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# Characterization of *Bacillus* spp. From the Gastrointestinal Tract of *Labeo rohita*—Towards to Identify Novel Probiotics Against Fish Pathogens

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**Abstract** The aim of the present study is to screen and characterize endogenous microbiota *Bacillus* spp. from the gastrointestinal (GI) tract of *Labeo rohita* in order to evaluate their probiotic attributes. A total of 74 isolates from the GI of *L. rohita* were evaluated for their antimicrobial properties by agar well-diffusion method against fish pathogens. Based on the better antibacterial features, three isolates (KADR1, KADR3, and KADR4) were selected for further delineation. The three selected isolates exhibited higher tolerance to bile salt, moderate tolerance to low pH, high surface hydrophobicity to solvents, and capable to autoaggregate. All three isolates demonstrated notable proteolytic, catalase activity and susceptibility to various antibiotics. Partial 16S rRNA sequencing revealed that the isolates exhibited 99 % sequence homology with *Bacillus subtilis*, *Bacillus aerophilus*, and *Bacillus firmus* of the database substantiating morphological and physiological characterization. Survivability in low pH and bile salt ensures their adaptability in the fish intestinal microenvironment. The ability to autoaggregate reveals colonization potential in the GI of the fish. Absence of hemolytic activity, antibiotic susceptibility to certain antibiotics, presence of protease and catalase activity, and non-pathogenic caliber of the above-mentioned isolates could be feasible characteristics when considering them as probiotics in the aquaculture industry.

**Keywords** Aquaculture · *Bacillus* spp. · Probiotics · Fish diseases · Antibiotics

## Introduction

The aquaculture industry stands to be one of the fastest growing animal food-producing sector of the world's economy, complementing the demand of animal protein food to the continuously growing human population. The major delimiting factor in aquaculture production is the

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emergence of infectious diseases caused by bacteria, fungi, virus, and protozoan parasites resulting in extensive sudden lose. Large quantity of chemicals and antibiotics are employed in fishery management in order to prevent fish diseases, which successfully warded off the issue to some extent. However, the continuous use of the antibiotics imposes a threat to the environment via the emergence of antibiotic-resistant pathogenic strains [1–4]. This stimulated the hunt for a novel prophylactic/treatment alternative to fishery biologist. In this line, probiotics are ascertained to be the suitable natural substitute with potent characteristics, which is accepted worldwide. Probiotics are live cell microorganisms imparting numerous health benefits to the host organism by improving innate immunity, antimicrobial activity, enzyme contribution for digestion, feed value, enhancing growth promoting factors, etc. [5].

Among the freshwater aquaculture commodities, the species belonging to Indian major carps such as *Labeo rohita*, *Cata catla*, and *Cirrhinus mirgala* are the most important cultivable fish species commonly found in the ponds, lakes, and rivers in Southeast Asia. These species are highly prone to *Aeromonas hydrophila* infection associated with tail and fin rot [6–11], motile aeromonad septicemia (MAS), and epizootic ulcerative syndrome [12], restricting the yield by causing high mortality and morbidity [13]. To overcome this issue, continuous use of antibiotics is unavoidable in farmers' routine protocol, resulting in causing major changes in the normal microbiota in the surrounding environment and the gastrointestinal tract of fish species and increasing the risks of emergence of multidrug resistance infectious pathogens [1–4].

Research on the live cell preparations in aquatic organisms is being raised to sustain the aquaculture industry. *Lactobacillus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., and *Saccharomyces cerevisiae* are the common probiotics employed for growth improvement in carps [14, 15]. Among *Bacillus* spp. the most widely used species includes *Bacillus subtilis*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus clausii*, *Bacillus megaterium*, *Bacillus licheniformis, Bacillus circulans*, and *Bacillus polymyxa* [16–18]. *Bacillus* spp. exhibit significant probiotics features than the *Lactobacillus* spp. owing to their inherent potential to produce spores which confers them tolerance to heat and longer shelf-life. In addition, production of diverse industrially significant secondary metabolites such as acetic acid, lactic acid, hydrogen peroxide, polymixin, bacitracin, gramicidin, bacteriocins etc. has been reported in *Bacillus* spp. [19–21] which make them an attractive target for research in recent years. Evidently, a number of *Bacillus* products were designated as GRAS by FAO recently and being licensed, conventionally applied for growth improvement and disease resistance in aquaculture. Some of the examples include BioPlus2B (*B. subtilis* and *B. licheniformis*) [22], BioStart™ HB-1 (*B. subtilis, B. megaterium*, and *B. polymyxa*), and BioStart™ HB-2 (*B. licheniformis*) [16]. Due to the increasing demand for aquaculture commodities, intensive research in the field is carried out by many researchers for identification of lead probiotics with improved characteristics [5, 14, 15, 22]. In this line, the present study was focused to identify and characterize *Bacillus* spp. from the intestinal microbiota of *L. rohita*. Based on the study, three novel species were identified and characterized to confirm probiotic properties using various biochemical examinations and 16S rRNA sequence; phylogenetic analysis illustrates the close relationship of the isolates KADR1, KADR3, and KADR4 with the commercial probiotic strains.

