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In vitro Profiling of Probiotic *Bacillus* Species Isolated from the Gut Microbiota of Brackish Water Fish, *Etroplus Suratensis* (Pearlspot)

Ethirajan Manimozhi[1], Arumugam Uma[2], Nathan Felix[3], Antony Cheryl[3]
Sethu Selvaraj[4], S. Arun Sudhagar[5], Harikrishan Lisha[1]

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ABSTRACT

Background: In aquaculture, antibiotic misuse has accelerated antimicrobial resistance (AMR), endangering fish health, ecosystems and public safety. Probiotics offer a sustainable alternative by enhancing immunity, growth and pathogen resistance without promoting AMR. The pearlspot (*Etroplus suratensis*), a valuable brackish water fish in South Asia, remains vulnerable to bacterial diseases, yet research on autochthonous probiotics for its health management is limited.

Methods: The present investigation aimed to assess the probiotic potential of two bacterial isolates, *Bacillus subtilis* (BLCET3/SRLAAH/2023) and *B. velezensis* (BLCET4/SRLAAH/2023) isolated from the gut of a brackish water fish, *E. suratensis*. The probiotic potential of the isolates was analyzed by in vitro assays, molecular analysis of probiotic marker genes and *in vivo* biosafety assay.

Result: Among the two isolates, BLCET3 exhibited stronger antimicrobial activity against *Streptococcus agalactiae*, *Aeromonas veronii* and *Enterococcus* sp. Both BLCET3 and BLCET4 tolerated a pH range of 2-9 and bile salt concentrations up to 10%, produced extracellular enzymes and formed biofilms. BLCET3 showing significantly higher ($P<0.05$) auto-aggregation, co-aggregation and cell surface hydrophobicity. Neither isolate displayed antibiotic resistance in ABST assays and *in vivo* biosafety tests confirmed them as non-pathogenic. PCR analysis revealed the presence of key probiotic marker genes. These findings confirm the probiotic potential of both isolates for aquaculture applications.

Key words: Autochthonous bacteria, Molecular profiling, Probiotic marker genes, Probiotics.

INTRODUCTION

Disease is a major constraint to aquaculture production, causing an estimated 21% loss of global aquaculture output, equivalent to approximately US$ 10 billion annually (Peeler *et al.,* 2023). In India, disease-related losses in aquaculture are estimated at US$ 2.48 billion, accounting for 14.95% of the annual production value (Patil *et al.,* 2025).

In aquaculture, antibiotics play a vital role in preventing and treating bacterial infections. While antibiotics are widely used to manage these infections, their overuse has led to antibiotic resistance, raising global health concerns (Mukhtar *et al.,* 2025). Probiotics have emerged as sustainable alternatives, offering health benefits such as improved digestion, pathogen inhibition and enhanced immunity in fish (Rathod *et al.,* 2025). They prevent pathogen colonization *via* competitive exclusion, organic acid production and antimicrobial metabolites like bacteriocins (Tabassum *et al.,* 2021).

Important probiotic selection criteria include their acid and bile tolerance for gut survival, adherence to epithelial surfaces and biofilm formation for colonisation (Hyronimus *et al.,* 2000). Genetic markers enable precise probiotic strain identification, complementing microbiological techniques (Papadimitriou *et al.,* 2015). Autochthonous probiotics from the fish gut are better adapted to aquatic environments than commercial probiotics sourced from terrestrial systems (Lalitha *et al.,* 2022; Hoseinifar *et al.,* 2024). Bacterial genera *viz*., *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Bacillus* and *Enterococcus* exhibit probiotic

[1]Department of Aquatic Animal Health Management, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.
[2]Directorate of Incubation and Vocational Training in Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, ECR-Muttukadu Chennai-600 001, Tamil Nadu, India.
[3]Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Vettar River View Campus, Nagapattinam-609 307, Tamil Nadu, India.
[4]Department of Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.
[5]Fish Health, Peninsular and Marine Fish Genetic Resources Centre, ICAR – National Bureau of Fish Genetic Resources, Kochi-682 001, Kerala, India.

