



Preliminary *in vitro* screening of gut isolates tentatively identified as *Lactobacillus delbrueckii* VR1 and *Bacillus subtilis* VR2 from Nile tilapia (*Oreochromis niloticus*) as potential probiotic candidates

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Abstract

We recovered two culturable isolates from pooled gut samples of three Nile tilapia (*Oreochromis niloticus*) collected in the Kaveri River, Tamil Nadu, India, on de Man, Rogosa and Sharpe (MRS) agar. They were tentatively identified as *Lactobacillus delbrueckii* VR1 and *Bacillus subtilis* VR2 (about 3.2×10^6 and 1.8×10^6 colony-forming units (CFU) g⁻¹ of gut content, respectively), based on 16S rRNA gene sequencing results. Neither isolate showed clear haemolysis. Turbidity (optical density at 600 nm, OD₆₀₀) after 3 h under acid, bile salt, NaCl and temperature challenge differed between the isolates and among conditions, but because these were single-endpoint readings made without viable counts they are reported as OD responses and not as growth, survival or tolerance. Auto-aggregation was low (6.8–11.9% for VR1 and 5.4–12.5% for VR2 after 1–3 h), whereas mixed cultures with four pathogens gave co-aggregation values of 49.7–62.5%, compared with 6.8–14.2% in the single-culture controls. Solvent-affinity values (47.8–76.5%) were obtained from an assay adapted from microbial adhesion to hydrocarbons/solvents (MATH/MATS) that used polar solvents and are not classical hydrophobicity values. Cell-free supernatants inhibited all six pathogens tested (zones of 7.8–14.1 mm, and 1.7–4.0 mm smaller after adjustment to pH 7); both isolates showed lipase and amylase activity on plates, and radical-scavenging activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was about 46% (VR1) and 51% (VR2), compared with 67.9% for ascorbic acid. These findings describe *in vitro* functional characteristics only. Genome-based identification, antimicrobial-resistance and virulence screening, and controlled feeding trials in Nile tilapia are needed before any probiotic efficacy or safety can be claimed.

Keywords: Aquaculture, Probiotic candidate, Cell surface properties, Antimicrobial activity, Tentative identification, 16S rRNA gene

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Introduction

Aquaculture has grown significantly in the last decades; outbreaks of bacterial diseases still stand out as one of the key limitations. The prevention of pathogens has relied mainly on antibiotic treatments and chemical agents, and long-term application of such methods is associated with environmental contamination by chemical residues as well as selection of antibiotic-resistant bacteria (Jolly *et al.*, 2023). Exposing fish to antibiotics could also negatively affect the composition of gut microbiota and increase fish's vulnerability to infections (Burgos *et al.*, 2024). Therefore, alternative approaches aimed at increasing hosts' resistance and improving gut microbiota are being sought. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al.*, 2014; Chaudhari and Dwivedi 2022; Gebeyehu 2023). In aquaculture, probiotics have been recommended as a replacement to chemical treatments with reported effects on immune response and disease resistance in finfish and crustaceans (Alvanou *et al.*, 2023; Wuertz *et al.*, 2023; Yousuf *et al.*, 2023). Probiotics based on host-associated strains of *Bacillus* were assessed for the ability to prevent *S. iniae* infection in Nile tilapia (*Oreochromis niloticus*) (Büyükdeveci *et al.*, 2023), and the effect of single-strain probiotic versus multi-strain probiotic supplementation on fish growth, gut health, and immunity was compared (Melo-Bolívar *et al.*, 2023). LABs and *Bacillus* are among the most

studied groups. Probiotics-related LABs have been isolated from fish gut microbiota, including tilapia (Patel *et al.*, 2020; Govindaraj *et al.*, 2021), while fish gut *Bacillus* isolates are used as antimicrobial compound producers and sources of species used in various biotechnology applications (Santos *et al.*, 2021; Herrmann *et al.*, 2023; Zhu *et al.*, 2023).

