the GI tract of studied freshwater fish might offer protection against some fish pathogens or *vice versa*.

Furthermore, the potential of the isolates to colonize the intestine was assessed in terms of their capacity to grow in fish intestinal mucus *in vitro*. All of the 27 primarily selected gut isolates could grow in intestinal mucus. However, minor differences were recorded in bacterial growth rate within mucus, which might be due to specific nutritional requirements of the bacteria or differences in oxygen concentration or pH of the medium as suggested by Geraylou et al. (2014). Vine, Leukes, and Kaiser (2004) determined high growing rate of five candidate probiotics bacteria isolated from the gut of the common clownfish (*Amphiprion percula*), while Geraylou et al. (2014) isolated different strains of *Bacillus* spp. and *Lac. lactis* having promising probiotic characteristics from the gut microbiota of Siberian sturgeon (*Acipenser baerii*). Among the isolates, *Lactococcus lactis* ssp. *lactis* STG45 and STG81 showed high viability and highest adhesion capacity to fish intestinal mucus. In accordance to the recent observations by Mukherjee and Ghosh (2016) and Dutta et al. (2018), the presently reported study also revealed that intestinal mucus is a good growth medium for the selected putative probiotics. Therefore, it might be apprehended that the putative probiotics characterized in the present report are likely to survive through the fish GI tract and colonize.

Bile usually exhibits specific and non-specific defense mechanisms of the gut against harmful bacteria (Charteris, Kelly, Morelli, & Collins, 1998). Therefore, higher tolerance to fish bile is an important criterion for the selection of a potential aquaculture probiotic to ensure their survival and growth in the small intestine of fish (Balcázar et al., 2008). In the present investigation, the primarily selected strains showed tolerance to high concentrations of bile (20% bile juice, pH 5.5–7). This finding may be related to the ability of the tested isolates to reduce the inhibiting effect of the bile salt via bile salt hydrolase (BSH) activity (De Smet, Hoorde, Woestyne, Christiaens, & Verstraete, 1995). Bile tolerance of probiotic bacteria intended for aquaculture application has been revealed in several studies (Buntin, Chanthachum, & Hongpattarakere, 2008; Dutta et al., 2015; Mukherjee & Ghosh, 2016; Nikoskelainen et al., 2001).

Among the 27 primarily selected isolates that showed antagonism towards pathogens as well as promising exoenzymes producers were subsequently tested for haemolytic activity. In the present investigation, 14 out of 27 isolates were hemolysis-negative strains (γ-hemolytic). Non-hemolytic strain of *B. subtilis* was reported by Nayak and Mukherjee (2011). Although there is no such correlation between haemolysis and the pathogenic nature of bacteria (Inamura, Muroga, & Nakai, 1984; Kodama, Moustofa, Mikami, & Izawa, 1985), haemolytic strains should be avoided as harmful haemolytic bacteria may cause serious infection in the skin and mucous membranes (Madigan, Martinko, & Parker, 2000; Ouwehand, Salminen, & Isolauri, 2002).

Intensive aquaculture has encouraged the growth of different infectious disease of fish, which leads to a raise in the use of different antimicrobial agents and chemotherapeutics (Defoirdt, Sorgeloos, & Bossier, 2011). However, fish do not actively metabolize antibiotic and pass them back to environment. According to Burridge, Weis, Cabello, Pizarro, and Bostick (2010), 75% of the antibiotics applied with feed are released into the water. Moreover, uncontrolled use of antibiotics in aquatic environment induces a selective pressure for emergence of drug-resistant bacteria, and thus, it was strictly criticized and restricted (Romero et al., 2012; Sørum, 2006). The emergence of multidrug-resistant pathogens leading to sudden infectious disease outbreaks is the most challenging problem in the aquaculture industry, resulting in heavy economic loss (Thankappan, Ramesh, Ramkumar, Natarajaseenivasan, & Anbarasu, 2015). Therefore, an ideal probiotic must be free of any plasmid-encoded antibiotic resistance genes (Merrifield et al., 2010). In the present study, antibiotic sensitivity of the selected 14 hemolysis-negative bacterial isolates was determined using 14 broad spectrum antibiotics commonly used in aquaculture. All of the strains were highly susceptible to the tested antibiotics, except for amoxycillin (10 mcg). The presence of antibiotic sensitive strains within the fish gut was in accordance with the previous studies (Nayak & Mukherjee, 2011; Thankappan et al., 2015; Sahoo et al., 2016). In contrast, Kim and Austin (2008) reported a broad spectrum of antibiotic resistance of *Carnobacterium* strains with probiotic properties isolated from rainbow trout (*Oncorhynchus mykiss*) intestine.

The selection of probiotic candidate should be given high precedence as inappropriate microorganisms might cause an imbalance in the ratio of good-to-bad bacteria (for review see, Lazado, Caipang, & Estante, 2015). The presently reported study might propose a selection criterion of the probiotics from autochthonous source that covers functional characterization, gut stability and bio-safety. Previous reports have indicated that probiotics could be linked to the modulation of gut microbiota in fish (Allameh et al., 2016; Gómez & Balcácar, 2008; Merrifield et al., 2010). Specifically, application of the probiotics might lead to alteration of microbial diversity (Ramos et al., 2013) and decrease in the abundance of pathogenic organisms (Pereira, Pereira, Soares, Mouriño, & Merrifield, 2019) within the gut. Thus, pathogen-inhibitory gut bacteria characterized as putative probiotics in the present report might be considered as a tool for manipulation of gut microflora in the hosts, as indicated elsewhere (Andani et al., 2012; Asaduzzaman et al., 2018). However, further studies are warned on this particular issue.

5. Conclusion

The present report pretends to help the aquaculture industry and scientific community by expanding the knowledge on potentiality and composition of the culture dependant gut bacteria in the IMCs. Among the pathogen inhibitory symbiotic bacteria detected in the present study, only 14 isolates were noticed with excellent exo-enzyme producing ability, γ-hemolytic and sensitive to the commonly used antibiotics. At present, there is an effort to emphasize host-associated microbiota as probiotics for the aquaculture industry (Lazado et al., 2015). Thus, the presently reported study might offer a scope to consider gut-associated bacteria as putative probiotics for the carps. A single probiotic candidate may not be used for all fish species, as a single strain may become ineffective for a particular species (Asaduzzaman et al., 2018; Lazado et al., 2015). These 14 isolates represented by genus *Bacillus* (13 nos.) and *Stenotrophomonas* (1 nos.), might form a probiotic consortium for prospective use in composite carp culture. However, further studies involving feeding trials with large number of fish in different commercial farm conditions are needed prior to application of these consortia in aqua-farming.

CRediT authorship contribution statement

Koushik Ghosh: Conceptualization, Supervision, Data curation, Writing - original draft, Writing - review & editing. **Anjan Mukherjee:** Investigation, Methodology, Formal analysis, Software. **Dipanjan Dutta:** Investigation, Methodology. **Sudeshna Banerjee:** Investigation, Methodology. **Eva Marie Breines:** Methodology. **Ellinor Hareide:** Methodology. **Einar Ringø:** Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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