## Materials and Methods

### Sample Collection and Isolation

Indian major carp, *L. rohita* (Hamilton) of average weight (>20 g) were collected from the Cauvery River, Tiruchirappalli District, Tamil Nadu, India and brought alive to the laboratory.

Ventral surface sterilization was done using double distilled water followed by 70 % ethanol. Under sterile conditions, the fish gut was dissected out and homogenized with 5 ml of normal saline. The homogenate was kept in a boiling water bath at 80 °C for 20 min to remove fungal contaminants. The homogenate was serially diluted and pour plated onto *Bacillus* agar medium pH 7.2, containing 6 g peptone, 3 g tryptone, 3 g yeast extract, 1.5 g beef extract, 1 mg $MnSO_4.4H_2O$, and 15 g agar-agar per liter purchased from Himedia (Mumbai, India). Plates were incubated at 37 °C overnight. Single colonies were picked and purified by streaking onto fresh *Bacillus* agar medium. Totally, 74 isolates were identified and stored at −80 °C in *Bacillus* broth supplemented with 30 % glycerol.

## In vitro Antimicrobial Activity

Agar well-diffusion method was carried out based on the protocol of Schillinger and Lucke [23] to detect the antimicrobial activity of all the isolates (74 in number) against target fish pathogens such as *Aeromonas hydrophila* ATCC 49140, *A. hydrophila* MTCC 1739, *Providencia rettgeri* JX136696, *Aeromonas* sp. JX136697, *Aeromonas* sp. JX136698, and *Aeromonas enteropelogenes* JX136699. Briefly, 0.1 ml of each above pathogen (~$10^5$ colony-forming units (CFU)$ml^{-1}$) was spread on Mueller-Hinton agar plates, wells with a diameter of 6 mm were made and filled with $10^6$–$10^7$ CFU $ml^{-1}$ live suspension of isolated culture (from the gastrointestinal tract (GI) of the fish). Plates were incubated at 37 °C for 24 h, and the zone of inhibition was recorded. The isolates demonstrating potent antagonistic activity against the tested pathogens were assessed for further probiotic properties.

## Tolerance to Low pH and Bile Salt

The selected three isolates named as KADR1, KADR3, and KADR4 were further characterized to identify their potent probiotic characteristics, such as tolerance to low pH and bile salt based on the method of Ahire et al. [24]. The isolates were grown overnight in *Bacillus* broth at 37 °C and pelleted at 8,000*g* for 5 min at 4 °C. Cells were washed twice with sterile phosphate buffer saline (PBS, pH 7.3) and resuspended in 1 ml PBS. Each isolate was diluted (1:100) in PBS of pH 1, 2, 3, and 4 followed by incubation at 37 °C in different time intervals such as 0, 1, 2, and 3 h, and the viability of the bacterial cells were determined in terms of CFU $ml^{-1}$ in *Bacillus* agar plate. The survivability of the isolates in different pH after 3 h of incubation has also been represented in percentage.

Bile salt resistance of the three isolates KADR1, KADR3, and KADR4 were determined by inoculating the bacterial isolates in bacillus broth containing 2.5, 5.0, 7.5, and 10 % of bile salt followed by incubation at 37 °C for 3 and 6 h. The growth medium with 0 % bile salt served as control. The treated cells were then evaluated by recording absorbance at 595 nm using ELISA reader (BioRad). Survivability of the isolates was represented by percentage.

## Hydrophobicity Assay

Hydrophobicity assay was conducted to evaluate the ability of the isolates adhered to solvents following the method of Thapa et al. [25]. Three different solvents, namely, xylene, chloroform, and ethyl acetate, were used to determine the isolates surface hydrophobicity. Briefly, the overnight grown cells were collected by centrifugation at 6000*g*, washed three times with PBS, resuspended in 10 ml Ringer's solution, and $OD_{600}$ was measured ($A_0$) as control. In tested sample, cell suspension was mixed with equal volume of solvent by vortexing for about 2 min and kept at room temperature for 30 min. The aqueous phase was removed and

absorbance measured at 600 nm ($A_1$). The hydrophobicity of bacterial adhesion to solvent was calculated using the formula $(1-A_1/A_0)\times 100$.

## Autoaggregation Assay

Autoaggregation assay was performed following the method of Patel et al. [26] with few modifications. Cells were collected from overnight culture by centrifugation, washed thrice with PBS (pH 7.3), and resuspended to obtain $OD_{595}$ 0.5, 4 ml of the cell suspension was gently vortexed for 10 s and incubated at 37 °C for 2 h. After incubation, supernatant was removed and absorbance measured at 595 nm using UV–Vis Spectrophotometer [27] (Jasco V-550, USA) was expressed in percentage following the formula: $1-(A_t/A_0)\times 100$, where $A_t$ represents the absorbance of cell suspension at time $t=2$ h and $A_0$ the absorbance at $t=0$.

