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In vitro assessment of probiotic potential and antagonistic activity of gut-associated bacteria from *Etroplus suratensis* against fish pathogens

Akhil Ignus, Fidha Aslam P.K., Anitha R. and Mithun Sukumaran*

Department of Aquatic Biology and Fisheries, University of Kerala
**E.mail: mithun@keralauniversity.ac.in*

ABSTRACT

The gastrointestinal tract (GIT) of aquatic organisms harbors diverse microbial communities with potential probiotic applications. In the present study, autochthonous bacterial isolates from the GIT of *Etroplus suratensis*, collected from Vellayani Lake, Kerala, India, were characterized for their antibacterial and probiotic potential. Fifteen morphologically distinct bacterial isolates were screened for antagonistic activity against key fish pathogens, including *Aeromonas hydrophila*, *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Vibrio fluvialis*, and *V. parahaemolyticus* using the agar double-layer method. Isolates demonstrating significant inhibition zones were further assessed for haemolytic activity, abiotic stress tolerance (bile salts, pH), cell surface hydrophobicity, and antibiotic susceptibility. Biochemical profiling and 16S rRNA gene sequencing revealed that the isolates predominantly belonged to the phyla *Bacillota*, *Pseudomonadota*, and *Actinomycetota*, with species such as *Bacillus cereus*, *Micrococcus luteus*, *Enterobacter cloacae* and *E. asburiae*. Further *in vivo* evaluations are recommended to validate these candidate strains as sustainable biocontrol agents.

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1. Introduction

Aquaculture is the world's fastest- growing industry and is the major animal protein producer (Santos *et al.*, 2021). The rapid demand for fish and fishery products led to the growth of this sector and has surpassed the capture fisheries production (FAO, 2024). The rapid intensification of aquaculture methods has led to the outbreak of bacterial diseases (Midhun *et al.*, 2018). The common practice of using antibiotics to counteract the bacterial outbreaks has led to the emergence of antibiotic resistance among pathogenic bacteria and the destruction of beneficial bacteria associated with the gut of the host organism (Romero *et al.*, 2012; Ringø, 2020). The leftover antibiotics in the system which later get discharged into the environment also pose a significant threat (Sugita *et al.*, 2002). The scientific community has developed many alternatives to antibiotics such as phage therapy, vaccines, phytobiotics, immunostimulants, probiotics, prebiotics and synbiotic concepts (Gatesoupe, 1999; Newaj-Fyzul *et al.*, 2014; Van Doan *et al.*, 2020). The microbial supplements used can also provide immunomodulatory effects along with the antagonistic effects produced by the secretion of metabolites (Etyemez and Balcazar, 2016). Probiotics are considered the major alternatives in this scenario as they provide improved growth and disease resistance in addition to the antagonism to pathogens (Verschuere *et al.*, 2000). Probiotics are defined as live microorganisms which, when administered in adequate amounts confer a health benefit on the host (FAO, 2016). When used in aquaculture systems probiotic bacteria significantly modulate the gut microbiota, enhance the host immunity by competing with pathogens for adhesion sites and nutrients or by the production of natural antimicrobial compounds (Hai, 2015; Wang *et al.*, 2024). Probiotics also enhance bioremediation and improve water quality by degrading the antibiotic residues in the environment (Van Doan *et al.*, 2020).

The commercial development of a probiotic requires in vitro and in vivo characterization, bio safety of isolates and proper taxonomic identification (Ganguly *et al.*, 2011). Non pathogenic bacteria associated with the host organisms are often selected as a good source of probiotics in aquaculture (Islam and Mithun, 2024). The autochthonous bacteria colonizing the gut of aquatic organisms have a higher chance to confer health benefits to the host than the allochthonous or non-fish bacteria (Ren *et al.*, 2019). Many studies have isolated and characterized gut-associated bacteria from fish having probiotic effects (Egerton *et al.*, 2018; Sweta *et al.*, 2024; Todorov *et al.*, 2024). The antibacterial properties of bacteria associated with the gut of freshwater fishes are rarely studied (Ghosh *et al.*, 2021; Mondal *et al.*, 2008).

Etroplus suratensis (Bloch, 1790), a major indigenous aquaculture species in Kerala. Its euryhaline nature makes it a suitable candidate for both freshwater and brackish water aquaculture. Despite the species being in high demand, its wild populations are subjected to various threats: overexploitation, exposure to pollutants in natural water bodies and low resistance to pathogenic infestations (Padmakumar *et al.*, 2012). The overuse of antibiotics has led to the emergence of antimicrobial resistance genes within the associated bacterial populations of *E. suratensis* (Ravi *et al.*, 2019). The gut microbiota associated with *E. suratensis* is an area to be explored in detail and literature on the development of a host associated probiotic is also scarce. In light of the context, this study aims to isolate, identify, and characterize potential probiotic bacteria from the gut of *E. suratensis* and evaluate their in vitro antagonistic properties against five well-known fish pathogens.

2. Materials and Methods

2.1. Sample collection and processing of specimens

Live specimens of *E. suratensis* were collected using a cast net from Vellayani Lake (8° 26' N and 76° 59' E), a fresh water lake in Thiruvananthapuram, Kerala, India.

The fish collected were starved for 48 hours to empty the gastrointestinal tract (GIT), following the recommendation of Nandi *et al.* (2017). They were then euthanized by lower cold shock. The surface of the fish was thoroughly scrubbed with 1% Iodine solution for decontamination (Trust and Sparrow, 1974). Fish were dissected aseptically within the laminar airflow and their GIT was removed under sterile conditions. Pooled samples were used to avoid erroneous conclusions due to individual variation in microflora (Kar and Ghosh, 2008).

2.2. Isolation of gut bacteria

The fish GIT was crushed and macerated in sterile saline (0.9%) to make a homogenized solution (Irianto and Austin, 2002). This stock solution was then made into 10-fold serial dilutions. Serially diluted samples (100 µl) were spread plated onto Tryptone Soy Agar (TSA, Himedia) and the plates were incubated at 30°C for 24-48 hours. After incubation, the colonies were selected based on morphological differences and pure cultures were stored in Nutrient Agar slants at 4°C until use.

2.3. Maintenance of Pathogenic Bacteria

Pure cultures of pathogenic bacteria were collected from the National Centre for Aquatic Animal Health (N.C.A.A.H.), Kochi. They were sub cultured and underwent biochemical characterization to ensure the purity of isolates. The following bacterial strains were used in the study: *Aeromonas hydrophila* MCCB-113, *Edwardsiella tarda* MTCC-2400, *Pseudomonas aeruginosa* MCCB-119, *Vibrio fluvialis* MRCS-26 and *V. parahemolyticus* MRCS-9. These are the most common pathogens affecting *E. suratensis* causing disease outbreaks during their culture. The bacterial cultures were stored in Nutrient agar slants supplemented with 1.5% NaCl at 4°C.

2.4. Detection of antimicrobial activity of Isolates

Antimicrobial activity of isolates was evaluated by the agar double layer method (Dopazo *et al.*, 1988) modified by (Mukherjee *et al.*, 2017). Briefly, the pathogens and isolated bacteria were inoculated on Muller Hinton broth (MH, Himedia) and were incubated at 30°C overnight. The pathogens were grown in MH broth supplemented with 1.5% NaCl. The inoculated medium of pathogenic bacteria was read at optical density 600 nm (Thermo- Gensys 150) and the absorbance was adjusted to 0.1 ($1x10^8$ cells/ml approximately) by dilution using the respective broth. The gut isolated bacteria were plated onto the surface of MH agar plate by agar spot method and incubated at 30°C for 24 hours. The bacteria were later killed by exposure to chloroform vapour (15 min). Later, these plates were overlaid with cultures containing pathogenic bacteria and further incubated for 48 hours at 30°C After incubation, the plates were analyzed for the zone of inhibition around the test cultures. The diameter of the zone around the colony is measured in mm. The activity score of the bacteria were presented as scores as follows; 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). Amikacin- 30 mcg (AK) and Meropenem- 10 mcg (MRP) were kept in each plate as positive controls and the sterile broth without bacterial inoculum was used as the negative control. The isolates showing higher activity scores among the groups were selected for characterization assays.

2.5. Safety assessment of isolates

2.5.1. Haemolytic activity

The haemolytic activity of selected isolates was determined by inoculating selected cultures on 5% Sheep blood agar (Himedia). After incubating the plates at 30^{O}C for 48 hours the plates were observed for the formation of haemolytic zones around the colony (Kuang *et al.*, 2024). The isolates which showed γ haemolysis (No clear zone around colonies) were selected for further studies.