Candidates taken from the gut of the intended host are generally expected to be better adapted to the host and its environment than commercial strains (Roobab *et al.*, 2020; Latif *et al.*, 2023), and screening of gut isolates from freshwater fish has been used as a first step to identify putative probionts (Ghosh *et al.*, 2007; Vijayaram and Kannan 2014). In vitro screening typically covers the absence of haemolysis, tolerance of acid, bile salts and salinity, cell surface properties relevant to adhesion, inhibition of pathogens and extracellular enzyme production (Ayyash *et al.*, 2021). The present study aimed to isolate and characterise culturable bacterial isolates recovered on MRS agar from pooled gut samples of Nile tilapia collected from the Kaveri River, Tamil Nadu, India. The isolates were examined for haemolytic activity, OD-based responses to acid, bile salts, NaCl and temperature, auto-aggregation, co-aggregation, solvent affinity, antimicrobial activity of cell-free supernatants, lipase and amylase activity, and DPPH radical-scavenging activity, and were identified tentatively by 16S rRNA gene sequencing. The

novelty of the work lies in bringing these assays together for two isolates from a river population of Nile tilapia in southern India, and in reporting the limits of each assay explicitly. The results are intended as an initial screening framework. Genomic characterisation, safety assessment and controlled in vivo feeding trials are still required before the isolates can be considered for use in aquaculture.

Materials and methods

Sample collection

Oreochromis niloticus specimens were obtained from routine harvests by licensed artisanal fishermen at the Kaveri River, Mohanur, Namakkal District, Tamil Nadu, India (11.059875° N, 78.142189° E), and not from retail markets. The site is a tropical, perennial freshwater system with surface-water temperatures of 24–32°C depending on the season. Three fish were selected at random and transported to the laboratory in sterile polyethylene bags containing habitat water during summer. Species identification followed the keys of Day (1878) and the pictorial guides of Jayaram (1999). The guts of the three fish were pooled and processed as a single sample.

Isolation and enumeration of gut bacteria

Fish were rinsed with sterile distilled water followed by 70% ethanol surface disinfectant. The gut contents were aseptically removed from the gastrointestinal tract, pooled and homogenized in 9 mL of sterile

physiological saline (0.85% NaCl). Serial dilutions were made and plated on MRS agar (HiMedia, India) by the pour-plate method (Patel *et al.*, 2020) and incubated at 37°C for 24–48 h. Colonies were counted and expressed as CFU g⁻¹ of gut content. Thirty-five colonies representing the culturable morphotypes recovered on MRS agar were purified by repeated streaking. Two representative isolates were then selected on the basis of colony morphology, Gram staining and biochemical profile. Because a single medium was used, they are described as selected culturable isolates and not as dominant members of the gut microbiota. Counts for the two isolates were about 3.2×10⁶ CFU g⁻¹ (VR1, tentatively *L. delbrueckii*) and 1.8×10⁶ CFU g⁻¹ (VR2, tentatively *B. subtilis*). MRS is not a selective medium for *Bacillus*, but *Bacillus* species can grow on it, and VR2 was recovered from the MRS plates. Both isolates were subcultured in Tryptic Soy Broth (TSB) for further work (Ghosh *et al.*, 2007).

Morphological, physiological and biochemical characterisation

The morphological, Gram staining, motility, endospore formation and biochemical tests (including catalase, oxidase, indole, methyl red [MR], Voges–Proskauer [VP] and carbohydrate fermentation) were performed according to the principles of Bergey's Manual of Systematic Bacteriology (De Vos *et al.*, 2009) in three independent experiments (n=3) (Table 1). Data are presented as an initial phenotypic profile. Gram staining: heat-

fixed smears from 24-h cultures were stained with crystal violet (1 min), Gram's iodine (1 min), decolourised with 95% ethanol (10–20 s) and counter-stained with safranin for 30 s under 100× magnification in oil immersion. Motility was tested by stab inoculating into motility test medium (HiMedia, India) and incubating at 37°C for 24 h; (+) diffuse growth away from the stab line indicated motile bacteria. For spore formation, cultures grown on nutrient agar slants (37°C, 24–48 h) were heat-shocked at 80°C for 10 min and plated onto nutrient agar; growth after heat shock was scored as positive. For the catalase test, a colony from a fresh culture was mixed with 3% (v/v) hydrogen peroxide and bubble formation within 10 s was scored as positive. A filter paper soaked with 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride was applied to an isolated colony after the application of H₂O, and a dark purple colour within 10–30 s was taken as positive for oxidase. For the indole test, cultures in tryptone broth (HiMedia, India) were incubated for 24 h at 37°C and then subjected to the addition of 5 drops of Kovac's reagent; a red ring within 1–2 min was scored as positive. Cultures in MR-VP broth (HiMedia, India) were incubated at 37 °C for 48 h for the MR and VP tests, with a positive result defined as red colour after addition of five drops of methyl red (0.02% w/v in 60% ethanol). For the VP test, add 0.6 mL of Barritt's reagent A (5% w/v α -naphthol in 95% ethanol) and 0.2 mL of reagent B (40% w/v KOH); a red colour

within 15 min is positive. Glucose, lactose, galactose, mannitol, cellobiose and raffinose fermentation were assessed in phenol red carbohydrate broth (2% w/v sugar) inoculated with 1% (v/v) culture and incubated at 37°C for 24–48 h with Durham tubes. The colour changes from red to yellow, as compared to the uninoculated control, was scored as acid production and gas in the Durham tube as gas production.