## Gastric Juice Tolerance

Gastric juice tolerance was estimated following the protocol described by Ahire et al. [24]. Cell suspension was diluted 1:10 in synthetic gastric juice (pH 2.5) and incubated at 37 °C. The survival rate of the isolates were measured at 0, 0.5, and 3 h by spreading on *Bacillus* agar plates, which were then incubated at 37 °C for 24 h. Tolerance of the isolates in the presence of gastric juice were represented in CFU $ml^{-1}$ and percentage.

## Antibiotic Susceptibility Assay

Antibiotic susceptibility of the selected three isolates was evaluated against the antibiotics such as ampicillin, amoxicillin, cephalaxin, streptomycin, penicillin-G, gentamycin, erythromycin, chloramphenicol, kanamycin, tetracycline, and rifampicin. Cells grown overnight at 37 °C, normalized to $OD_{595}$ 0.5 were spread on Muller-Hinton agar (Himedia, Mumbai, India) to check the antibiotic susceptibility test. Antibiotic discs were dispensed on to the plates and incubated at 37 °C for 24 h. Zone of inhibition was measured (mm), and the antibiotic sensitivity was recorded as different grades based on their activity.

## Pathogenicity Test

The pathogenicity of the three isolates, KADR1, KADR3, and KADR4 were tested against Rohu (*L. rohita*, Hamilton) of average weight 12–16 g obtained from commercial fish farms in the Thanjavur district of the Cauvery delta. Twelve tubs were maintained, each containing 15 fish for four sets of experiment in triplicates. The fish were acclimatized for a week, supplemented with commercial feed at ~5 % of the body weight for each fish group prior to experimentation and maintained in de-chlorinated freshwater. Each group was injected intra-peritoneally with 0.5 ml of fresh culture of either KADR1, KADR3, or KADR4 in triplicates containing $10^8$ cells $ml^{-1}$. The group administered with PBS served as the control in triplicates. Mortality (if any) was observed in the groups of control and treated fish daily for 10 days.

## Proteolytic and Amylolytic Activity

The overnight-grown bacterial isolates, KADR1, KADR3, and KADR4, were inoculated on casein hydrolysis milk powder agar for determination of proteolytic activity. Plates were incubated at 37 °C for 48 h, and the halos or clear zone around the colonies were recorded and tabulated.

Amylolytic activities were determined according to Keleke et al. [28]. Briefly, the three isolates were streaked onto total amylolytic bacteria (TAB) agar media supplemented with starch and incubated at 37 °C for 48 h. Lugol solution was overlaid on the surface of cultured plate for visualization of the clear zone around the colonies.

### Catalase and Hemolytic Assay

Catalase assay was performed as described by Barbosa et al. [29]. Overnight bacterial culture was resuspended with 3 % of hydrogen peroxide solution to check the formation of gas bubbles, indicating positive results (Catalase-positive). Hemolysis was determined on nutrient agar supplemented with 5 % RBC.

### Strain Characterization

Morphological and biochemical characterization of the isolates were carried out for preliminary identification of the strain. Further, for molecular analysis, genomic DNA of each isolates was extracted from the 12 h culture following the phenol-chloroform method [30]. The 16S rRNA gene of the isolates were amplified using Universal 27 F Forward (5′-CCAGAATTCAGAGTTTGATCMTGGCTCA-3′), and 1492R reverse (5′-ACCAAGCT TTACGGYTACCTTGTTAGGACTT-3′) primers in a Thermal cycler (Eppendorf) under the following condition: 95 °C (5 min) initial denaturation, followed by 34 cycles of denaturation at 94 °C (1 min), annealing at 58 °C (1 min), extension at 72 °C (3 min), and a final extension at 72 °C (7 min). The PCR products were separated on 1 % agarose gels and imaged. Amplicons were eluted and the purified DNA products were sequenced (Eurofins Genomics India Pvt. Ltd., Bangalore). The chromatograms were compared with the available nucleotide sequence of *Bacillus* species in the National Center for Biotechnology Information (NCBI) database. The gene sequences of KADR1, KADR3, and KADR4 were identified and subsequently submitted to NCBI and accession numbers were obtained.

For phylogenetic tree construction, the 16S rRNA gene sequences of the strains in FASTA format were downloaded from the NCBI database along with the gene of commercial strain, *B. subtilis* AF142577, in order to determine the sequence homology of the identified isolate in this study with the latter. All the collected sequences were aligned using the multiple sequence alignment program, CLUSTAL W. Raw form of the phylogenetic tree was downloaded, saved, and employed to construct the tree using Fig Tree v1.3.1.

### Statistical Analysis

All experiments were performed in triplicates, and results were expressed as mean±standard deviation using SPSS for Windows version 11.5 (SPSS, Chicago, IL).