Corresponding Author: Arumugam Uma, Directorate of Incubation and Vocational Training in Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, ECR-Muttukadu Chennai-600 001, Tamil Nadu, India. Email: uma@tnfu.ac.in

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potential (Nayak *et al.,* 2010), with *Bacillus* species gaining prominence due to their resilience and effectiveness in aquaculture (Cheng *et al.,* 2017). In brackish water aquaculture, *Bacillus sp.* enhanced growth, water quality, stress tolerance and disease resistance in finfish and shellfish species *viz*., *Oreochromis niloticus, Labeo rohita, Penaeus vannamei* and *Scylla tranquebarica* (Hortillosa *et al.,* 2022; Jamil *et al.,* 2025; Gunarto *et al.,* 2023; Monier *et al.,* 2023).

E. suratensis (Pearlspot), native to South Asian brackish water habitats, is commercially significant and suitable for polyculture systems. Despite its aquaculture importance, bacterial diseases remain a challenge in their culture activities (Swaminathan *et al.,* 2022). Studies on autochthonous probiotics characterization from *E. suratensis* remain largely unexplored. To address this, two *Bacillus* species were isolated and characterized from the gut of *E. suratensis* and their probiotic potential were evaluated.

MATERIALS AND METHODS

Sample collection and isolated of gut-associated *Bacillus* species

Seventy-five healthy specimens of *E. suratensis* (46±2.4 g; 11.4±1.2 cm) were collected from Pulicat lagoon (13.4177° N, 80.3185°E) and transported alive to the State Referral Laboratory for Aquatic Animal Health, Chennai. After 48 h of starvation, the guts were aseptically dissected, homogenized and serially diluted in sterile saline (10^{-1} to 10^{-6}). Samples were spread plated on Hichrome *Bacillus* agar and incubated at 37°C for 24-48 h. Morphologically distinct colonies were sub-cultured to obtain pure isolates, yielding 37 bacterial strains from the gut of *E. suratensis*.

Screening for antibacterial activity

Fish bacterial pathogens *viz*., *Streptococcus agalactiae* (NCBI Accession no. PP580699), *Aeromonas veronii* (Accession no. OR487464), *Enterococcus* sp. (Accession no. PP600907) obtained from the microbial archive in the State Referral Laboratory of Aquatic Animal Health - Madhavaram, Chennai was used. The antibacterial potential for the isolates was screened using disc diffusion method (Lapenda *et al.,* 2015). The zones of inhibition (mm) against the bacterial pathogens were measures and the effective isolates., BLCET3 and BLCET4 were further characterised biochemically and molecular confirmated by PCR and sequencing.

Biochemical characterization

Morphological and biochemical characterization of the bacterial isolates BLCET3 and BLCET4 were carried out by gram-staining, motility test, oxidase, catalase, methyl red, voges-proskauer test, growth at 5% NaCl, indole, citrate utilization, carbohydrate fermentation and amino acid utilization tests (Berkeley *et al.,* 1984).

Molecular confirmation of bacterial isolates

Total DNA was extracted from bacterial isolates BLCET3 and BLCET4 using commercial DNA extraction Kit (Qiagen, Germany). 16S rRNA gene of the bacteria was amplified following Weisburg *et al.* (1991). Amplified products (1484 bp) were visualized on a 1.5% ethidium bromide-stained agarose gel and documented (Bio-Rad). Sequenced products (Eurofins, India) were analyzed using NCBI BLAST, submitted to GenBank for accession numbers and a phylogenetic tree was constructed *via* Maximum Likelihood in MEGA 11.

