Screening of autochthonous intestinal microbiota as candidate probiotics isolated from four freshwater teleosts

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In this study altogether 109 autochthonous gut bacteria were screened from 4 fish species (*Labeo rohita, Labeo bata, Catla catla* and *Puntius javanicus*), of which 13 isolates displayed antagonism to 4 fish potent pathogens, namely *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Aeromonas sobria*. Eight promising isolates were further evaluated for extracellular enzyme production, non-hemolytic activity, bile tolerance and identified by 16S rRNA sequencing. Strains CCF7 (identified as *Bacillus* sp.), CCH9 and PJH1 (identified as two strains of *Bacillus amyloliquefaciens*) exhibited high score in antagonism assay and fulfilled other probiotic criteria, including safety aspects. However, application of these probiotics in aquaculture industries requires *in vivo* experiments and other information like immune modulating efficiency and binding ability on gut.

Keywords: Antagonism, *Bacillus* species, fish gut bacteria, pathogen, probiotics.

THE production of fish in countries like China, India, Norway, etc. has increased significantly and its demand is also expected to increase in the coming years[1]. Among several issues, the infection in aquatic animals is the most dangerous, which results in massive economic losses[2]. These disease outbreaks are mostly caused by pathogenic microorganisms of the genera *Aeromonas*, *Vibrio*, *Edwardsiella*, *Pseudomonas* and *Streptococcus*[3–5]. There are several routes through which pathogens can enter inside the body; however, entry via the mucosal surface of the gastrointestinal (GI) tract of fish is considered as the major one[6].

Conventionally, to reduce disease risk, different types of antibiotics and medicines are used in the aquaculture sector[7,8]. However, for the past few years, the use of antibiotics and chemical antimicrobials in aquaculture industries has been restricted, as they could cause major risks to human health by promoting the selection of resistant strains[5,9]. In this direction, probiotics might be an alternative tool due to their environment-friendly nature and antagonistic activity against infectious microorganisms[2,10]. According to Verschuere *et al.*[11], probiotics are live microorganisms (usually bacteria or yeast) that may confer several beneficial effects (enhance innate immunity, produce extracellular enzymes for host nutrition, produce bacteriocins and enhance growth promoting factors) to the host. The application of probiotics first started in terrestrial animals; however, their efficacy in the aquatic environment was unknown[12]. During the last decade, attempts have been made by researchers to isolate probiotic bacteria from indigenous or exogenous microbiota of aquatic animals[13]. Although several studies have reported the efficiency of probiotics in aquaculture isolated from marine fish and shellfish[14–18], only a few reports have been published regarding probiotic bacteria isolated from the gut of freshwater teleosts[19,20].

In this study, four freshwater teleosts were selected for isolation, screening and molecular identification of autochthonous gut bacteria having favourable probiotic properties such as antagonistic characteristics, production of extracellular digestive enzymes, bile tolerance, non-haemolytic nature and bio-safety aspects.

Materials and methods

Fish sampling, post-mortem examination and isolation of autochthonous bacteria

In order to isolate autochthonous gut bacteria, four fish species, namely catla (*Catla catla*), bata (*Labeo bata*), rohu (*Labeo rohita*) and java barb (*Puntius javanicus*) were collected from a fish farm near Santiniketan, West Bengal, India. The fish were kept in glass aquarium without food (starved condition) for two days to clean their digestive tracts before dissection[21]. Following sacrifice, the intestine of fish was aseptically removed within laminar airflow on ice and autochthonous bacteria were isolated according to Banerjee *et al.*[22].

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Selection of pathogenic (indicator) bacteria

Four opportunistic aquatic pathogens, *Aeromonas salmonicida* (MTCC 1945), *Aeromonas hydrophila* (MTCC 1739), *Pseudomonas fluorescens* (MTCC 103) and *Aeromonas sobria* (MTCC 3613) were selected due to their proven disease-causing ability in fish. In order to confirm the pathogenicity of these bacterial strains, immersion method with adult *L. rohita* was used[23]. All the bacterial strains trigger pathogenic effect in young fish. The progression of disease was monitored daily (data not shown).