## Results

### Screening and Identification of the Isolates

Seventy four isolates obtained from the GI tract of healthy *L. rohita* were screened based on gram staining (Gram +) and best antibacterial activity against the target fish pathogens. Three isolates named as KADR1, KADR3, and KADR4 exhibiting notable antimicrobial activity

against the target fish pathogens (Table 1) were selected for further probiotic characterization, very low (<1 mm)/no antagonistic activity was observed in the remaining seventy one strains tested against the target fish pathogens. Partial 16S rRNA sequences of the isolates demonstrated high sequence homology with *Bacillus* species (99 %). The sequences were submitted to the Genbank database and the following accession numbers were obtained, *B. subtilis* KADR1 JQ302302, *Bacillus aerophilus* KADR3 JQ312663, and *B. firmus* KADR4 JQ822106. Using Fig Tree v1.3.1 software, the phylogenetic tree was constructed with strains exhibiting 99 % similarity such as *B. megaterium* EU147197.1, *B. subtilis* EU137641.1, *B. pumilus* EU147184, *B. aerophilus* JQ312663, *B. licheniformis* EF156868, *Bacillus atrophaeus* EU138516.1 etc. (Fig. 1). *B. subtilis* KADR1 JQ302302 depicted very close relationship (99 %) with the commercial strain *B. subtilis* AF142577.

## Tolerance to Low pH and Bile Salt

The identified probiotic isolates KADR1, KADR3, and KADR4 presented wide range of pH tolerance. Among the three isolates, KADR1 showed highest percentage viability of 90.90 % after 3 h of exposure compared to 85.71 and 84.61 % demonstrated by the isolates, KADR3 and KADR4, respectively, at highly acidic pH 2. At pH 3 and 4, KADR1 showed 94.23 and 96.14 % survivability, respectively after 3 h incubation. KADR3 displayed 96.22 % (pH 3) and 96.40 % (pH 4) survivability; however, the survivability of KADR4 at pH 3 was comparably lesser, 92.10 % and it turned out to be 98.34 % at pH 4. The survivability of the isolates expressed in terms of CFU $ml^{-1}$ and percentage is shown in Table 2. At pH 1, neither of the isolates were able to survive.

All the tested isolates were able to grow at increasing concentration of bile salt (Fig. 2). KADR4 recorded greater survivability of 78.75, 76.41, 66.86, and 67.44 % at 2.5, 5.0, 7.5, and 10 % of bile salt, respectively after 3 h of incubation. However, after 6 h the survivability was reduced to 25.71, 25.71, 23.80, and 25.39 %, respectively at increasing concentration of bile salt. KADR3 exhibited 67.43, 55.47, 61.57, and 60.81 % survivability, respectively after 3 h of incubation, while 41.12, 34.63, 32.32, and 31.31 % survivability was recorded after 6 h of incubation. Survivability percentage of KADR1 was 58.05, 49.93, 49.43, and 47.06 % after 3 h and 27.35, 25.06, 20.77, and 19.68 % after 6 h, respectively at increasing concentration of bile salt.

**Table 1** Antibacterial activities of the probiotic *Bacillus* sp. isolated from *Labeo rohita* against reference fish pathogens

| Name of the fish pathogens | Test probiotic organisms | | |
|---|---|---|---|
| | KADR1 (JQ302302) | KADR3 (JQ312663) | KADR4 (JQ822106) |
| *Aeromonas hydrophila* (ATCC 49140) | +++ | +++ | + |
| *A. hydrophila* (MTCC 1739) | +++ | ++ | ++ |
| *Providencia rettgeri* KADR11JX136696 | ++ | ++ | +++ |
| *Aeromonas* sp. KADR12 JX136697 | +++ | +++ | + |
| *Aeromonas* sp. KADR13 JX136698 | ++ | ++ | ++ |
| *A. enteropelogenes* KADR14 JX136699 | +++ | ++ | +++ |

+ zone of inhibition between 1 and 2 mm, ++ zone of inhibition between 2 and 4 mm, +++ zone of inhibition above 4 mm