2.5.2. Abiotic stress tolerance assay

The isolates were screened to study their tolerance to varying bile salt concentrations and pH. Bile tolerance assay of selected isolates was done by culturing isolates in liquid media (Nutrient broth) supplemented with bile salt (0.1, 0.3, 0.5, 1%) following standard methodology (Vinderola and Reinheimer, 2003) and nutrient broth without bile salt was used as control. The pH tolerance was examined by growing the isolates in liquid culture medium with different pH gradients 2 – 9 and pH 7 was used as control (Kuang *et al.*, 2024). All cultures were incubated overnight and the optical density was measured at 600 nm using spectrophotometer (Thermo- Gensys 150).

2.5.3. Cell surface hydrophobicity

The degree of cell surface hydrophobicity of the selected isolates was assessed by microbial adhesion to hydrocarbons, which was determined by xylene extraction according to the method of Divyashree *et al.* (2021), with slight modifications. Briefly, the selected isolates were grown in nutrient broth (Himedia). The bacterial suspension was prepared by centrifuging the overnight-grown culture of selected bacterial isolates at 5000 rpm for 10 minutes. The bacterial pellets were washed twice with P.B.S solution and were later resuspended in 5 ml P.B.S. 3 ml of the cell suspension was mixed with an equal volume of xylene and further vortexed for 3 min, to mix the two-phase system. After one hour of incubation at room temperature, the upper aqueous layer was removed, and OD was measured at 600 nm. Hydrophobicity was evaluated as the percentage decrease in the OD of a bacterial suspension at the initial aqueous layer and calculated using the equation:

% Hydrophobicity = $(A_o\text{-}A_t/A_o) * 100$.

A_o represents the absorbance before extraction with organic solvent, and A_t represents the absorbance after extraction with organic solvent.

2.6. Antibiotic Susceptibility of Isolates

Susceptibility of the isolates to antimicrobial agents was analyzed by the disc diffusion test (Bauer *et al.*, 1966). For this, the isolates were grown overnight in Muller Hinton broth and dilutions of broth culture of all the isolated bacteria were made equivalent to 0.5 McFarland standard and were lawn cultured on the surface of MH Agar (Himedia Laboratories) plate. Then the selected antibiotic discs (Himedia) according to CLSI standards were placed over this. The antibiotics tested were Ampicillin 10 mcg (AMP), Piperacillin and Tazobactam 100/10 mcg (PIT), Cefazolin 30 mcg (CZ), Meropenem 10 mcg (MRP), Amikacin 30 mcg (AK), Azithromycin 15 mcg (AZM), Ciprofloxacin 5

mcg (CIP), Vancomycin 30 mcg (VA), Penicillin-G 10 units (P), Tetracycline 30 mcg (TE), Cefuroxime 30 mcg (CXM), Chloramphenicol 30 mcg (C), Erythromycin (E) 15 mcg. After overnight incubation, the degree of sensitivity was determined by measuring the zone of inhibition around the discs using Biogram interpretative chart and the Multiple Antibiotic Resistance (MAR) indexing of the isolates was determined by calculating the ratio between the number of antibiotics to which the isolate is resistant and the total number of antibiotics to which the isolate was exposed (CLSI, 2018).

2.7. Biochemical analysis of isolates

The biochemical characteristics of the selected isolates were studied according to Bergey's manual of determinative bacteriology (Bergey, 1994). Gram staining, Oxidase, Catalase, Indole, Methyl Red, Voges-Proskauer, Citrate and Urease tests were done.

2.8. DNA isolation and 16s rRNA sequencing

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. The eluted DNA was checked for quality using nanodrop (Thermofisher). The DNA was amplified for 16S rRNA region using universal primers 16S-RS-F (CAGGCCTAACACATGCAAGTC) and 16S-RS-R (GGGCGGWGTGTACAAGGC) (Donio *et al.*, 2013). After verifying the PCR product on 1% agarose gel electrophoresis, the amplicons were purified and sequenced at the DNA sequencing facility (R.G.C.B., Thiruvananthapuram, India) using ABI 3500 DNA Analyzer (Applied Biosystems) following Sanger DNA sequencing method. The sequences obtained were aligned using BioEdit (Version 7.7). The sequences were then analyzed for homology search in NCBI using the BLAST search algorithm. Sequence identities of 99% and 97% were used as criteria for species and genus assignments, respectively (Janda and Abbott, 2007). The perplexity in assigning the species/genus was overcome by comparing with the biochemical tests results. Each species' representative 16S rRNA gene sequences were then submitted to GenBank (NCBI).

2.9. Phylogenetic analysis

The sequences of the 16S rRNA gene in the isolates of each species were used for phylogenetic study. The sequences were aligned using ClustalW and a Maximum likelihood (ML) tree was constructed by MEGA version X using Kimura 2-parameter model (Kimura, 1980). The confidence in the ML tree was estimated by 1000 bootstrap replicates (Tamura *et al*., 2021).

3. Results

3.1. Isolation of Gut associated Bacteria

Twenty fish were collected for isolation of gut associated bacteria. The morphometric parameters of fish and water quality data of the study site are given in Table 1.

3.2. Enumeration of gut-associated bacteria

The total viable count (TVC) of the fish GIT-associated bacteria ranged between 1.8 x 10^4 to 2.09 x 10^6 CFU g^{-1} of the GIT. A total of 56 isolates were selected based on their colony morphology, colour and characteristic growth patterns. The selected isolates were screened for antagonistic activity against five fish pathogens.

3.3. Antagonistic activity of isolates

Fifteen isolates showed activity against *A. hydrophila, P. aeruginosa* and *V. parahaemolyticus*. In case of *E. tarda* 11 isolates were having antagonistic activity and *V. fluvialis* with 13 isolates showing activity (Fig. 1 – 4). The activity is represented as zone of inhibition (mm) ± standard deviation against respective pathogens in Table 2 and the activity scores of isolates are represented in Fig. 6. The highest activity score was shown by isolates D6, C17, C15, C5, C14, C13, B7 and A4.

3.4. Safety Assessment of Isolates

3.4.1. Haemolytic activity

The isolates were tested for haemolysis activity on blood agar (Fig. 7). Four isolates out of 15 showed γ-haemolysis on blood agar. The isolates B7, C5, C9 and C14 were selected for further analysis.

3.4.2. Abiotic stress tolerance assay

The isolates B7, C5, C9 and C14 were selected for abiotic stress tolerance assays. All the isolates could grow in the physiological conditions of the GIT. The bile salt assay (Fig. 8) proved that isolates grow at all concentrations from 0.3 to 1%. In pH tolerance assay (Fig. 9), all the isolates showed growth in the tested pH range (2-9).The control group is represented as C in the graph.

3.4.3. Cell surface hydrophobicity Assay

All the isolates showed good adherence to the non-polar solvent Xylene (Table 3). The cell surface hydrophobicity

Table 1. Hydrographic parameters and morphometry of fish collected from Vellayani lake

	Coordinates	8°25'32 N 76°59'38 E
Hydrographic parameters	Temperature (°C)	24
	Dissolved Oxygen (ppm)	5.7
	pH	7.5
	Ammonia (ppm)	0.20
	Nitrite (ppm)	0.16
	Nitrate (ppm)	BDL
	Phosphate (ppm)	0.01
	Salinity (ppt)	1
Morphometric Parameters of Fish	Length (cm)	16.39±3.57
	Weight (g)	127.90 ±65.73
	Gut Weight (g)	2.28±0.88

Table 2. Showing the spectrum of antimicrobial activity of isolates against fish pathogens