In vitro determination of antagonistic activity of gut bacteria

The antagonistic assay was done following the double agar layer method described elsewhere[24]. Briefly, macro colonies of the test candidates were spread on Mueller Hinton agar (pH 7.0) plates, grown for 48 h in an incubator (30° ± 1°C) and killed by chloroform vapour. Then 15 ml of 1.0% agar (pH 7.0) mixed with 15 ml log phase culture of pathogens was prepared. The chloroform-killed colonies were overlaid with the above-mentioned soft agar and incubated for 12 h (30° ± 1°C). The diameter of the transparent zone around the colonies indicates the degree of inhibition.

Extracellular enzyme production by selected isolates

Extracellular enzyme-producing ability of the bacterial strains showing high antagonistic activity was confirmed both by qualitative and quantitative methods. The qualitative enzyme assay of protease, amylase, cellulase and lipase was done on agar plates supplemented with the respective substrates (peotone-gelatin, starch, carboxymethyl-cellulose and tributyrin) according the methods described elsewhere[22,25]. The quantitative enzyme activities exhibited by these bacterial isolates were measured following the method of Bairagi *et al.*[21]. In brief, the selected bacterial candidates were cultured in continuous shaking mode (30° ± 1°C, 48 h), centrifuged (10,000 *g*), and the supernatant was used for amylase[26], protease[27], cellulase[28] and lipase[29]. The concentration of protein in the supernatant was measured following the method of Lowry *et al.*[30].

Bile tolerance

Bile tolerance ability of the selected strains was checked following the method of Nikoskelainen *et al.*[31]. In brief, bacterial strains were cultured (tryptone soya broth), centrifuged (10,000 *g* for 20 min at 4°C), and the bacterial mass were collected and washed twice in 0.1 mM phosphate buffer saline (pH 7.4). The bacterial count was adjusted to 10^7 CFU/ml. Bacterial cells were re-centrifuged and re-suspended in PBS containing 2.5%–10% (v/v) bile juice (collected from rohu gall bladder)[31]. Incubation was done at 30° ± 1°C for 2 h. The bacterial viable count on agar plate was done using the serial dilution method.

Hemolytic assay

The promising bile-tolerating strains were further subjected to hemolytic activity[32]. Briefly, the selected bacterial candidate was grown on blood agar plate. The transparent zone around the colony indicates the positive sign of hemolytic activity.

Growth inhibition study

The growth inhibition efficiency of the selected probiotic candidates against these potent fish pathogens was determined according to Ringø[33] with modification. Overnight cultures of the test isolates in TSB were centrifuged, and the supernatant was collected and sterilized using syringe filters (0.22 µm pore size). In order to check the inhibitory efficacy, 5 ml of the supernatant was added to 95 ml of fresh medium inoculated with 100 µl of pathogenic bacteria culture (10^6 CFU/ml). Medium without supernatant but inoculated with the pathogens was taken as control. All flasks were incubated (30° ± 1°C, 120 rpm) and OD of the bacterial culture was determined at 600 nm (OD_{600}) at 1 h interval for 0–36 h.

Bio-safety assay of putative probiotic bacteria

This is an important step before using a probiotic candidate for commercial purpose. In this study, bio-safety experiment was done through *in vivo* trial using healthy *L. rohita* (18–20 g) as the model organism. The putative probiotic candidates were grown in TSB (pH 7.0), biomass was collected using centrifugation (3000 *g* for 20 min) and suspended in 0.1 mM PSB. Then 100 µl solution (approximately 10^9 CFU/ml) was intraperitoneally injected to experimental fish, whereas 100 µl PBS solution (without bacteria) was injected to control fish[19]. The swimming behaviour and health status were monitored up to 14 days. Both control and experimental fish were sacrificed and the degree of disease symptoms was compared.

Identification of the selected strains using 16S rRNA sequencing analysis

The promising probiotic candidates were identified by 16S rRNA sequence analysis using forward primer 27F (AGAGTTTGATCMTGGCTCAG) and reverse primer 1491R (GGTTACCTTGTTACGACTT)[22]. The obtained

raw sequence was edited, aligned and submitted to NCBI. The neighbour-joining tree was constructed using MEGA 6.0 software.