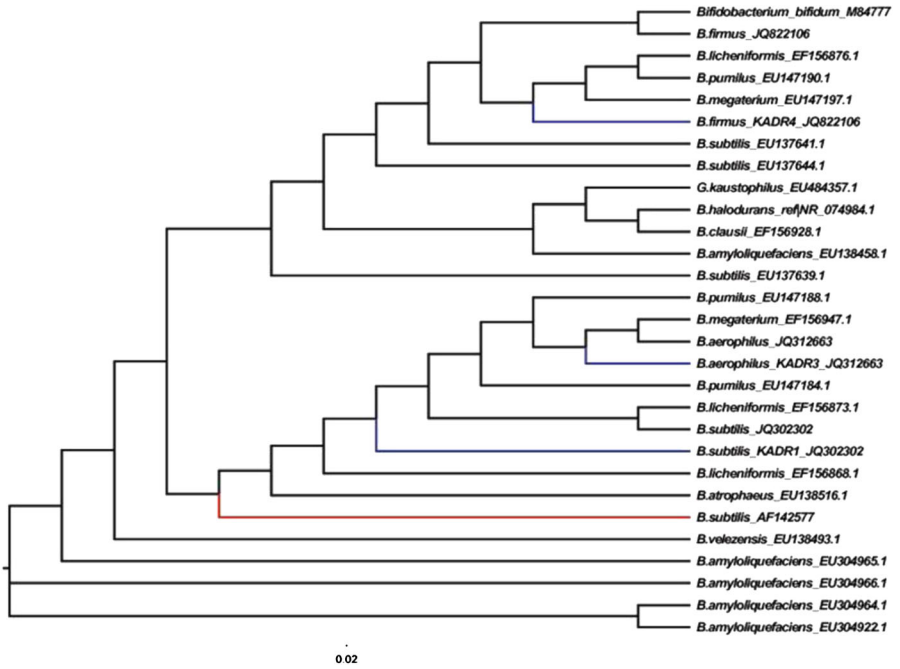


**Fig. 1** Phylogenetic tree showing species relatedness of *Bacillus* isolates identified from gastrointestinal tract of *Labeo rohita*. The *red colored line* represents the commercial *Bacillus* strain and *the blue colored line* denotes the *Bacillus* isolates identified in the present study

**Table 2** Survivability of the probiotic isolates in different pH over different time-points in (CFU $ml^{-1}$)

| Name of the isolates | pH | Viability of bacteria in CFU $ml^{-1}$ ($\times 10^6$) | | | | % of survivability after 3 h |
|---|---|---|---|---|---|---|
| | | 0 h | 1 h | 2 h | 3 h | |
| KADR1 (JQ302302) | 1 | 0.07±0.011 | 0±0.00 | 0±0.00 | 0±0.00 | 0 |
| | 2 | 0.22±0.011 | 0.21±0.008 | 0.21±0.004 | 0.20±0.011 | 90.90 |
| | 3 | 2.43±0.081 | 2.35±0.050 | 2.31±0.096 | 2.29±0.105 | 94.23 |
| | 4 | 2.85±0.141 | 2.78±0.091 | 2.75±0.121 | 2.74±0.091 | 96.14 |
| KADR3 (JQ312663) | 1 | 0.25±0.009 | 0±0.00 | 0±0.00 | 0±0.00 | 0 |
| | 2 | 0.28±0.013 | 0.25±0.009 | 2.24±0.016 | 2.24±0.007 | 85.71 |
| | 3 | 2.65±0.079 | 2.61±0.045 | 2.58±0.110 | 2.55±0.060 | 96.22 |
| | 4 | 2.78±0.095 | 2.75±0.078 | 2.72±0.113 | 2.68±0.100 | 96.40 |
| KADR4 (JQ822106) | 1 | 0.06±0.006 | 0±0.00 | 0±0.00 | 0±0.00 | 0 |
| | 2 | 0.13±0.004 | 0.12±0.007 | 0.11±0.004 | 0.11±0.012 | 84.61 |
| | 3 | 2.28±0.173 | 2.25±0.098 | 2.18±0.085 | 2.10±0.080 | 92.10 |
| | 4 | 2.42±0.115 | 2.41±0.096 | 2.40±0.101 | 2.38±0.085 | 98.34 |

Each value is the mean±standard deviation of three separate experiments

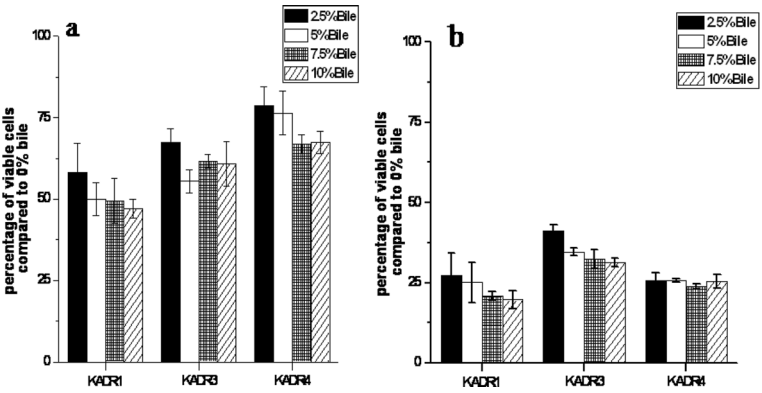


**Fig. 2** **a** Probiotics isolates bile salt tolerance after 3 h at 37 °C. **b** Probiotics isolates bile salt tolerance after 6 h at 37 °C. Values are presented as mean±SD and in terms of percentage

## Hydrophobicity and Autoaggregation

Adhesion ability of the three probiotic isolates were examined in xylene, chloroform, and ethyl acetate (Fig. 3). KADR1 was found to adhere with xylene at 23.90 %, chloroform at 41.15 %, and ethyl acetate at 14.35 %, whereas least adhesion was observed with the isolate KADR3, which displayed 3.44 % in xylene, 8.8 % in chloroform, and 4.96 % in ethyl acetate. The percentage of hydrophobicity for KADR4 was 33 % in xylene, 34.86 % in chloroform, and 6 % in ethyl acetate.