Isolate code	Zone of inhibition (mm)				
	AH	**ET**	**PA**	**VPA**	**VF**
A4	14 ± 0^{efghi}	9 ± 1.73^{bc}	13.33 ± 1.53^{defg}	9.33 ± 1.53^{b}	10.33 ± 0.58^{bcdefg}
B7	15.33 ± 0.58^{ghi}	10.33 ± 1.53^{bcd}	11 ± 1^{bcd}	10 ± 1^{bc}	10.33 ± 1.53^{bcdefg}
C2	12 ± 1^{cdefgh}	0 ± 0^{a}	12.33 ± 1.16^{cdef}	20 ± 2^{e}	0 ± 0^{a}
C4	12.67 ± 0.58^{defgh}	0 ± 0^{a}	13 ± 1^{defg}	20.33 ± 2.09^{e}	0 ± 0^{a}
C5	11.67 ± 1.53^{cdefg}	9.67 ± 0.58^{bcd}	9.33 ± 0.58^{bc}	11.33 ± 0.58^{bc}	7.67 ± 0.58^{b}
C7	17.33 ± 2.52^{ij}	8.33 ± 1.53^{b}	10.33 ± 2.09^{bcd}	9.33 ± 0.58^{b}	10.33 ± 0.58^{bcdefg}
C9	17 ± 1.74^{ij}	0 ± 0^{a}	18.33 ± 1.53^{ijk}	8.33 ± 1.16^{b}	8 ± 1^{bc}
C13	16.67 ± 0.58^{i}	9.67 ± 1.53^{bcd}	14.67 ± 0.58^{efgh}	10.67 ± 0.58^{bc}	9.33 ± 0.58^{bcde}
C14	13.67 ± 1.16^{efghi}	10.67 ± 0.58^{bcd}	16.33 ± 1.53^{ghi}	11 ± 1^{bc}	8.33 ± 1.16^{bcd}
C15	15.67 ± 2.08^{hi}	8.67 ± 1.53^{b}	11.67 ± 0.58^{bcde}	16 ± 1.73^{d}	11.33 ± 1.16^{efg}
C17	11.67 ± 2.08^{cdefg}	10.67 ± 1.16^{bcd}	21.33 ± 1.53^{kl}	11 ± 1^{bc}	11 ± 1^{defg}
D5	10.67 ± 0.58^{cdef}	12 ± 1^{cd}	8.67 ± 1.53^{b}	11.33 ± 1.53^{bc}	11 ± 1^{defg}
D6	14.33 ± 1.16^{fghi}	12.67 ± 1.53^{d}	20.33 ± 0.58^{jkl}	13.33 ± 1.53^{cd}	12.67 ± 1.16^{d}
L2	10.67 ± 1.53^{cdef}	8.33 ± 1.16^{b}	21.67 ± 0.57^{kl}	11 ± 1^{bc}	9.33 ± 0.58^{bcde}
L3	6.33 ± 0.58^{b}	0 ± 0^{a}	10.33 ± 0.58^{bcd}	8.67 ± 0.58^{b}	8.33 ± 1.53^{bcd}
AK	17.33 ± 0.56^{ij}	20.67 ± 0.58^{e}	22.33 ± 1.16^{l}	24.67 ± 0.58^{f}	19.67 ± 0.58^{h}
MRP	20.67 ± 0.58^{j}	23.33 ± 0.58^{e}	17.67 ± 0.58^{hij}	24.67 ± 0.58^{f}	28.67 ± 1.53^{i}
F-Value	58.851	135.114	63.748	94.259	117.891
P-Value	<0.001	<0.001	<0.001	<0.001	<0.001
Inference	Significant	Significant	Significant	Significant	Significant

*All values are expressed in Mean ± SD

*Different superscript letters in the same column indicate statistical significance at 5% level

AH – *A. hydrophila*, ET – *E. tarda*, PA – *P. aeruginosa*, VPA - *V. parahaemolyticus*, VF - *V. fluvialis*, AK - Amikacin- 30 mcg and MRP - Meropenem- 10 mcg