Statistical analysis

In order to understand the significance difference, all the data were subjected to one-way ANOVA, followed by Duncan's multiple range tests at the significance level $P = 0.05$.

Results and discussion

Table 1 presents the log viable count of cultural bacteria (autochthonous) in the GI tract of these fish species. Bacterial count was primarily high in the distal intestine (DI) region.

Antimicrobial activity of bacterial isolates against pathogenic bacteria has been proposed as the major criterion for the selection of probiotics in many studies[11,34]. Initially, we isolated 109 bacterial strains from the GI tract of the examined fish species and screened their antagonistic ability against four opportunistic fish pathogens, namely *A. sobria*, *A. hydrophila*, *P. fluorescens* and *A. salmonicida*. Among these 109 candidates, 13 have been primarily selected based on their ability to inhibit pathogens (Table 2). All the selected 13 isolates exhibited antagonistic activity against *P. fluorescens*, whereas 5, 11 and 8 strains were recorded to be active against *A. hydrophila*, *A. salmonicida* and *A. sobria* respectively. Based on the activity score, the strain CCF7 isolated from the proximal intestine (PI) of catla was taken as a promising one (score 14), followed by PJH1 (13) and CCH9 (11) respectively.

Eight isolates with total score ≥5 in the antagonism assay were considered as promising and further assayed for their extracellular enzyme-producing ability (Table 3). The strain CCF7 showed the highest amylase and protease activity (33.5 ± 1.15 U and 3.66 ± 0.12 U respectively), whereas cellulase (22.03 ± 0.72 U) and lipase (5.15 ± 0.20 U) activities were detected to be maximum in the strains LRH3 and PJH1 respectively. In recent years, several reports have been published regarding the enzyme (protease, amylase, lipase, cellulose, etc.) producing ability of the GI tract bacteria of fish[22,35–37]. It has been suggested that enzyme-secreting GI tract bacteria play an important role in digestion of the host[38]. Therefore, from a nutritional point of view, probiotic bacteria gain special attention in aquaculture industries[39].

Table 1. Log viable count of autochthonous bacteria in the gut of examined fish

Fish species	Log total viable count (per g of intestinal tissue)	
	Proximal intestine	Distal intestine
Labeo rohita	5.17 ± 0.09	5.85 ± 0.07
Catla catla	5.79 ± 0.12	5.61 ± 0.17
Labeo bata	4.91 ± 0.16	5.23 ± 0.15
Puntius javanicus	6.3 ± 0.11	7.41 ± 0.08

Data are presented as mean ± SD of five determinants.

Table 4 presents the bile tolerance efficiency exhibited by the eight selected isolates. Interestingly, three bacterial strains – CCF7, CCH9 and PJH1 showed higher tolerance to bile, and hence were selected for further studies. According to Ramesh *et al.*[40], the bile tolerance ability of probiotic candidate is an important characteristic, as it has to work in the gut environment (high bile concentration). Similar observations have also been reported by probiotic bacteria[19,39]. The evaluation of haemolytic activity is another important selection criterion of probiotic strain, as haemolytic bacteria may cause malfunction in the defence system[41,42]. Surprisingly, none of the selected candidates exhibited haemolytic activity.

Until now, reports regarding *in vitro* inhibition of aquatic pathogens in liquid media are scanty, and thus we have determined the growth pattern of these pathogens in the presence of culture broth of probiotic candidates. Figure 1 shows the growth inhibition of *A. hydrophila*, *A. salmonicida*, *A. sobria* and *P. fluorescens* by the sterile filtered supernatant of CCF7, CCH9 and PJH1. Among three probiotic candidates, CCF7 was recorded to be most effective against all the four pathogens. An early stationary phase was detected in *A. hydrophila* treated with cell-free soup of these three isolates compared to positive control (Figure 1 *a*), whereas in the case of *A. salmonicida*, *A. sobria* and *P. fluorescens*, the growth curve patterns were quite different. The culture supernatant of probiotic candidates inhibited growth of these pathogens at the primary stage and as a result lag phase was extended in each case (Figure 1 *b–d*). However, in a similar study, Ringø[33] isolated several probiotic candidates from Arctic Atlantic salmon (*Salmo salar*), charr (*Salvelinus alpinus*), and wolf fish (*Anarhichas lupus*) and tested their antagonistic activity against several pathogens. Similarly, Askarian *et al.*[43] confirmed antimicrobial activity of gut bacterium (*S. salar*) *Bacillus thuringiensis* against some fish pathogens.