In vitro screening for probiotic potential

The probiotic potential of *Bacillus* isolates was assessed through multiple in vitro assays. pH and bile tolerance were evaluated following Khan *et al.* (2021), with bacterial cultures adjusted to OD_{600} 0.25 (10w CFU/mL). For pH tolerance, cultures were incubated in TSB (pH 2-9) at 37°C for 24 hours. For bile tolerance, isolates were exposed to bile salts (0%, 2.5%, 5% and 10% w/v) in PBS (pH 7.4) at 30°C for 1 hour and growth was measured at 600 nm (Shimadzu, Japan). Salt and phenol tolerance were tested in MRS broth with varying concentrations of NaCl (0-5% w/v) and phenol (0-5% v/v), incubated at 30°C for 24 hours and measured at 600 nm (Aswathy *et al.,* 2008). Extracellular enzymatic activity was evaluated using nutrient agar supplemented with specific substrate: starch for amylolytic activity, carboxymethyl cellulose for cellulase activity, gelatin for gelatinase activity and skimmed milk for proteolytic activity. The bacterial isolates, BLCET3 and BLCET4 were streaked onto respective media and incubated at 30°C for 24 h. Amylase activity was detected by flooding with 1% iodine solution, gelatinase with 15% mercuric chloride and cellulase with 1% Congo red followed by washing with 1 M NaOH (Balan *et al.,* 2012; Kasana *et al.,* 2008). Auto-aggregation and co-aggregation assays followed Rastogi *et al.* (2020) and Handley *et al.* (1987). Cell hydrophobicity was tested using xylene, chloroform and ethyl acetate following the method of Patel *et al.* (2009). Biofilm formation was screened using congo red agar method (Ramesh *et al.,* 2015). The isolates, BLCET3 and BLCET4 were streaked on Muller Hinton agar (Himedia, India) containing 0.8 g/l of Congo red dye and incubated for 48h at 37°C.

Detection of probiotic marker genes

The probiotic properties of BLCET3 and BLCET4 were confirmed by PCR amplification of marker genes, including 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (2,3-BIP), arginine/ornithine antiporter (ArcD), choloylglycine hydrolase (CGH), LuxS and the E1 β-subunit of the pyruvate dehydrogenase complex (E1-β) (Khan *et al*., 2021). DNA quality and concentration were checked spectrophotometrically ($OD_{260/280}$ ~1.8-2.0) prior to amplification. PCR reactions included standard positive and negative controls to ensure specificity and reliability. Amplicons were resolved on 1.5% agarose gels and documented using a gel documentation system (Bio-Rad, Germany).

Safety evaluation of the probiotic isolates

The antibiotic susceptibility of BLCET3 and BLCET4 was evaluated using the Kirby-Bauer disk diffusion test on Mueller-hinton Agar (MHA) with sterile antibiotic discs

Table 1: Antibacterial activity of the bacterial isolates against targeted fish pathogens.

Pathogenic bacteria	Zone of Inhibition (ZOI) - mm		Antibiotic control - Chloramphenicol
	BLCET3	BLCET4	
S. agalactiae	23±1.00^{ab}	20±2.00^{b}	25±2.00^{a}
Enterococcus sp.	18±1.00^{b}	13±1.00^{c}	28±2.00^{a}
A. veronii	14±1.00^{b}	11±1.00^{c}	21±1.00^{a}

(Himedia, India) following CLSI standards (Bauer *et al.,* 1996). Zones of inhibition (ZOI in mm) were measured after 24 - 48 hrs of incubation at 37°C. For in vivo biosafety assessment, groups of 10 *E. suratensis* (23.5±2 g) in triplicates were intraperitoneally injected with 0.1 mL bacterial suspensions (10^9 CFU/mL) of each isolate. A control group received an equivalent volume of sterile PBS (pH 7.2). Fish were monitored daily for 10 days for signs of disease and mortality.

Statistical analysis

The results of *in vitro* tests were analyzed using the IBM SPSS Statistic 26 software. All the data were analyzed using one-way ANOVA employing Tukey's post hoc test (p=0.05) to identify the significant differences among the groups. The results were represented as the mean ± standard error, with statistical significance at P<0.05.

RESULTS AND DISCUSSION

Bacillus spp. are promising probiotics in aquaculture due to their spore-forming nature, ease of large-scale production, enzyme synthesis, beneficial metabolites and immunostimulatory properties (Nayak *et al.,* 2010; Li *et al.,* 2023). The healthy fish gut provides host-adapted autochthonous probiotics that enhance fish and ecosystem health while reducing the environmental risks linked to non–host-specific strains (Li *et al.,* 2024; Fadel *et al.,* 2025). A total of 37 bacterial isolates from the gut of 75 healthy *E. suratensis* specimens were screened for their antibacterial efficacy. Among them, two isolates BLCET3 and BLCET4, exhibited significant antibacterial activity against fish pathogens, including *S. agalactiae*, *A. veronii* and *Enterococcus* sp. The zones of inhibition (ZOI) were measured and are presented in Table 1. The antibacterial activity observed is likely attributed to the production of bacteriocins, enzymes and other antimicrobial metabolites (Naeem *et al.,* 2018) which enhance the ability of these isolates to inhibit pathogenic bacterial growth effectively.