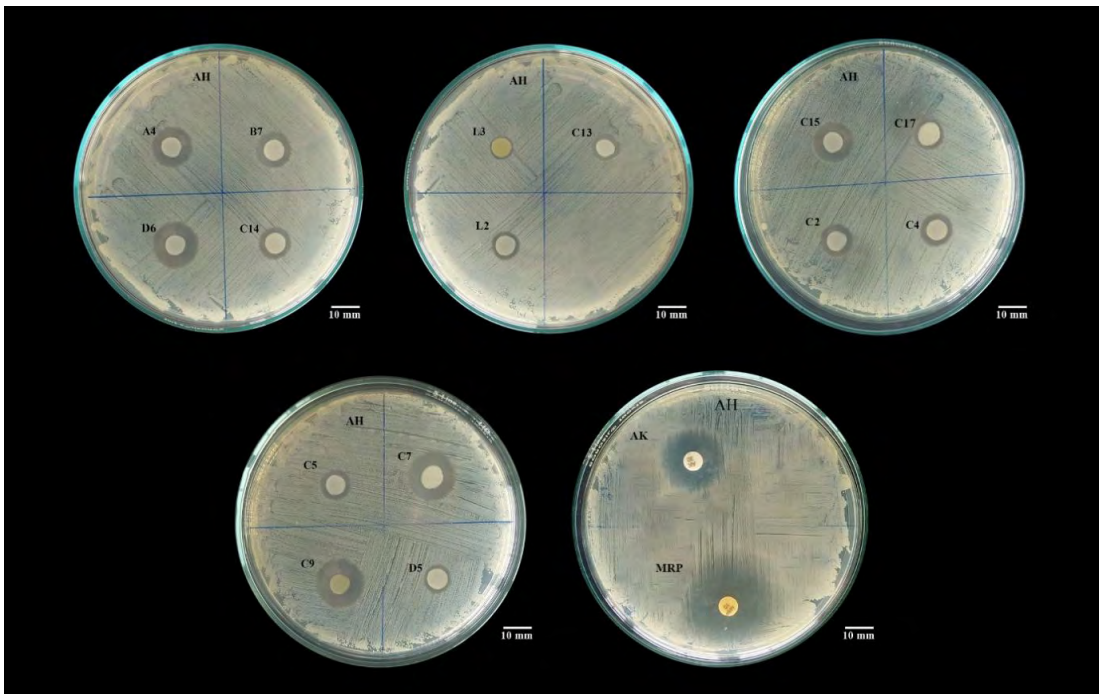


Fig. 1. Antimicrobial activity of isolates against *Aeromonas hydrophila*

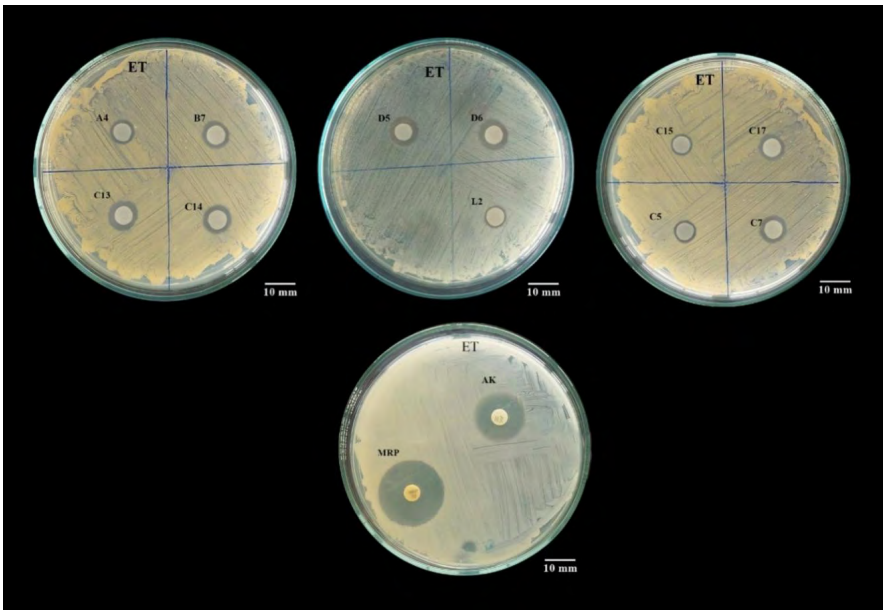


Fig. 2. Antimicrobial activity of isolates against *Edwardsiella tarda*

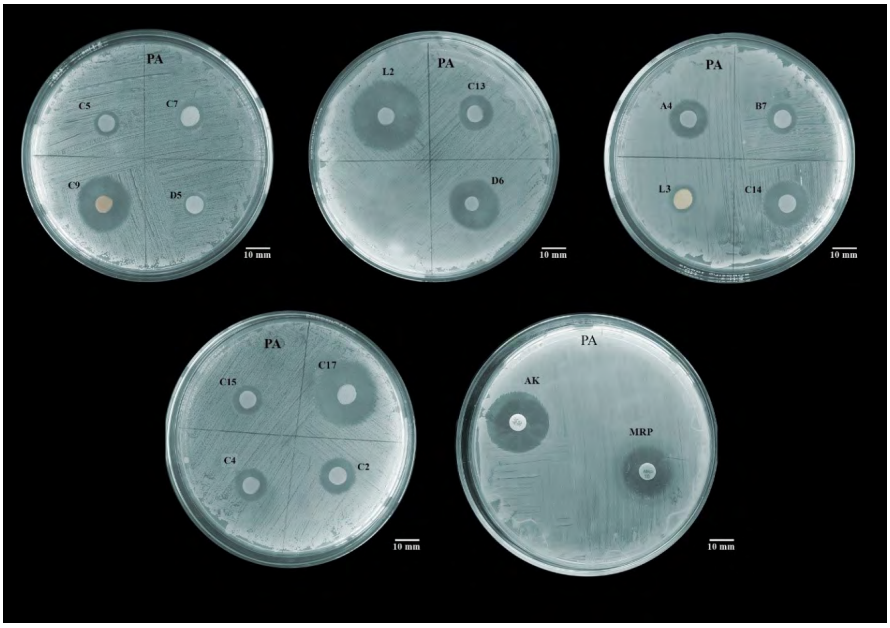


Fig. 3. Antimicrobial activity of isolates against *Pseudomonas aeruginosa*

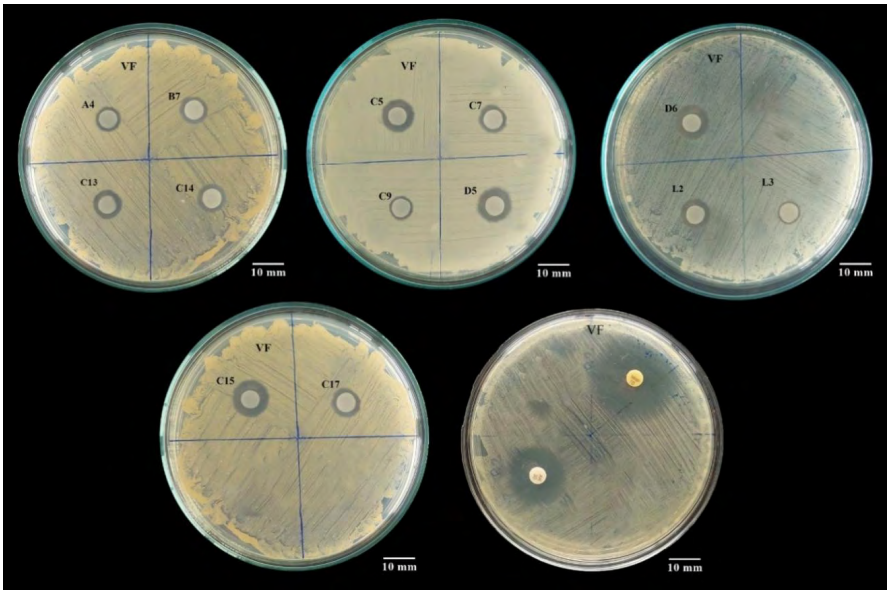


Fig. 4. Antimicrobial activity of isolates against *Vibrio fluvialis*

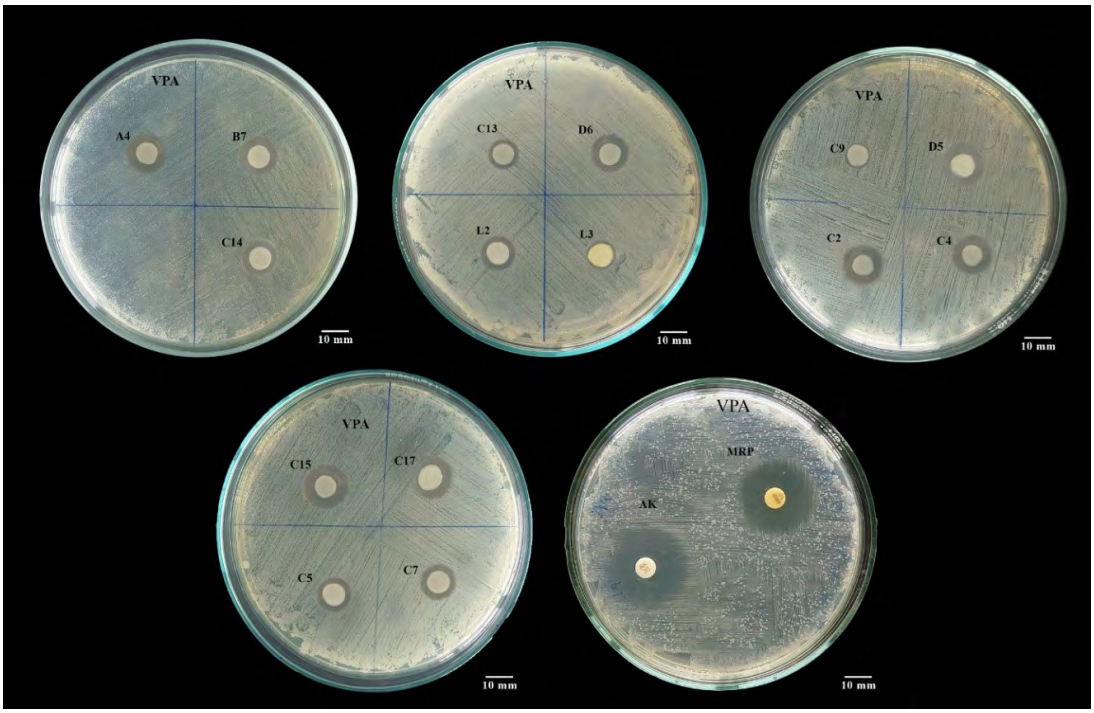


Fig. 5. Antimicrobial activity of isolates against *Vibrio parahaemolyticus*

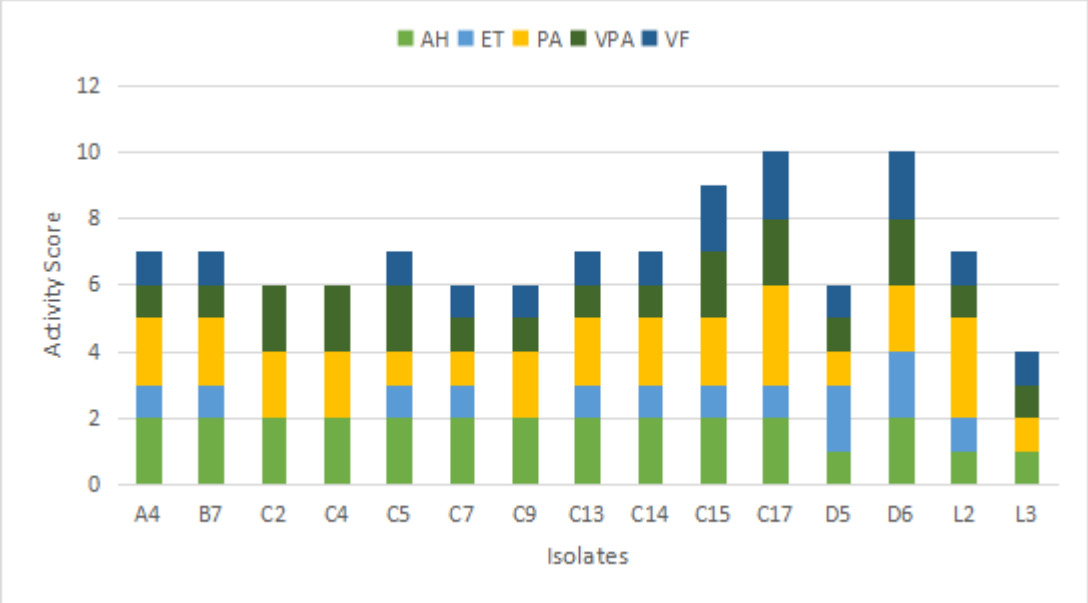


Fig. 6. Activity Scores of isolates against pathogenic bacteria; 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–25 mm) and 4 (very high, ≥26 mm)

Table 3. Cell surface hydrophobicity of isolates towards Xylene

Bacterial isolates	Hydrophobicity %
B7	78.81 ± 2.65^{b}
C5	77.16 ± 1.79^{b}
C9	94.08 ± 1.60^{c}
C14	89.56 ± 1.75^{a}
F - Value	138.5
P-Value	<0.001
Inference	Significant