Bio-safety towards the host is a vital prerequisite for any probiotic bacteria to be used for commercial purposes[11]. To confirm the bio-safety nature of the selected probiotic candidates (CCF7, CCH9 and PJH1), we conducted a small-scale *in vivo* trial using healthy *L rohita*. After 14 days, no external or internal signs of disease or mortality were recorded in the experimental fish. Thus, these three candidates are considered to be safe for fish and might be useful in aquaculture industries.

Finally, three selected isolates CCF7, CCH9 and PJH1 were identified by partial 16S rRNA sequence analysis (Table 5), which revealed that all three isolates belong to *Bacillus* species, which is fairly common in the gut

Table 2. *In vitro* antagonistic activity of gut bacterial isolates against potent fish pathogens

Fish species examined	Bacterial strains	Inhibition of pathogenic strains (score)				Total score
		AH	AS	ASO	PF	
L. rohita	LRF1	1	2	2	3	8
	LRH2	0	1	0	2	3
	LRH3	0	2	1	4	7
	LRH4	0	4	0	4	8
C. catla	CCF5	0	1	0	2	3
	CCF7	3	4	3	4	14
	CCH9	2	3	2	4	11
L. bata	LBF3	1	3	0	3	7
	LBF4	0	1	3	2	6
	LBH1	0	0	2	2	4
P. javanicus	PJH1	3	4	3	3	13
	PJH2	0	0	1	2	3
	PJH5	0	2	0	2	4

AH, *Aeromonas hydrophila*; AS, *Aeromonas salmonicida*; ASO, *Aeromonas sobria* and PF, *Pseudomonas fluorescens*. 4, Very high (above 20 mm inhibition zone diameter); 3, High (15–20 mm inhibition zone diameter); 2, Moderate (10–14 mm inhibition zone diameter); 1, Low, (5–9 mm inhibition zone diameter) and 0, No inhibition.

Table 3. Quantitative extracellular enzyme activity of selected bacterial candidates

Bacterial strains	Amylase activity (U)[1]	Protease activity (U)[2]	Cellulase activity (U)[3]	Lipase activity (U)[4]
LRF1	19.49 ± 0.75^{d}	1.37 ± 0.14^{c}	11.87 ± 0.37^{c}	3.51 ± 0.16^{c}
LRH3	24.34 ± 0.57^{e}	1.09 ± 0.15^{d}	22.03 ± 0.72^{a}	3.9 ± 0.10^{b}
LRH4	8 ± 0.50^{f}	0.14 ± 0.03^{e}	–	3.97 ± 0.14^{b}
CCF7	33.5 ± 1.15^{a}	3.66 ± 0.12^{a}	8.77 ± 0.31^{e}	3.67 ± 0.11^{c}
CCH9	32.97 ± 0.61^{a}	3.44 ± 0.12^{a}	10.08 ± 0.41^{d}	3.49 ± 0.13^{c}
LBF3	18.65 ± 0.96^{d}	3.12 ± 0.27^{b}	10.8 ± 0.44^{d}	4.02 ± 0.18^{b}
LBF4	26.78 ± 0.66^{b}	3.2 ± 0.18^{b}	13.17 ± 0.54^{b}	5.15 ± 0.20^{a}
PJH1	9.45 ± 0.54^{e}	1.6 ± 0.09^{c}	2.4 ± 0.09^{f}	–

Data are mean ± SD of five determinants. Means in the same column with different superscript letters are significantly different ($P < 0.05$).
(U)[1] – µg maltose liberated/mg of protein/ml of culture filtrate. (U)[2] – µg tyrosine liberated/mg of protein/ml of culture filtrate. (U)[3] = µg glucose liberated/mg of protein/ml of culture filtrate. (U)[4] – µmol of fatty acid liberated/mg of protein/ml of culture filtrate.