Biochemical characterization revealed metabolic variations between BLCET3 and BLCET4, particularly in their ability to ferment carbohydrates and utilize amino acids (Table 2). These findings suggest that both isolates possess a versatile metabolic profile, contributing to their survivability and functionality in the gut environment (Kuebutornye *et al.,* 2020). Molecular characterization through 16S rRNA sequencing identified BLCET3 as *B. subtilis* (Accession No. PP851370) and BLCET4 as *B. velezensis* (Accession No. PP657343) (Fig 1).

Table 2: Biochemical characterization of the bacterial isolates.

Test	BLCET3	BLCET4
Gram staining	+	+
Cell morphology	Rod	Rod
Motility	+	+
Oxidase	-	-
Catalase	+	+
MR test	-	-
VP test	-	-
Indole test	-	-
Citrate utilisation test	+	-
Xylose (Xy)	+	+
Cellobiose (Ce)	+	+
Rhamnose (Rh)	-	+
Galactose (Ga)	-	
Salicin (Sa)	+	+
Trehalose (Te)	+	+
Mannitol (Mn)	-	+
Raffinose (Rf)	+	-
Inulin (In)	+	+
Arabinose (Ar)	+	+
Lactose (La)	+	+
Inositol (Is)	-	+
Arginine	+	+
Lysine	-	+
Histidine	-	-
Ornithine	+	+
Serine	+	+

The ability to withstand harsh gastrointestinal conditions is fundamental probiotic trait (Ghosh *et al.,* 2017). Both BLCET3 and BLCET4 exhibited pH tolerance across a wide range (2-9), with optimal growth observed at pH 7-8 (Fig 2a). Notably, BLCET3 displayed slightly greater resilience at extreme pH levels compared to BLCET4. Similarly, both isolates tolerated bile concentrations up to 10% (w/v), although growth gradually decreased with increasing bile concentrations (Fig 2b). These findings are consistent with earlier report of Nayak *et al.* (2024), which highlighted the importance of pH and bile tolerance in probiotic bacteria.

Both isolates demonstrated salt tolerance up to 5% NaCl and phenol tolerance up to 0.6%, with growth declining beyond these thresholds (Fig 2c, 2d). The ability to withstand salt stress aligns with observations by Emam and Dunlap (2020) who reported the role of osmolyte regulation in maintaining cellular balance under hypertonic

conditions. Phenol tolerance, an indicator of resilience in the gastrointestinal environment, further validates the robustness of these isolates (Yadav *et al.,* 2016).

Extracellular enzymes production *viz*., amylase, cellulase, gelatinase and protease were observed in both isolates, indicated by clear halo zones around colonies on substrate- specific agar plates. These enzymatic activities facilitate the breakdown of complex dietary components, improving nutrient absorption in the host (Banerjee *et al.,* 2013). The enzymatic profile observed in BLCET3 and BLCET4 suggests their potential to enhance the digestive efficiency of *E. suratensis*.