*All values are expressed in Mean ± SD

*Different superscript letters in the same column indicate statistical significance at 5% level

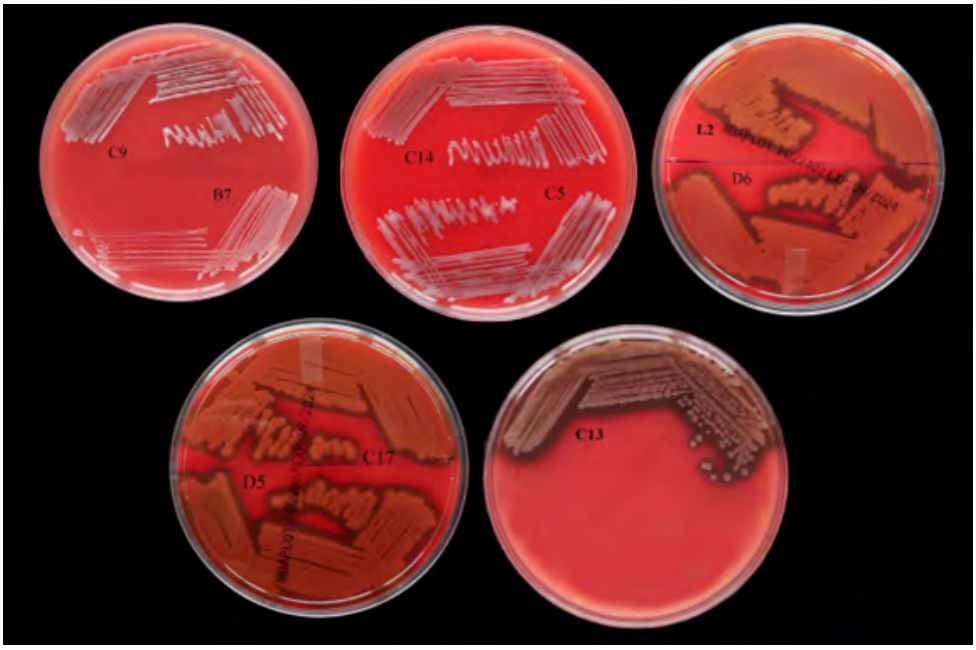


Fig. 7. Haemolytic activity of isolates on blood agar
(Zone around colonies showing β haemolysis)

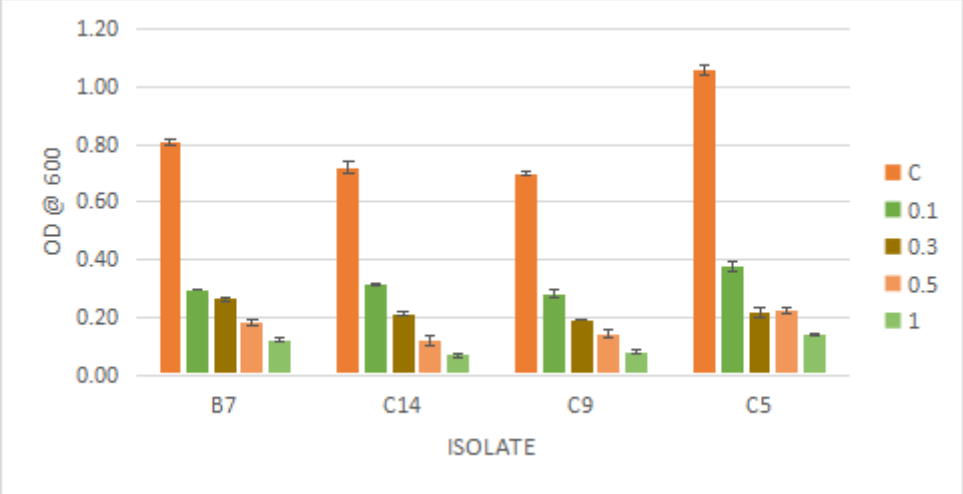


Fig. 8. Graph showing the bile salt tolerance assay of isolates

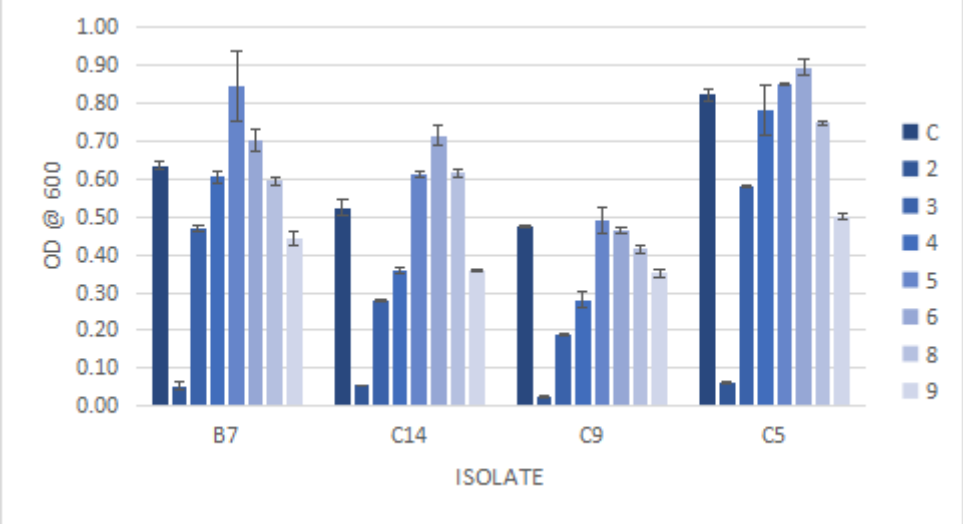


Fig. 9. Graph showing the pH tolerance assay of isolates

of strains varied between 77.16 % (C5) and 94.08 % (C9).

3.5. Antibiotic Susceptibility of Isolates

The antibiogram of selected isolates was done using 13 antibiotics belonging to different classes. Isolate C14 showed susceptibility to all antibiotics. All the other isolates showed resistance to Ampicillin. The isolates C5 and C9 showed resistance to Penicillin. The MAR index ranged from 0.08 to 0.15. The MAR index of isolates was less than 0.2 showing the absence of multiple drug resistance. The antibiogram of isolates is represented in Table 4.

3.6. Biochemical characterization of isolates

Table 5 gives the biochemical characterization of the selected isolates. Out of four isolates, two were gram-positive and two were gram-negative. The species-level identification was confirmed later by 16S rRNA sequencing.

3.7. Identification and phylogeny of Isolates

The 16S rRNA sequences of the isolates were tested for homology using BLAST analysis. The results showed that the bacteria comes under Phyla Pseudomonadota (2), Actinomycetota (1) and Bacillota (1). The accession numbers of sequences submitted to GenBank are given in Table 6.

To elucidate the evolutionary relationships among bacterial isolates, a molecular phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on 16S rRNA gene sequences (Fig.10).

The tree includes representatives of *Enterobacter cloacae, E. asburiae, Bacillus oceanisediminis, Micrococcus luteus* and an archaeal outgroup, *Sulfolobus acidocaldarius* to root the tree.

***Enterobacter* Clade**: This cluster comprises *Enterobacter cloacae* (PQ237709, AJ251469.1) and *Enterobacter asburiae* (PV554188, AB004744.1). The proximity and strong bootstrap support (98%) confirm their close genetic relationship. Sub-clustering within the clade shows *E. cloacae* and *E. asburiae* forming separate, well-supported subgroups (bootstrap values of 90% and 99%, respectively), suggesting species-level divergence.

***Bacillus* Clade**: Isolates PQ227381 and GQ292772.1 form a robust clade (bootstrap 99%), representing *Bacillus oceanisediminis*. The high similarity between these sequences supports their conspecific status and suggests minimal genetic divergence within this species in the sampled environment.

***Micrococcus* Clade**: Isolates PQ227385 and AJ536198.1 form another highly supported clade (bootstrap 98%), corresponding to *Micrococcus luteus*. This grouping indicates a close evolutionary relationship between the isolate and the type strain DSM 20030T.

The basal position of *Sulfolobus acidocaldarius* (AF130853.1) serves as an effective outgroup, providing a root for the tree and distinguishing the bacterial taxa from the archaeal lineage.