Screening of probiotic-candidate properties

Haemolytic activity

Haemolytic activity was determined on 5% sheep blood agar. The streaked isolates were incubated for 48 h at 37°C; the absence of any zone was recorded as γ -haemolysis (non-haemolytic).

Acid, bile salt, NaCl and temperature challenge

For the acid challenge (Erkkilä and Petäjä 2000), cells were suspended in PBS adjusted to pH 1.5, 2.5, 3.5 and 4.5 and incubated at 30°C for 3 h, after which turbidity (OD₆₀₀) was read. For the bile salt challenge (Ahire *et al.*, 2011), peptone water containing 0.3%, 0.6% or 1.0% (w/v) ox bile was inoculated at a volume ratio of approximately 1:100 and incubated at 30°C for 3 h before the OD₆₀₀ reading. For the NaCl challenge, cells were incubated at 30°C for 3 h in TSB supplemented with 0.3%, 0.6% or 1.0% (w/v) NaCl, and TSB without added NaCl served as the reference (0%). For the temperature challenge, cultures were incubated at 4, 15, 25 and 37°C for 3 h

and OD₆₀₀ was measured. Uninoculated medium was included as a control in the acid and NaCl assays only; no bile-salt-free control was included. All four assays used a single 3-h endpoint reading and no viable counts were made, so the readings are reported as OD (turbidity) responses and are not interpreted as growth, survival or tolerance.

Cell surface properties

Auto-aggregation

Auto-aggregation was assessed by the method of Del Re *et al.* (2000). Following overnight culture, samples (8,000×g, 10 min, 4°C) were pelleted through sterile PBS two times and resuspended in PBS to an OD₆₀₀ of 0.5 (approximately equivalent to 1×10⁸ CFU mL⁻¹). In sterile glass tubes, four millilitres of each suspension were introduced and incubated at 30°C without agitation. At 0, 1, 2 and 3 h, 1 mL of the upper layer was withdrawn, and OD₆₀₀ was read using PBS as the blank. Replicates: three biological replicates (n=3) of all treatments were incubated. Auto-aggregation (%)=(A₀-A_t)/A₀×100, where A₀ is OD₆₀₀ (0 h), and A_t is OD₆₀₀ at 1, 2 or 3 h.

Co-aggregation

Co-aggregation was determined by the method of Handley *et al.* (1987). *L. delbrueckii* VR1 was paired with *Klebsiella pneumoniae* and *Vibrio parahaemolyticus*, and *B. subtilis* VR2 with *Escherichia coli* and *Staphylococcus aureus*. Each isolate and pathogen was standardised in PBS, and

control tubes contained either the probiotic candidate or the pathogen alone. Equal volumes of the two suspensions were mixed, all tubes were incubated at 30°C, and OD₆₀₀ was recorded at 0, 1, 2 and 3 h. Co-aggregation (%)=[(A_{probiotic} + A_{pathogen})/2 - A_{mix}]/[(A_{probiotic} + A_{pathogen})/2] × 100. Values are reported for each pair separately (n=3 biological replicates).

Solvent affinity

A test based on the principle of microbial adhesion to hydrocarbons/solvents (MATH/MATS) (Rosenberg *et al.*, 1980; Bellon-Fontaine *et al.*, 1996) was adapted to evaluate cell surface affinity for three solvents: ethanol, methanol and ethyl acetate. These are polar solvents and different from the hydrocarbons used in the classical MATH assay; consequently, the output is a solvent-affinity index instead of cell surface hydrophobicity. Equal volumes of solvent were mixed with washed cells in PBS, allowed to stand, and then the OD₆₀₀ of the aqueous phase was compared to that at time 0. It is calculated using the formula (A₀-A)/A₀×100, where A₀=initial OD₆₀₀ of the suspension; and A=OD after exposure to solvent.