Table 4. Bile tolerance ability of the selected strains

Bacterial strains	Initial mean counts (log CFU/ ml)	log CFU/ml at different bile concentrations (%)			
		2.5	5.0	7.5	10.0
LRF1	7.05 ± 0.02	6.91 ± 0.03	6.85 ± 0.03	6.7 ± 0.02	6.52 ± 0.05
LRH3	7.14 ± 0.04	7.08 ± 0.02	6.94 ± 0.02	6.82 ± 0.03	6.75 ± 0.03
LRH4	6.88 ± 0.02	6.79 ± 0.06	6.65 ± 0.04	6.53 ± 0.02	6.38 ± 0.05
CCF7	7.15 ± 0.05	7.1 ± 0.03	7.08 ± 0.03	7.02 ± 0.03	6.98 ± 0.02
CCH9	7.03 ± 0.03	7.01 ± 0.01	6.95 ± 0.05	6.91 ± 0.03	6.87 ± 0.02
LBF3	6.9 ± 0.01	6.83 ± 0.04	6.75 ± 0.03	6.58 ± 0.02	6.35 ± 0.03
LBF4	7.08 ± 0.05	7.02 ± 0.04	6.94 ± 0.01	6.89 ± 0.04	6.75 ± 0.03
PJH1	7.16 ± 0.02	7.11 ± 0.03	6.98 ± 0.06	6.93 ± 0.01	6.89 ± 0.02

Data are presented as mean ± SD of three determinants.

mucosa of tropical freshwater fish[32,36,37,44]. Strain CCF7 (GenBank accession no. KP256501.1) exhibited high similarity with *Bacillus* species (GenBank accession no. KM974803.1), whereas CCH9 and PJH1 (GenBank accession nos KP256502.1 and KT719406.1 respectively) showed close similarity with *Bacillus amyloliquefaciens* at 95% and 98% respectively. Few recent studies have reported the antipathogenic potential of gut-adherent *B. amyloliquefaciens* from freshwater fish[36,45]. Figure 2 presents the phylogenetic relationship/tree of these three

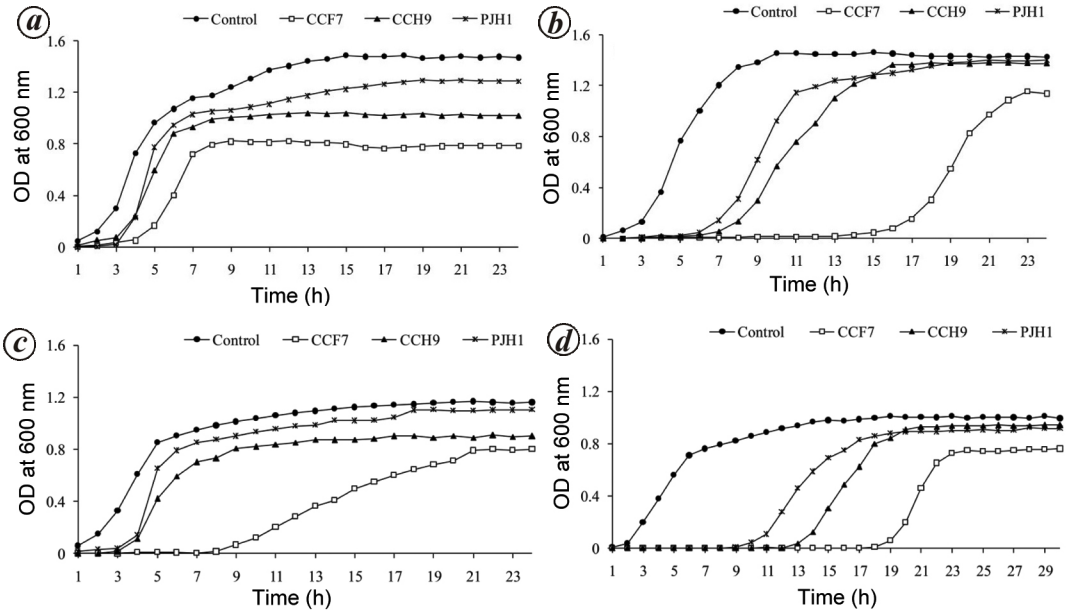

Figure 1. *In vitro* growth inhibition of (***a***) *Aeromonas hydrophila*, (***b***) *Aeromonas salmonicida*, (***c***) *Aeromonas sobria* and (***d***) *Pseudomonas fluorescens* by extracellular extracts of CCF7, CCH9 and PJH1.