Table 4. The antibiogram of isolates

	AMP	PIT	CZ	MRP	AK	AZM	CIP	VA	P	TE	CXM	C	E	MAR Index
B 7	R	S	S	S	S	S	S	S	S	S	S	S	S	0.08
C 5	R	S	S	S	S	S	S	S	R	S	S	S	S	0.15
C 9	R	S	S	S	S	S	S	S	R	S	S	S	S	0.15
C 14	S	S	S	S	S	S	S	S	S	S	S	S	S	0.00

(R; Resistant, S; Susceptibility)

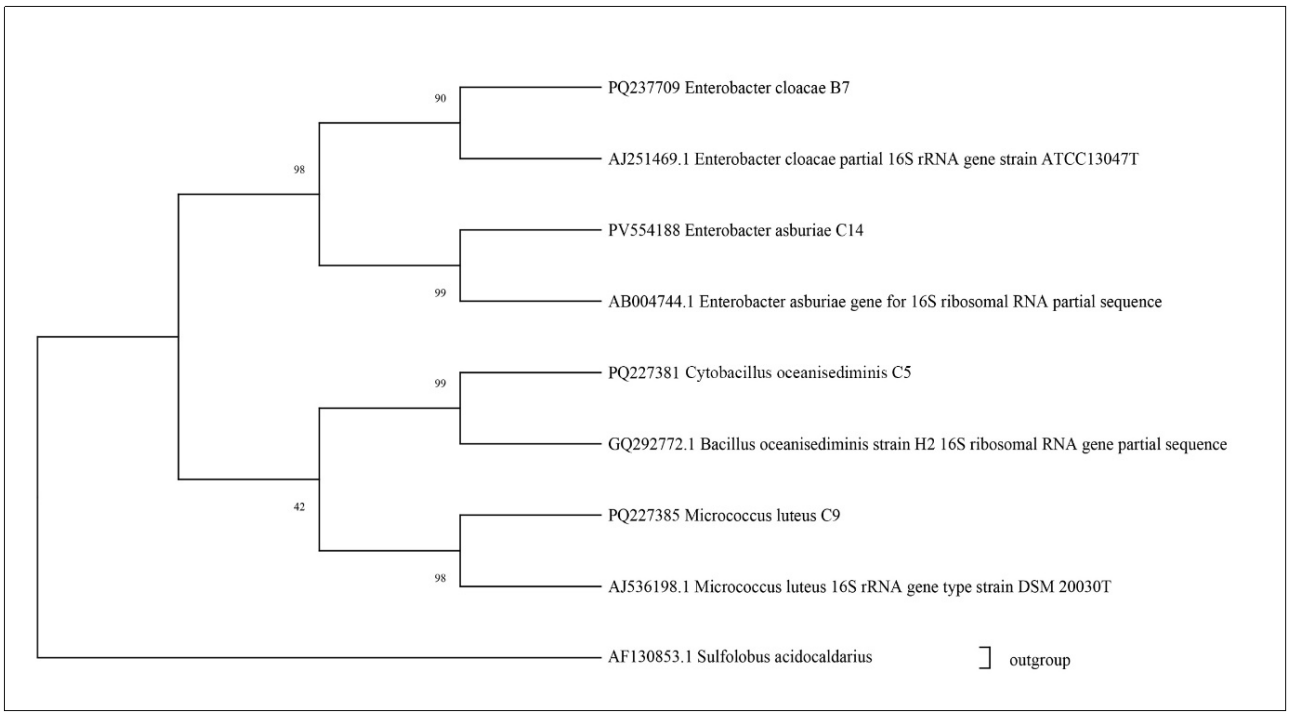


Fig. 10. Maximum likelihood tree of gut isolates based on 16S rRNA gene sequencing

Table 5. Showing the biochemical characterization of isolates (+; Positive, -; Negative)

	Gram stain	Oxidase	Catalase	Indole	MR	VP	Citrate	Urease
B 7	-	-	+	-	-	+	+	+
C 5	+	+	+	+	+	+	+	+
C 9	+	+	+	-	+	-	-	+
C 14	-	-	+	-	-	+	+	+

Table 6. Showing the 16S rRNA identification of isolates

Code	Nearest neighbor	Accession number	Class
B 7	*Enterobacter cloacae*	PQ237709	Gammaproteobacteria
C 5	*Cytobacillus oceanisediminis*	PQ227381	Bacilli
C 9	*Micrococcus luteus*	PQ227385	Actinomycetes
C 14	*Enterobacter asburiae*	PV554188	Gammaproteobacteria

4. Discussion

The fish gastrointestinal tract has become an area of interest in probiotics in recent times. In the present study, the autochthonous gut microbes of *E. suratensis* were isolated and tested for their antibacterial properties against major bacterial fish pathogens. The selected strains were later characterized for probiotic evaluation for their bio-control applications in managing disease outbreaks. The total viable count of GIT associated bacteria ranged from 1.8 x 10^4 to 2.09 x 10^6 CFU/g. The gut associated microbes in the GIT of *E. suratensis* enumerated in earlier studies were found to be 0.09 x 10^5 to 1.45 x 10^6 CFU/g (Maya *et al.*, 1995; Immanuel *et al.*, 2003; Das *et al.*, 2014).

The bacteria showing antibacterial activity was differentiated on the basis of biochemical methods. The major contributors were gram-positive, which forms the major group in gut-associated bacteria of freshwater fish (Verner - Jeffreys *et al.*, 2003). Among the 15 isolates showing antagonism towards different pathogenic bacteria, isolates showing higher activity scores were selected for further characterization. The identity of the isolates was confirmed by 16S rRNA sequencing and the results showed that the isolates belonged to the Phyla Bacillota, Pseudomonadota, Actinomycetota. Presence of *Bacillus*, *Micrococcus* and *Enterobacter* species was earlier reported in the studies associated with the GIT of *E. suratensis* (Immanuel *et al.*, 2003; Asla *et al.*, 2016; Ravi *et al.*, 2019). Most of these bacteria are found freely in the aquatic environment, influencing the formation of gut-associated bacteria in aquatic organisms rather than terrestrial organisms (Sugita *et al.*, 1983). The antagonism of the bacterial isolates against fish pathogens is mediated by competitive exclusion, production of bacteriocins or enzymes (Verschuere *et al.*, 2000). The use of indigenous bacteria as probiotics in aquaculture has been found effective in recent years. *Bacillus* and *Micrococcus* species have been earlier tested as probiotics in enhancing the digestive enzymes and growth of *E. suratensis* (Sankar *et al.*, 2017) and this shows their ability to improve the health of the host.

Isolate *C. oceanisediminis* were isolated from the marine environment and found to be effective in modulating the health of *Ctenopharyngodon idella* (Gao *et al.*, 2024). *C. oceanisediminis* has been isolated and used as an animal and human probiotic (Yadav *et al.*, 2022). *E. cloacae* have been isolated from the gut of *Arapaima gigas* and its probiotic efficiency was tested against *A. hydrophila* and *Plesiomonasshigelloides* (Aparecido *et al.*, 2021; Girijakumari *et al.*, 2018). Li *et al* (2023) isolated a novel *E. asburiae* from the intestine of healthy common carp and displayed an extensive antibacterial spectrum against *Aeromonas hydrophila, A. veronii, A. caviae, A. media, A. jandaei, A. enteropelogenes, A. schubertii, A. salmonicida*, *Pseudomonas aeruginosa*, *P. putida*, *Plesiomonas shigelloides*, and *Shewanella*. The strain was also characterized to grow between pH 4 and 7 and was highly resistant to 4% (wt/vol) bile salts. The isolate C14 from the present study also shows antagonistic effects and characteristic properties similar to the above findings.

Bacteria having antibacterial properties are generally used in aquaculture to control bacterial infection and have been an efficient alternative for the overusing of antibiotics (Van Doan *et al.*, 2020). The use of bacteria in live form was formerly considered as probiotics. However, later it was changed to the use of live or dead bacterial isolates or their metabolites which incur health benefits to the host (Wang *et al.*, 2018). Some of the bacterial isolates, like *B. cereus,* which generally causes disease in fish when given in dead or inactive form or as adjuvants in the case of vaccines said to have stimulated the innate immune system of the host (Irianto and Austin, 2002). Thus, isolates like *B. cereus* (C 15) from this study, which have antibacterial effect but did not qualify the probiotic parameters can be used in their inactive form as immune modulators in fish vaccines.