Antibacterial, enzymatic and antioxidant activities

Antibacterial activity

Agar well diffusion was used for the screening of antibacterial activity (Mohsin and Ali 2021). Cell-free supernatant (CFS) of overnight cultures

was collected by centrifugation (10,000×g, 15 min, 4°C) and sequentially filtered through 0.22 µm syringe filters to obtain CFS. CFS was investigated both as-is (unadjusted, acidic, pH<7) and adjusted to a standard of pH 7. The test pathogens were grown overnight in aerobic TSB cultures at 37°C (*Klebsiella pneumoniae*, *Vibrio parahaemolyticus*, *V. vulnificus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella Typhimurium*) and adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU mL⁻¹). Each suspension was swabbed onto Mueller-Hinton agar plates (HiMedia, India), and then 6 mm diameter wells were punched (2 for each suspension), each well containing 50 µL of CFS. The negative control was Sterile TSB. As the experiment was performed in triplicate (independent replicates), inhibition zones were measured in mm after 48 h incubation of plates at 28±1°C.

Enzyme activity

Amylolytic activity was screened on starch agar containing 1% (w/v) soluble starch. Overnight cultures were spot-inoculated (5 µL), incubated at 30 °C for 48 h and flooded with Gram's iodine solution (0.5% w/v iodine in 1% w/v potassium iodide) for 1 min; a clear zone against a dark blue background indicated amylase activity. Lipolytic activity was screened on agar with 1% (v/v) coconut oil or canola oil, following the method of Nespolo and Brandelli (2010); 5 µL spots were incubated overnight at 30°C, reading lipase activity as clear zones around the colonies. Amylase and lipase

screening of fish-gut isolates on agar plates follows the method used for other isolated gut bacteria (Bairagi *et al.*, 2002; Ray *et al.*, 2012). Clearance zones (in mm) were recorded with uninoculated plates as negative controls; all tests were repeated in triplicate (n=3).

Antioxidant activity

Radical-scavenging activity was determined by the DPPH method (Brand-Williams *et al.*, 1995) with minor modifications. Cells from overnight cultures were harvested by centrifugation (8,000×g, 10 min, 4 °C), washed twice with sterile PBS and resuspended in PBS to an OD₆₀₀ of 0.5 (about 1×10^8 CFU mL⁻¹). Equal volumes of cell suspension and DPPH solution (freshly prepared in 95% ethanol) were mixed and kept at room temperature in the dark for 30 min, and absorbance was read at 517 nm. Three controls were run in parallel: a sample blank (cells in 95% ethanol without DPPH), a reagent control (PBS without cells, mixed with DPPH) and a positive control. In the positive control, ascorbic acid (1 mg mL⁻¹) replaced the cell suspension, was mixed with DPPH in the same volume ratio and was treated identically. Scavenging activity (%) = $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$, where A_{control} is the absorbance of the reagent control and A_{sample} is the absorbance of DPPH plus cell suspension (or ascorbic acid) after subtraction of the corresponding blank. Treatments were performed in triplicate (n=3). Ascorbic acid is a pure compound at a defined concentration, whereas the

isolates were tested as intact cells at about 10^8 CFU mL⁻¹, so the positive control shows that the assay responds to a scavenger but does not allow a like-for-like comparison.

Molecular identification by 16S rRNA gene sequencing

We performed isolation of genomic DNA as per the protocol given by Babu *et al.* (2013). The 16S rRNA gene was amplified with primers 27F and 1492R. Purified PCR products were then sequenced using Sanger sequencing provided by Eurofins Genomics. The sequences were deposited in GenBank/INSDC with submission IDs SUB16250843 and SUB16252060. Similarity to type strains was evaluated by BLASTn against the NCBI 16S rRNA sequence database, restricted to type material. Because 16S rRNA gene similarity cannot reliably resolve species-level identity in these groups, the assignments are provisional.

Phylogenetic analysis

The 16S rRNA gene sequences of VR1 and VR2 were aligned with closely related type-strain and reference sequences retrieved from NCBI GenBank in MEGA X. The tree was constructed by the neighbour-joining method, with evolutionary distances calculated using the Kimura 2-parameter model, and branch support was assessed by bootstrap analysis with 1,000 replicates. Maximum-likelihood analysis was not used.