Autoaggregation was investigated on the basis of sedimentation characteristics of the three isolates. KADR4 showed a maximum autoaggregation of 46.78 % followed by KADR1 and KADR3 which showed 42.18 and 39.59 %, respectively (Fig. 4).

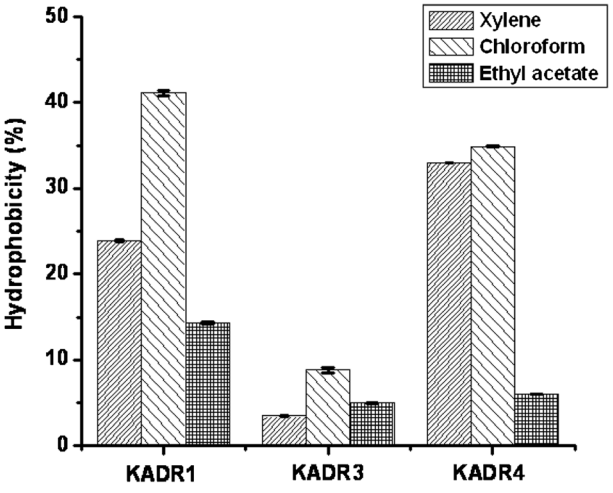


**Fig. 3** Probiotics isolates cell surface hydrophobicity against various solvents. Each value is the mean±SD of three separate experiments

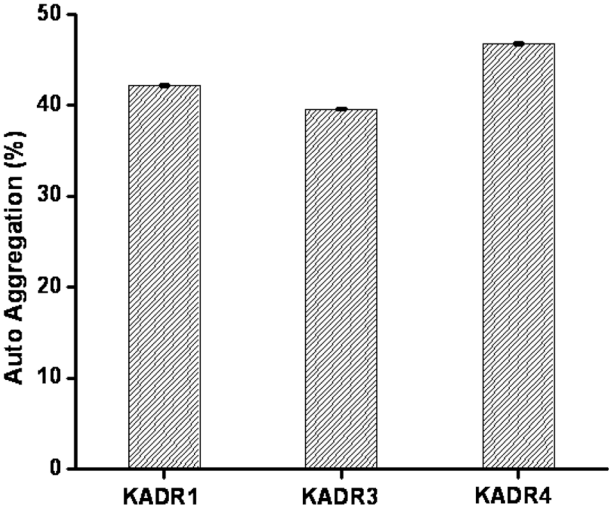


**Fig. 4** Autoaggregation of the probiotic isolates in PBS. Each value is the mean±SD of results from three separate experiments

## Gastric Juice Tolerance

Tolerance of probiotic isolates to gastric juice were evaluated at different time period such as 0.5 and 3 h and compared with control at 0 min. The viability of the isolates in the presence of gastric juices at different time period is shown in Table 3. KADR1 and KADR3 showed highest tolerance of 59.01 and 40.83 % when compared to KADR4 with 35.36 % tolerance after 3 h of incubation when compared to control at 0 min.

## Antibiotic Susceptibility

All the three selected isolates were highly susceptible (more than 11 mm of zone of inhibition) to antibiotics ampicilllin, amoxicillin, penicillin-G, erythromycin, chloramphenicol, kanamycin, tetracycline, and rifampicin, moderate to streptomycin and gentamycin and exhibited resistance to the antibiotic cephalaxin (Table 4).

## Pathogenicity Test

Non-pathogenicity was observed for all the tested isolates against the experimental fish, *L. rohita* compared to control. In addition, no-impact on growth rate was also observed in between the experimental groups (data not shown).

## Proteolytic and Amylolytic Activity

Proteolytic and amylolytic activities were determined and tabulated for all the three isolates (Table 5). Among the three isolates, KADR3 exhibited strong both proteolytic and amylolytic activities as observed by clear zone of inhibition (6–7 mm) around the colony; however, the isolates KADR1 and KADR4 possess only moderate amylolytic activity and no proteolytic activity.

**Table 3** Gastric juice tolerance analysis of *Bacillus* isolates in terms of CFU $ml^{-1}$

| Name of the isolates | Viability of bacteria in CFU $ml^{-1}$ ($\times 10^3$) | | | % of survivability after 3 h |
|---|---|---|---|---|
| | 0 h | 0.5 h | 3 h | |
| KADR1 (JQ302302) | 0.61±0.115 | 0.44±0.50 | 0.36±0.020 | 59.01 |
| KADR3 (JQ312663) | 2.62±0.175 | 1.49±0.272 | 1.07±0.145 | 40.83 |
| KADR4 (JQ822106) | 0.82±0.051 | 0.54±0.041 | 0.29±0.066 | 35.36 |

Each value is the mean±standard deviation of three separate experiments

### Catalase and Hemolytic Activity

All the three identified probiotic isolates were found to be catalase-positive and non-hemolytic bacteria (Table 6).