Antibiotic resistance is commonly associated with the GIT-associated bacteria of fish and is mediated by the overuse of antibiotics in aquaculture systems (Austin and Sharifuzzaman, 2022). The isolates were tested for antibiotic resistance using different classes of antibiotics. The antibiotic ampicillin showed resistance to all the isolates and this phenomenon is reported in earlier studies and is commonly associated with gram-negative bacteria (Serrano, 2005). The isolates were tested for their ability to withstand the physiological conditions of the GIT and

the isolates survived all the parameters like pH and Bile salts. The isolates showed a high level of cell surface hydrophobicity to non-polar solvents. This shows their ability to colonize in the GIT of the host and can form biofilms (Ganguly *et al.*, 2019). Consequently, this study reveals that the selected isolates obtained from the GIT of *E. suratensis* can be used as putative probiotics in aquaculture. The application of transcriptomics in this area can be beneficial in future research.

In summary, bacterial isolates associated with the GIT of *E. suratensis* possessed significant antibacterial activity against aquaculture pathogens and survived diverse physiological stresses. Future studies focusing on the characterization of probiotics and in vitro experiments will facilitate the use of live, inactivated, or dead elements from these isolates to enhance aquatic animals' growth and disease resistance.

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5. References

Aparecido, P.A., Sampaio L.L., Duarte G.V., Rocha P.B., Prazeres R.D., and Tavares-Dias, M., 2021. Cultivable autochthonous bacteria of the intestinal mucosa of *Arapaima gigas* (Pisces: Arapaimidae) with probiotic potential. *Aquaculture Research*, 52(**4**), 1788–1796. https://doi.org/10.1111/are.15025

Asla, V., Neethu, K.P., Athira, V., Nashad, M., and Mohamed H. A., 2016. Prevalence of Antibiotic Resistance among the Gut Associated Bacteria of Indigenous Freshwater Fishes *Aplocheilus lineatus* and *Etroplus maculatus*. *International Journal of Aquaculture*, 6(**3**) 1-9. https://doi.org/10.5376/ija.2016.06.0003

Austin, B. and Sharifuzzaman, S.M., 2022. Probiotics in Aquaculture. 1–313. https://doi.org/10.1007/978-3-030-98621-6

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Turck, M., 1966. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *American Journal of Clinical Pathology,* 45(**4**), 493–496. https://doi.org/10.1093/ajcp/45.4_ts.493

Bergey, D.H., 1994. Bergey's manual of determinative bacteriology. Lippincott Williams and Wilkins.

CLSI., 2018. Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplement M100. (P. Wayne (ed.); 28th ed.): Clinical and Laboratory Standards Institute.

Das, P., Mandal, S., Khan, A., Manna, S.K., and Ghosh, K., 2014. Distribution of extracellular enzyme-producing bacteria in the digestive tracts of 4 brackish water fish species. *Turkish Journal of Zoology*, 38(**1**), 79–88. https://doi.org/10.3906/zoo-1205-3

Divyashree, S., Anjali, P.G., Somashekaraiah, R. and Sreenivasa, M.Y., 2021. Probiotic properties of *Lactobacillus casei* – MYSRD 108 and *Lactobacillus plantarum*-MYSRD 71 with potential antimicrobial activity against *Salmonella paratyphi*. *Biotechnology Reports*, 32. https://doi.org/10.1016/j.btre.2021.e00672

Donio, M.B.S., Ronica, F.A., Viji, V.T., Velmurugan, S., Jenifer, J.S.C.A., Michaelbabu, M., Dhar, P., and Citarasu, T., 2013. *Halomonas sp*. BS4, A biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance. *SpringerPlus,* 2(**1**), 1–10. https://doi.org/10.1186/2193-1801-2-149

Dopazo, C.P., Lemos, M.L., Lodeiros, C., Bolinches, J., Barja, J. L. and Toranzo, A.E., 1988. Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. *Journal of Applied Bacteriology*, 65(**2**), 97–101. https://doi.org/10.1111/j.1365-2672.1988.tb01497.x

Egerton, S., Culloty, S., Whooley, J., Stanton, C. and Ross, R.P., 2018. The gut microbiota of marine fish. *Frontiers in Microbiology*, 9(**873**), 1–17. https://doi.org/10.3389/fmicb.2018.00873

Etyemez, M. and Balcazar, J.L., 2016. Isolation and characterization of bacteria with antibacterial properties from Nile tilapia (*Oreochromis niloticus*). *Research in Veterinary Science,* 105, 62–64. https://doi.org/10.1016/j.rvsc.2016.01.019

FAO., 2024. The state of world fisheries and aquaculture 2024blue transformation in action. FAO. PP - Rome: https://digitallibrary.un.org/record/4050926/files/1408750-EN.pdf

FAO.,(2016. The State of World Fisheries and Aquaculture 2016, Rome. 200.

Ganguly, A., Banerjee, A., Mandal, A., Khan, M. A. and Mohapatra, P.K.D., 2019. Isolation and Characterization of Bacteria from the Intestine of *Clarias batrachus* for Probiotic Organism. *Proceedings of the Zoological Society*, 72(**4**), 411–419. https://doi.org/10.1007/s12595-018-0283-x

Ganguly, N.K., Bhattacharya, S.K., Sesikeran, B., Nair, G.B., Ramakrishna, B.S., Sachdev, H.P.S., Batish, V.K., Kanagasabapathy, A.S., Muthuswamy, V., Kathuria, S.C., Katoch, V.M., Satyanarayana, K., Toteja, G.S., Rahi, M., Rao, S., Bhan, M.K., Kapur, R. and Hemalatha, R., 2011. ICMR-DBT Guidelines for Evaluation of Probiotics in Food. Indian *Journal of Medical Research,* 134(**1**). https://journals.lww.com/ijmr/fulltext/2011/34010/icmr_dbt_guidelines_for_evaluation_of_probiotics.7.aspx

Gao, Y., Qiang, L., Wu, N., Tan, R., Sun, Y., Li, Z., Shen, X. and Cai, Y., 2024. Study on the Potential Probiotics Isolated from Marine Aquaculture System and Evaluation for Aquaculture Application. *Aquaculture Research*, 2024. https://doi.org/10.1155/2024/9555271

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. *Journal of Applied Microbiology*, 180, 147–165.

Ghosh, K., Mukherjee, A., Dutta, D., Banerjee, S., Breines, E.M., Hareide, E. and Ringø, E., 2021. Endosymbiotic pathogen-inhibitory gut bacteria in three Indian Major Carps under polyculture system: A step toward making a probiotics consortium. *Aquaculture and Fisheries,* 6(**2**), 192–204. https://doi.org/10.1016/j.aaf.2020.03.009

Girijakumari, R.N., Ethiraja, K. and Marimuthu, N.P., 2018. In vitro and in vivo evaluation of probiotic properties of *Enterobacter cloacae* in Kenyi cichlid, *Maylandia lombardoi. Aquaculture International*, 26(**4**), 959–980. https://doi.org/10.1007/s10499-018-0262-2

Hai, N.V., 2015. Research findings from the use of probiotics in tilapia aquaculture: A review. *Fish and Shellfish Immunology*, 45(**2**), 592–597. https://doi.org/10.1016/j.fsi.2015.05.026

Immanuel, G., Menenthira, V., Prema, P. and Palavesam, A., 2003. Influence of probionts on the gut microbial diversity in pearl spot, *Etroplus suratensis* (Bloch). *Indian Journal of Fisheries,* 50: 499-508.

Irianto, A. and Austin, B., 2002. Probiotics in aquaculture. *Journal of Fish Diseases,* 25(11), 633–642. https://doi.org/10.1046/j.1365-2761.2002.00422.x

Islam, R. and Mithun, M.H., 2024. Probiotics in aquaculture: A pathway to safer and healthier fish farming. *Archives of Agriculture and Environmental Science*, *9*(**4**), 847-857. https://doi.org/10.26832/24566632.2024.0904030

Janda, J.M. and Abbott, S.L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology,* 45(**9**), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Kar, N. and Ghosh, K., 2008. Enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* (Hamilton) and *Channa punctatus* (Bloch). *Turkish Journal of Fisheries and Aquatic Sciences*, 120(**1**), 115–120.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*(2), 111–120. https://doi.org/10.1007/BF01731581

Kuang, X., Fang, Z., Xiong, N., Ou, J., Wang, F. and Luo, S., 2024. Probiotic characterization of a novel *Bacillus cereus* strain fkW8-1-2 isolated from intestine of white crucian carp (*Carassius cuvieri*). *Reproduction and Breeding*, 4(**2**), 95-101. https://doi.org/10.1016/j.repbre.2024.03.003.