Ethical statement

Fish samples were collected from routine harvests (see Section 2.1) in accordance with Tamil Nadu, India, local fisheries regulations by licensed artisanal fishermen at the site of collection. The gut contents of three individual fish were pooled. Formal Institutional Animal Ethics Committee (IAEC) approval was not required as no experimental procedures from live vertebrate animals were performed in the laboratory. We can give you a written notice of exemption upon request.

Statistical analysis

Each experiment was carried out with three independent biological replicates ($n=3$), that is, separately grown cultures assayed on different days. Where duplicate wells, plates or readings were made within a replicate (for example, two wells per pathogen in the agar well diffusion assay), these technical replicates were averaged and the mean was treated as a single observation. All isolates came from one pooled gut sample, so replication is at the level of culture and not of individual fish. Data are presented as mean $\pm$ SD. Differences were considered significant at $p<0.05$.

Results

Isolation and preliminary characterisation of the isolates

From the pooled gut sample, thirty-five colonies corresponding to the different morphotypes that could be cultured on MRS agar were screened. Two

representative isolates were chosen according to the colony morphology, Gram staining and biochemical profile: VR1 (3.2×10^6 CFU g⁻¹) and VR2 (1.8×10^6 CFU g⁻¹) (Fig. 1; Table 1). Both were Gram-positive rods, indole-negative and MR-positive and both fermented lactose, galactose and cellobiose. VR1 was non-motile, non-spore-forming, catalase-negative,

oxidase-negative and VP-negative, in addition to being a non-mannitol fermenter. VR2 was motile, spore-forming, catalase-positive, oxidase-positive and VP-positive, fermenting mannitol but not raffinose. Neither isolate produced a haemolytic zone on blood agar (γ -haemolysis, non-haemolytic).

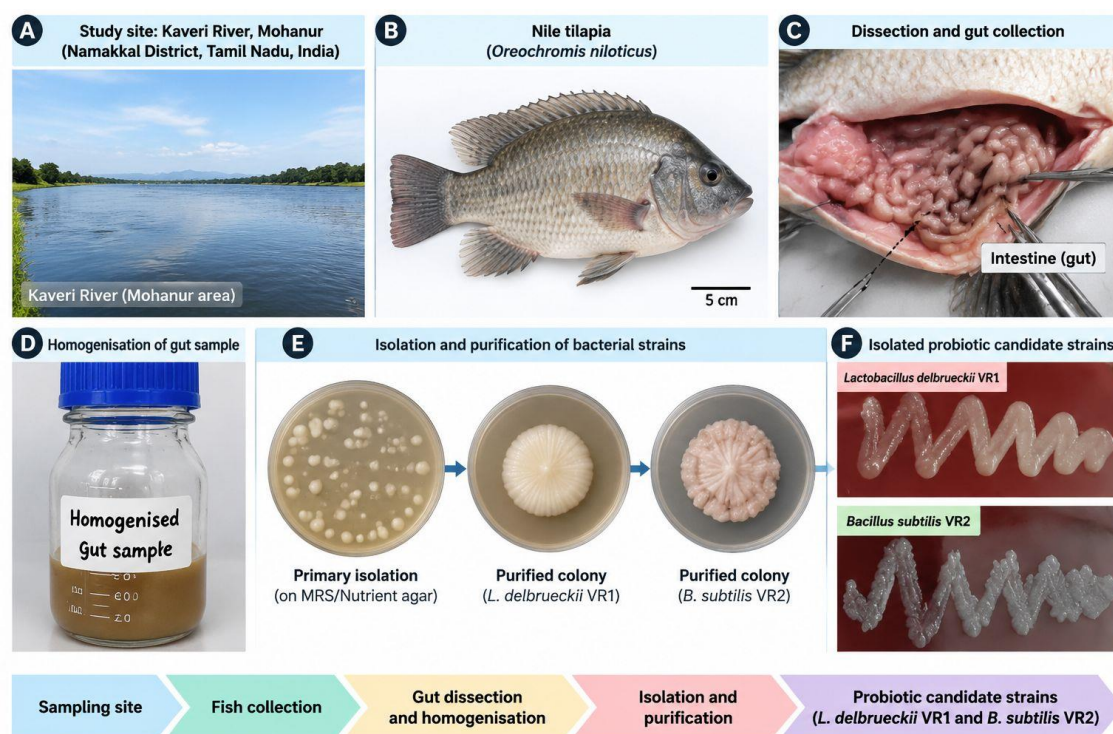


Figure 1: *O. niloticus* collected from the Kaveri River, Mohanur area, Namakkal District, Tamil Nadu, India, and isolation of the two bacterial isolates from the gut.