## Discussion

Fish gut has unique and diverse unexplored microorganisms known to have a profound effect on the health status of the animal. Consequently, growth, development, and innate immunity of the host most probably depends on these gut microbes. In order to exploit the beneficial aspects of these microbes otherwise known as probiotics, it is crucial to isolate and characterize them.

The application of probiotics has been gaining momentum since they are widely employed as natural alternatives to antibiotics to control the multidrug resistance characteristics of infectious pathogens in humans and animals. In recent years, probiotics are supplemented along with feed in many livestock production sectors including human food to protect the host organisms for various beneficial effects, such as reducing pathogenic microbial infection,

**Table 4** Antimicrobial susceptibility and resistance pattern of the probiotic isolates, KADR1, KADR3, and KADR4 against the selective antibiotics

| Antibiotics (mcg) | Probiotic isolates | | |
|---|---|---|---|
| | KADR1 | KADR3 | KADR4 |
| Ampicillin (10) | +++ | +++ | +++ |
| Amoxicillin (10) | +++ | +++ | +++ |
| Cephalaxin (30) | R | R | R |
| Streptomycin (10) | ++ | ++ | ++ |
| Penicillin-G (10) | +++ | +++ | +++ |
| Gentamycin (10) | ++ | ++ | ++ |
| Erythromycin (15) | +++ | +++ | +++ |
| Chloramphenicol (30) | +++ | +++ | +++ |
| Kanamycin (10) | +++ | +++ | +++ |
| Tetracycline (30) | +++ | +++ | +++ |
| Rifampicin (5) | +++ | +++ | +++ |

++ zone of inhibition between 2 and 4 mm, +++ zone of inhibition above 4 mm, *R* resistant

**Table 5** Amylolytic and proteolytic activity of *Bacillus* spp.

| Test | Probiotic isolates | | |
|---|---|---|---|
| | KADR1 (JQ302302) | KADR3 (JQ312663) | KADR4 (JQ822106) |
| Amylolytic activity | ++ | +++ | ++ |
| Proteolytic activity | − | +++ | − |

++ zone of inhibition 4 mm, +++ zone of inhibition between 6 and 7 mm, − no activity

enhancing innate immunity, reducing LDH cholesterol level, immunostimulation, production of digestive enzymes etc. Literature reveals that discovery of indigenous probiotics organism incessantly shows promising results than the commercial isolates in different genus and location [31]. With this background, the present study was an attempt made to isolate and characterize three novel indigenous probiotics isolates named as KADR1, KADR3, and KADR4 from Indian major carps of the river Cauvery in the context of promoting the aquaculture industry.

The isolated probiotics organisms were identified as *Bacillus* sp., an industrially important model microorganism next to *Escherichia coli*, ubiquitous in nature, and possess high tolerance to a wide range of extreme environments owing to its endospore forming adaptability. *Bacillus* sp. is identified as potent probiotics since at least 50 years with Enterogermina®, an Italian product [32]. However, extensive research on *Bacillus* was initiated 15 years back [33–35] and subsequently a number of *Bacillus* sp. were evaluated for their efficacy in various livestock production sectors such as poultry, cattle, and fishery.

The isolated bacteria were characterized by various morphological, biochemical, and molecular biological techniques and named as KADR1 for *B. subtilis*, KADR3 for *B. aerophilus*, and KADR4 for *B. firmus*. Morphological and biochemical attributes showed that all the isolates were Gram-positive, rod shaped, endospore-forming, catalase-positive, indole negative, capable of hydrolyzing starch, and utilizes sucrose as carbon source. KADR1 and KADR3 were able to reduce nitrate, whereas KADR4 was unable to reduce. KADR3 and KADR4 were able to utilize mannitol as carbon source whereas KADR1 was not. Isolates demonstrated significant antibacterial activity against the fish pathogens *A. hydrophila* ATCC 49140, *A. hydrophila* MTCC 1739, *A. enteropelogenes* JX136699, and *P. rettgeri* JX136696. Based on the characteristic features such as survival in low pH, bile salt tolerance, non-pathogenecity, ability to adhere on surface using different solvents, autoaggregation etc., these isolates were confirmed as potent probiotics.

The ability of the isolates to survive and grow in the high concentration of bile in the stomach passage time and adherence on the fish gut epithelium are important aspect to be analyzed. Isolates identified in the present study were able to survive in wide range of bile

**Table 6** Catalase and hemolytic activity of *Bacillus* spp.

| Test | Probiotic isolates | | |
|---|---|---|---|
| | KADR1 (JQ302302) | KADR3 (JQ312663) | KADR4 (JQ822106) |
| Catalase activity | + | + | + |
| Hemolytic activity | − | − | − |

+ presence of activity, − absence of activity

concentration of 6 % and even higher up to 10 % as reported earlier [36]. The bile tolerance of the isolates were comparable to the commercial isolates *Lactobacillus acidophilus* LA-1 and *Lactobacillus rhamnosus* GG that exhibited a survivability of $10^8$ cfu/ml (>80 %) at 1 % of bile (*w*/*v*) [37]. Further, the isolates were found to exhibit survivability at very low pH of 2 and 3 demonstrating their use as dietary adjuncts. Acid tolerance to such low pH organisms has been earlier demonstrated in the *Lactobacillus* spp. from *L. rohita* [38, 39] as potent probiotics. Further, the pH tolerance level of the Bacillus isolates were comparable to commercial isolates, *L. acidophilus* LA-1 and *L. rhamnosus* GG, which demonstrated >80 % survivability at pH 3 [37].