Li, J., Zhang, Z., Wu, Z., Qu, S., Wang, G., Wei, D., Li, P. and Ling, F., 2023. *Enterobacter asburiae* E7, a novel potential probiotic, enhances resistance to *Aeromonas veronii* infection via stimulating the immune response in common carp (*Cyprinus carpio*). *Microbiology Spectrum, 11*(**2**), e04273-22. https://doi.org/10.1128/spectrum.04273-22

Maya, R., Dhevendaran, K., Mathew, A., Georgekutty, M. and Natarajan, P., 1995. Seasonal variations of bacteria in fish *Etroplus suratensis* and *Etroplus maculatus* (Pisces: Cichlidae). *Indian Journal of Marine Sciences*, 24(**4**), 225–228.

Midhun, S.J., Neethu, S., Vysakh, A., Radhakrishnan, E.K. and Jyothis, M., 2018. Antagonism Against Fish Pathogens by Cellular Components Preparations of *Bacillus coagulans* (MTCC-9872) and It's In Vitro Probiotic Characterisation. *Current Microbiology*, 75(**9**), 1174–1181. https://doi.org/10.1007/s00284-018-1506-0

Mondal, S., Roy, T., Sen, S.K. and Ray, A.K., 2008. Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. *Acta Ichthyologica et Piscatoria*, 38(**1**), 1–8. https://doi.org/10.3750/AIP2008.38.1.01

Mukherjee, A., Dutta, D., Banerjee, S., Ringø, E., Breines, E.M., Hareide, E., Chandra, G. and Ghosh, K., 2017. Culturable autochthonous gut bacteria in rohu, *Labeo rohita*. In vitro growth inhibition against pathogenic *Aeromonas* spp., stability in gut, bio-safety and identification by 16S rRNA gene sequencing. *Symbiosis*, 73(**3**), 165–177. https://doi.org/10.1007/s13199-017-0474-7

Nandi, A., Banerjee, G., Dan, S.K., Ghosh, P., Ghosh, K. and Ray, A.K., 2017. Screening of autochthonous intestinal microbiota as candidate probiotics isolated from four freshwater teleosts. *Current Science,* 113(**4**), 767–773. https://doi.org/10.18520/cs/v113/i04/767-773

Newaj-Fyzul, A., Al-Harbi, A.H. and Austin, B., 2014. Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*, 431, 1–11. https://doi.org/10.1016/j.aquaculture.2013.08.026

Padmakumar, K.G., Bindu, L., and Manu, P.S., 2012. *Etroplus suratensis* (Bloch), the state fish of Kerala. *Journal of Biosciences*, 37(**6**), 925–931. https://doi.org/10.1007/s12038-0129271-x

Ravi, A., Das, S., Basheer, J., Chandran, A., Benny, C., Somaraj, S., Sebastian, K.S., Mathew, J. and Krishnankutty, E.R., 2019. Distribution of antibiotic resistance and virulence factors among the bacteria isolated from diseased *Etroplus suratensis*. *3 Biotech*, 9(**4**), 0. https://doi.org/10.1007/s13205-019-1654-3

Ren, X., Wu, B., Zhao, F., Qi, L., Qiu, X., Li, R., Yang, S., Liu, F., Yi, G., Ding, X., Xia, L. and Sun, Y., 2019. Antagonistic activity and protective effect of a *Bacillus subtilis* isolate against fish pathogen *Edwardsiella piscicida*. *Fisheries Science,* 85(**6**), 1011–1018. https://doi.org/10.1007/s12562-019-01346-8

Ringø, E., 2020. Probiotics in shellfish aquaculture. *Aquaculture and Fisheries*, 5(**1**), 1–27. https://doi.org/10.1016/j.aaf.2019.12.001

Romero, J., Feijoo, C.G. and Navarrete, P., 2012. Antibiotics in Aquaculture – Use, Abuse and Alternatives. In Carvalho, E.D., David, G.S. and Silva, R.J. (Eds.), Health and Environment in Aquaculture. IntechOpen. https://doi.org/10.5772/28157

Sankar, H., Philip, B., Philip, R. and Singh, I.S.B., 2017. Effect of probiotics on digestive enzyme activities and growth of cichlids, *Etroplus suratensis* (Pearl spot) and *Oreochromis mossambicus* (Tilapia). *Aquaculture Nutrition*, 23(**4**), 852–864. https://doi.org/10.1111/anu.12452

Santos, R.A., Oliva-Teles, A., Pousão-Ferreira, P., Jerusik, R., Saavedra, M.J., Enes, P. and Serra, C.R.,(2021. Isolation and Characterization of Fish-Gut *Bacillus* spp. as Source of Natural Antimicrobial Compounds to Fight Aquaculture Bacterial Diseases. *Marine Biotechnology,* 0123456789. https://doi.org/10.1007/s10126-021-10022-x

Serrano, P., 2005. Responsible use of antibiotics in aquaculture. https://api.semanticscholar.org/CorpusID:85792575

Sugita, H., Okano, R., Suzuki, Y., Iwai, D., Mizukami, M., Akiyama, N. and Matsuura, S., 2002. Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. *Fisheries Science*, 68(**5**), 1004–1011. https://doi.org/10.1046/j.1444-2906.2002.00525.x

Sugita, H., Oshima, K., Tamura, M. and Deguchi, Y., 1983. Bacterial Flora in the Gastrointestine of Fresheater Fishes in the River. *Nippon Suisan Gakkaishi*, 49(**9**), 1387–1395. https://doi.org/10.2331/suisan.49.1387

Sweta, P., Shubham, H., Mottakunja, B., Shalini, D. and Tony, K., 2024. Influence of gut microbiome on health and development of penaeid shrimps. *Aquatic Sciences*. https://doi.org/10.1007/s00027-023-01018-x

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution, 38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Todorov, S.D., Marcos, J., Lima, S., Enrique, J., Bucheli, V., Popov, I.V., Kumar, S., Michael, T. and Chikindas, L., 2024. Probiotics for Aquaculture: Hope, Truth, and Reality. *Probiotics and Antimicrobial Proteins*, 0123456789. https://doi.org/10.1007/s12602-024-10290-8

Trust, T.J. and Sparrow, R.A.H., 1974. The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Canadian Journal of Microbiology*, 20(**9**), 1219–1228. https://doi.org/10.1139/m74-188

Van Doan, H., Hoseinifar, S.H., Ringø, E., Ángeles, E.M., Dadar, M., Dawood, M.A.O. and Faggio, C., 2020. Host-Associated Probiotics: A Key Factor in Sustainable Aquaculture. *Reviews in Fisheries Science and Aquaculture,* 28(**1**), 16–42. https://doi.org/10.1080/23308249.2019.1643288

Verner-Jeffreys, D.W., Shields, R.J., Bricknell, I.R. and Birkbeck, T.H., 2003. Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture,* 219(1–4), 21–42. https://doi.org/10.1016/S0044-8486(02)00348-4

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and molecular biology reviews : MMBR*, *64*(**4**), 655–671. https://doi.org/10.1128/MMBR.64.4.655-671.2000

Vinderola, C.G. and Reinheimer, J.A., 2003. Lactic acid starter and probiotic bacteria: A comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International*, 36(9–10), 895–904. https://doi.org/10.1016/S0963-9969(03)00098-X

Wang, A.R., Ran, C., Ringø, E., and Zhou, Z.G., 2018. Progress in fish gastrointestinal microbiota research. *Reviews in Aquaculture*, 10(**3**), 626–640. https://doi.org/10.1111/raq.12191

Wang, C., Li, F., Wang, D., Lu, S., Han, S., Gu, W., Jiang, H., Li, Z. and Liu, H., 2024. Enhancing Growth and Intestinal Health in Triploid Rainbow Trout Fed a Low-Fish-Meal Diet through Supplementation with *Clostridium butyricum*. *Fishes*, *9*(**5**), 178. https://doi.org/10.3390/fishes9050178

Yadav, M., Kumar, T., Kanakan, A., Maurya, R., Pandey, R. and Chauhan, N.S., 2022. Isolation and Characterization of Human Intestinal Bacteria *Cytobacillus oceanisediminis* NB2 for Probiotic Potential. *Frontiers in Microbiology*, 13(**7**). https://doi.org/10.3389/fmicb.2022.932795