OD-based responses to acid, bile salt, NaCl and temperature

The acid, bile salt, NaCl and temperature assays were single-endpoint (3 h) measurements made without viable counts; the acid assay was run in PBS, the bile salt assay in peptone water and the NaCl assay in TSB.

Table 1: Biochemical and physiological characteristics of the isolates tentatively identified as *L. delbrueckii* VR1 and *B. subtilis* VR2.

Test	<i>L. delbrueckii</i> VR1	<i>B. subtilis</i> VR2
Gram stain	+ve	+ve
Cell morphology	Rod	Rod
Motility	-ve	+ve
Spore formation	-ve	+ve
Catalase test	-ve	+ve
Oxidase test	-ve	+ve
Indole test	-ve	-ve
Methyl red (MR) test	+ve	+ve
Voges-Proskauer (VP) test	-ve	+ve
Glucose fermentation	+ve (acid)	+ve (acid/gas)
Lactose fermentation	+ve	+ve
Galactose fermentation	+ve	+ve
Mannitol fermentation	-ve	+ve
Cellobiose fermentation	+ve	+ve
Raffinose fermentation	+ve	-ve
Lipase activity (coconut and canola oil agar)	+ve	+ve

+ve, positive reaction; -ve, negative reaction

The values below are therefore turbidity (OD₆₀₀) readings, which may reflect changes in cell density but do not show growth, survival or tolerance as such. Uninoculated medium gave OD₆₀₀ values of 0.30 (pH 1.5), 0.20 (pH 2.5), 0.40 (pH 3.5) and 0.70 (pH 4.5) (Fig. 2A), so the blank itself varied with pH. VR1 gave 0.25, 0.50, 0.30 and 0.10 at pH 1.5, 2.5, 3.5 and 4.5, respectively, with the highest reading at pH 2.5 and the lowest at pH 4.5. VR2 gave 0.60, 0.65, 0.33 and 0.60, with the highest reading at pH 2.5 and the lowest at pH 3.5. At pH 1.5 the VR2 reading (0.60) was twice that of the uninoculated medium (0.30), but this cannot be interpreted as growth in an acidic, non-growth buffer and needs confirmation by viable counts. In the bile salt assay, OD₆₀₀ values for VR1 at 0.3%, 0.6% and 1.0% bile salt were 0.80, 0.20 and 0.40, respectively, and those for VR2 were 0.55, 0.78 and 0.30 (Fig. 2B). VR1 was highest at 0.3% and lowest at 0.6%, whereas VR2 was highest at 0.6% and

lowest at 1.0%. The isolates therefore responded differently across concentrations, and neither showed a consistent concentration-dependent pattern. No bile-salt-free control was included, so these readings cannot be used to estimate a loss of tolerance relative to unexposed cultures. In the NaCl assay (Fig. 2C), uninoculated medium gave OD₆₀₀ values of 0.40, 0.40, 0.70 and 0.20 at 0, 0.3, 0.6 and 1.0% NaCl, respectively. VR1 gave 0.70, 0.52, 0.39 and 0.35, and VR2 gave 0.85, 0.72, 0.35 and 0.10, at 0, 0.3, 0.6 and 1.0% NaCl, respectively. The readings of both isolates decreased as NaCl increased, with the highest reading without added NaCl and the lowest at 1.0% NaCl. This pattern is consistent with a lower turbidity response at higher salt concentrations, but viability was not assessed. The OD₆₀₀ measured after 3 h at 4, 15, 25 and 37°C is shown in Figure 2D. For VR1, it was 0.10, 0.40, 0.33 and 0.58, with the highest value at 37 °C. For VR2, it was 0.75, 0.15, 0.20 and 0.39,

with the highest value at 4°C and a much lower value at 15°C. With only four temperatures and a single 3-h endpoint, these values give the highest response among the temperatures tested, not a growth optimum. The high reading of

VR2 at 4°C is unexpected in view of its low values at 15 and 25°C and needs to be repeated and confirmed by viable counts.