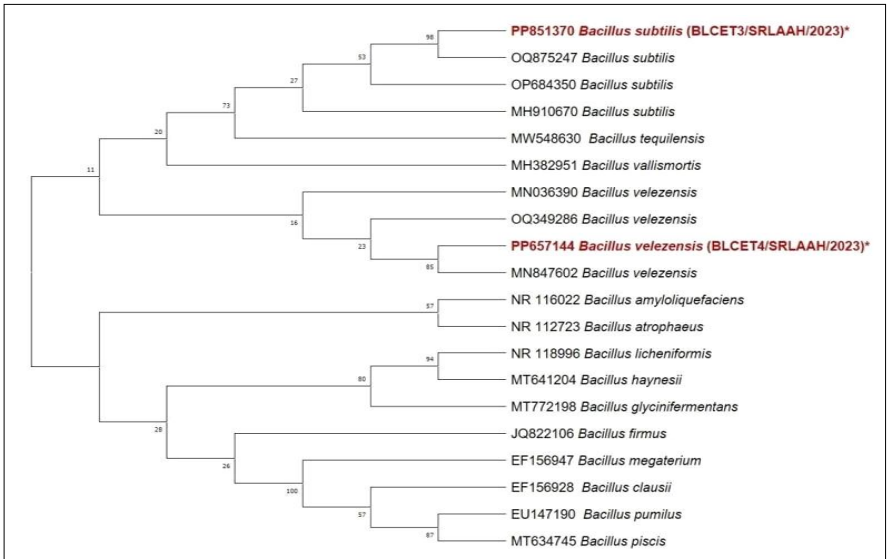
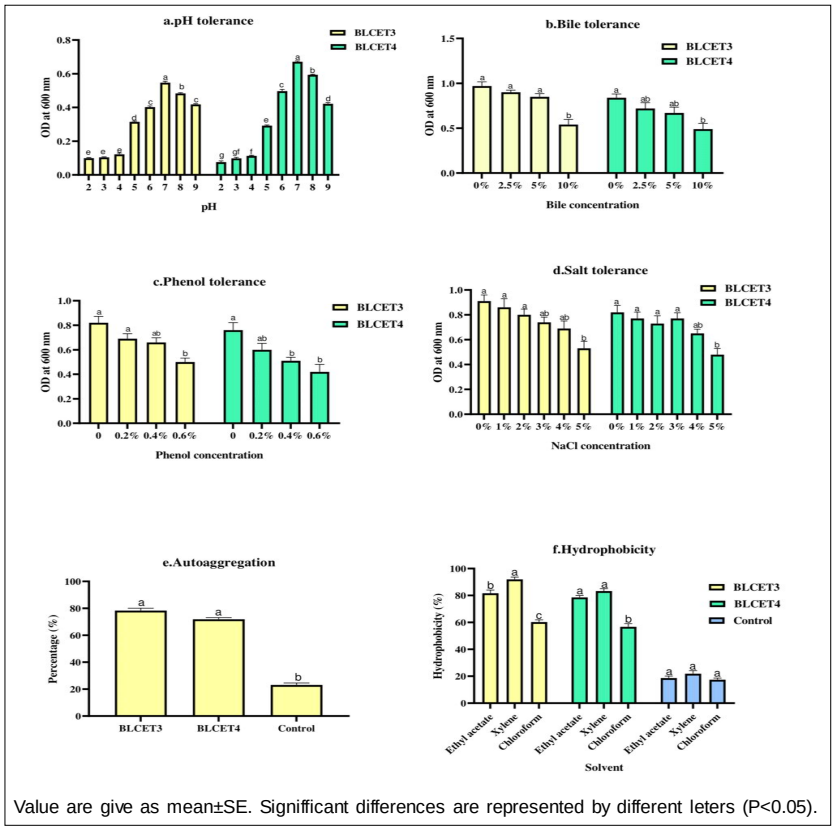


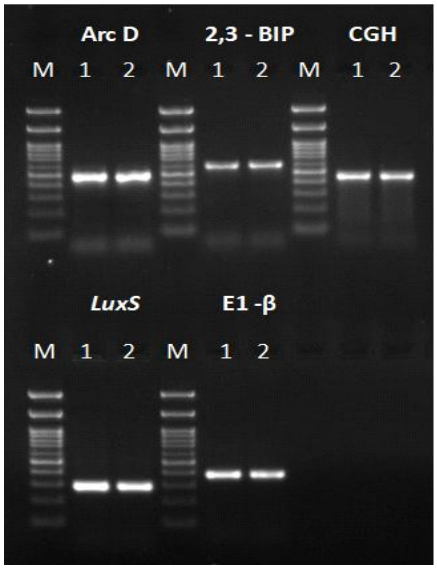
Fig 1: Phylogram constructed using the neighbour-joining tree for *Bacillus* species.



Value are give as mean±SE. Signifficant differences are represented by different leters (P<0.05).

Fig 2: *In vitro* assays for probiotic screening of bacterial isolate.