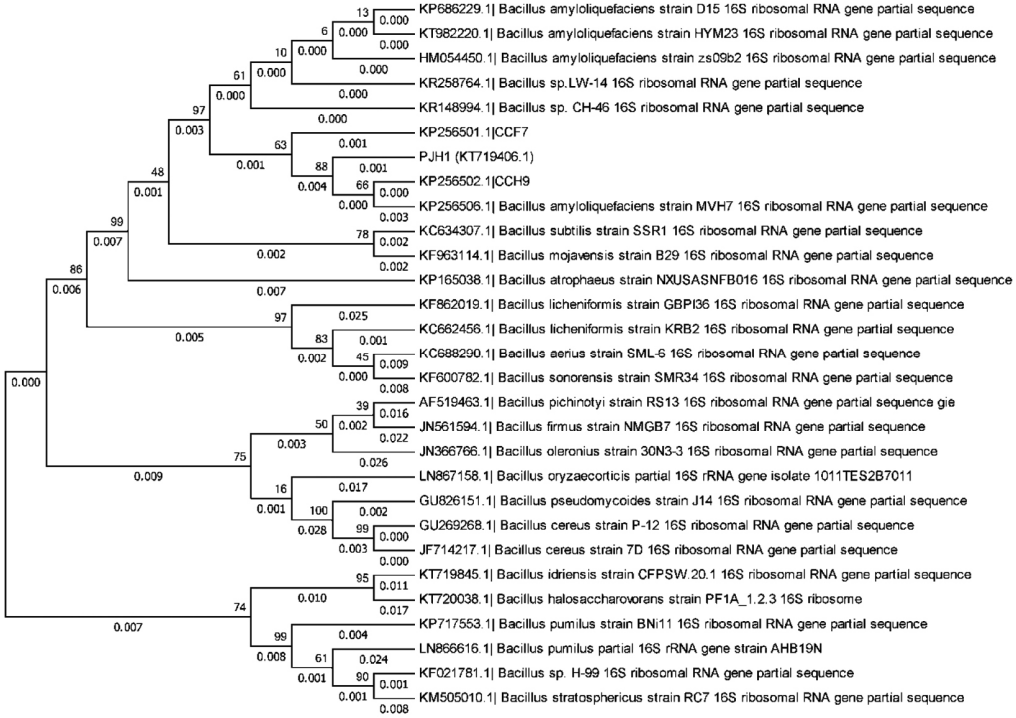

Figure 2. Dendrogram showing phylogenetic relations of CCF7, CCH9 and PJH1 with other closely related strains available at NCBI database.

Table 5. The closest homologues of isolates obtained by blasting partial 16S rRNA gene sequences with NCBI GenBank database

Studied sample	Accession no.	Sequence similarity (%)	Sequence description (GenBank accession no.)
CCF7	KP256501.1	98	*Bacillus* sp. ap-1(KM974803.1)
CCH9	KP256502.1	95	*Bacillus amyloliquefaciens* subsp. *plantarum* (JN700124.1)
PJH1	KT719406.1	99	*Bacillus amyloliquefaciens* strain zs09b2 (HM054450.1)

promising strains. The bootstrap values represent the similarity and homology of the neighbouring sequences.

Conclusion

The use of probiotics (as an alternative to antibiotics) in the aquaculture sector is increasing rapidly due to its low cost and environment-friendly nature. In the present study, we have tested the probiotic efficiency of three fish gut bacterial candidates (*Bacillus* sp. CCF7, *B. amyloliquefaciens* CCH9 and *B. amyloliquefaciens* PJH1). Along with major probiotic properties (antimicrobial activity, enzyme production, bio-safety, etc.), these strains also have endospore-forming ability which will enhance the storage duration of fish feed. However, the commercial use of these probiotics candidates needs further studies. Further, long-term *in vivo* studies are required to determine their applicability in aquaculture environment.

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