Adhesion to epithelial cells is a vital parameter to be a potent probiotic, since it provides the ability to resist the flux of the intestinal content [40, 41]. Colonization in the intestinal epithelial cell wall and mucosal surfaces are an important desirable property of probiotic bacteria in order to prevent the pathogenic bacteria adhesion, invading all the available space of the intestine and preventing the inflammatory reactions. The surface properties like autoaggregation and hydrophobicity exhibited by the isolates may contribute on its adhesion property [27]. In this study, we report that *Bacillus* isolate KADR4 showed high autoaggregation percentage (46.78 %). This property could confer a competition to pathogen and colonization of *Bacillus* in the gastrointestinal tract.

*Bacillus* species produce proteases (namely subtilisin), which assist digestion and reduce allergenicity. Protease enhances the protein digestion in the GI tract of the fish and involves in defense mechanism against pathogens by cleaving their receptor sites in the intestinal epithelial cells [42]. Remarkably, *Bacillus* sp. KADR3 displayed significant level of proteolytic activity among the three isolates.

The enzyme catalase is well-known to play a crucial role in scavenging the free oxygen generated during metabolic processes; *Bacillus* spp. are endowed to produce catalase which can reduce various harmful effects caused due to ROS [43]. The catalase producing potential of the isolates is an indication of antioxidant producing characteristic feature of the isolate, since it may be a good candidate to use as probiotics. *Bacillus cereus* family is a highly threatening hemolytic bacteria causing diarrhea to the host organism. The present study confirms that all the three identified probiotics were non-pathogenic and non-hemolytic, tested against the fish *L. rohita* and human RBC, respectively, which testifies to their safety and efficacy for aquaculture and other livestock production purpose. Furthermore, the pathogenicity test carried out against the experimental fish, rohu, demonstrated that the isolates do not induce any mortality in the fish affirming the safety of the probiotic isolates.

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. In order to overcome the issue, a heap of prophylactic and therapeutic alternatives are identified but the success rate is very limited. Moreover, the therapeutic agents, particularly antibiotics, were heavily applied in the aquaculture industry before the invention of vaccine for health management as well as better growth; however, the residues persistence in the environment induces multidrug resistance to the host organism at an alarming issue. The application of vaccines success rate is significantly higher only to the particular pathogens, and administration of vaccine to aquatic organism is a great challenging task, the route of immunization decides their potency. In this context, identification of potential probiotic microbe to stimulate the host organism's innate immunity via naturally producing a number of secondary metabolites, antagonistic to the intestinal pathogens, preventing the inflammatory disorders caused due to pathogens, produce various immunostimulants, neutralizing various bacterial endotoxins etc. are helpful to the host organism overall health status.

The isolated and characterized probiotic isolates possess the ability to kill the fish pathogens identified from the diseased native fish (*L. rohita*) such as *P. rettgeri* JX136696, *Aeromonas* sp.

JX136697, *Aeromonas* sp. JX136698, and *A. enteropelogenes* JX136699 and reference fish pathogenic strains *A. hydrophila*ATCC 49140 and *A. hydrophila* MTCC 1739. *A. hydrophila,* an important fish pathogen causing epizootic uncreative syndrome, fin rot, tail rot, hemorrhagic septicemia etc. in Indian major carps [10, 13, 44]. This study proves that the three identified isolates posses better antibacterial activity against fish pathogens.

Similarly, susceptibility of the isolates to the antibiotics ampicillin, amoxicillin, streptomycin, penicillin-G, gentamycin, erythromycin, chloramphenicol, kanamycin, tetracycline, and rifampicin and resistant to the antibiotic cephalaxin reveals that these probiotics microbes are safe to use against fish pathogens in the aquaculture industry. The commercial isolates, *B. subtilis* VKPM B2335 (BS3) and *B. licheniformis* VKPM B2336 (BL31), were also found sensitive to the antibiotics ampicillin, kanamycin vancomycin, streptomycin, gentamycin etc. [45]. However, BS3 was resistant to oxacillin and showed intermediate resistance to amoxicillin, methicillin, and some cephalosporins, and strain BL31 was resistant to chloramphenicol and clindamycin [45]. Based on the above good attributes, the present study concludes that the identified and characterized three isolates, KADR1, KADR3, and KADR4, are novel, possess notable probiotics properties, and thus could be safe to host organism to use as probiotics for enhanced livestock production and better health management practice in particular to the aquaculture industry.

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