Auto-aggregation and co-aggregation assays revealed strong aggregation abilities in both isolates. BLCET3 exhibited auto-aggregation of 78.34% and co-aggregation of 45.73%, while BLCET4 showed slightly lower values (71.92% and 39.47% respectively) (Fig 2e). These findings suggest that both strains can adhere to intestinal surfaces and competitively exclude pathogens, consistent with the results of Thankappan *et al.* (2015). Additionally, cell hydrophobicity assays demonstrated strong affinities for xylene, ethyl acetate and chloroform with BLCET3 showing higher hydrophobicity than BLCET4 (Fig 2f). Hydrophobicity is critical for bacterial adhesion to host intestinal mucosa, which supports colonization and persistence in the gut (Patel *et al.,* 2009). Biofilm formation enhances bacterial survival, resistance to stress and pathogen exclusion (Guo *et al*., 2016) and the ability of probiotic strains to form biofilms enables them to outcompete pathogens for nutrients and colonization sites, thereby conferring protection to the host (Puvanasundram *et al.,* 2022). Both the isolates produced black crystalline colonies on Congo red agar indicating their ability to form biofilm.



(Lane M- DNA Marker (100 bp); 1- BLCET3, 2 – BLCET4; Amplicon size: ArcD gene – 486 bp; 2,3 BIP – 587 bp; CGH – 447 bp; *LuxS* -291bp; E1-β - 394 bp).

Fig 3: Agarose gel electrophoresis of PCR products of probiotic markers genes of bacterial isolates.

Table 3: Antibiotic susceptibility of the bacterial isolated from the gut of *E. suratensis.*

Antibiotics	BLCET3	BLCET4
Ampicillin (10 µg)	S	I
Amoxicillin (30 µg)	S	S
Rifampin (5 µg)	S	S
Co-trimoxazole (10 µg)	S	S
Ciprofloxacin (5 µg)	S	S
Penicillin (10 µg)	I	I
Erythromycin (15 µg)	S	S
Oxytetracycline (10 µg)	S	S
Meropenem (10 µg)	S	S
Sulphafurazole (300 µg)	S	S
Chloramphenicol (30 µg)	S	I
Clindamycin (2 µg)	S	S
Cefoxitin (30 µg)	S	S
Cefotaxime (30 µg)	I	S

The functional probiotic attributes of BLCET3 and BLCET4 were validated through PCR amplification of key marker genes (Fig 3). These included 2,3-Bisphosphoglycerate-independent phosphoglycerate mutase (gpmM) for acid stress tolerance (Kapse *et al.,* 2019), Choloylglycine hydrolase (bsh) for bile salt deconjugation (Khan *et al.,* 2021), Arginine/ornithine antiporter (ArcD) for acid and bile salt resistance (Mazhar *et al.,* 2023), *LuxS* for quorum sensing and colonization efficiency (Jiang *et al.,* 2021) and E1 β-subunit of pyruvate dehydrogenase complex (fbpA) for adhesion to intestinal epithelial cells (Oliveira *et al.,* 2017).

The presence of these marker genes in the bacterial isolates further affirms their probiotic nature in molecular level and similar findings was reported by Khan *et al.* (2021).

Antibiotic susceptibility is considered an important attribute of probiotics (Yu *et al.,* 2023). Both the isolates demonstrated susceptibility to the majority of tested antibiotics (Table 3). The antibiotic susceptibility of the *Bacillus* isolates indicates a lower risk of AMR transfer, further supporting their potential as safe probiotic candidates. Our biosafety evaluation revealed that neither *B. subtilis* (BLCET3) nor *B. velezensis* (BLCET4) induced any signs of disease or mortality in the injected fish, suggesting their safety for in vivo applications. Our in vitro study will serve as a strongbase line for future in vivo trials assessing the immunomodulatory effects, while optimized downstream processing, formulation, encapsulation and delivery are critical for successful large-scale application of these probiotics in aquaculture (Ordanel *et al*., 2025).

CONCLUSION

The isolates *B. subtilis* (BLCET3) and *B. velezensis* (BLCET4) exhibited strong antimicrobial activity, enzymatic capabilities, pH and bile tolerance, biofilm formation, adhesion properties and absence of AMR. Probiotic marker genes and safety assessments further confirmed their potential as probiotics. Future studies are needed to evaluate their large-scale *in vivo* efficacy for fish health management.

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Conflict of interest

The authors declare that there is no conflict of interest.

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