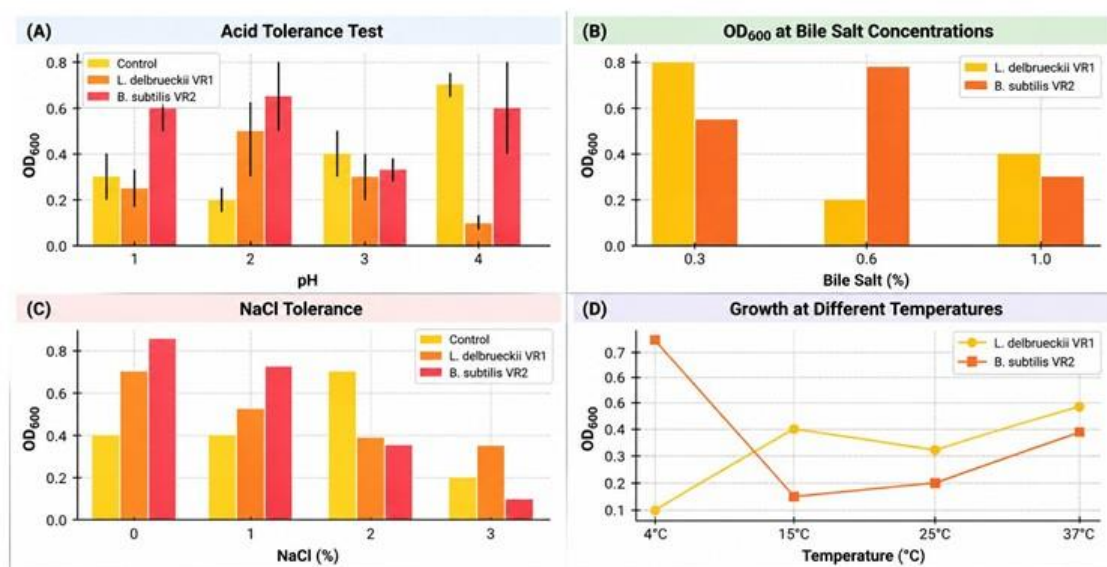


Figure 2: OD₆₀₀ responses of *L. delbrueckii* VR1 and *B. subtilis* VR2 after 3 h under (A) acid (pH 1.5, 2.5, 3.5 and 4.5), (B) bile salt (0.3, 0.6 and 1.0%, w/v), (C) NaCl (0, 0.3, 0.6 and 1.0%, w/v) and (D) temperature (4, 15, 25 and 37 °C). Control indicates uninoculated medium (A, C); no unexposed control was included in (B). Values are means $\pm$ SD of three biological replicates.

Cell surface properties

The OD₆₀₀ of VR1 fell from 1.18 at 0 h to 1.10, 1.08 and 1.04 after 1, 2 and 3 h, which corresponds to auto-aggregation of 6.8%, 8.5% and 11.9%, respectively (Fig. 3A). For VR2 the OD₆₀₀ fell from 1.12 to 1.06, 1.03 and 0.98, corresponding to 5.4%, 8.0% and 12.5%. The percentages were recalculated from these OD readings with the equation in Section 2.5.1. Auto-aggregation of both isolates was therefore modest and should not be described as high. Co-aggregation was measured separately for each pair (Fig. 3B). In mixed culture, *L. delbrueckii* VR1 gave 58.3% with *K.*

pneumoniae (VR1 alone 12.4%; pathogen alone 8.6%) and 49.7% with *V. parahaemolyticus* (9.7%; 6.8%). *B. subtilis* VR2 gave 62.5% with *E. coli* (VR2 alone 14.2%; pathogen alone 9.1%) and 55.6% with *S. aureus* (10.8%; 7.3%). In every pair, the mixed-culture value was about 4.4–5.2 times the higher of the two single-culture control values, which were not zero (6.8–14.2%). This shows cell association between each isolate and each pathogen under the assay conditions. It does not show competitive exclusion or colonisation. Solvent affinity (Fig. 3C) for *L. delbrueckii* VR1 was 68.7 $\pm$ 4.0% with

ethanol, $54.2 \pm 4.0\%$ with methanol and $76.5 \pm 3.5\%$ with ethyl acetate, and for *B. subtilis* VR2 it was $61.3 \pm 4.0\%$ with ethanol, 47.8% with methanol and 69.7% with ethyl acetate. For both isolates, ethyl acetate gave the highest and methanol the lowest values, and VR1 values were higher than the

corresponding VR2 values for all three solvents. These are solvent-affinity values from an adapted MATH/MATS assay and not hydrophobicity values from a classical hydrocarbon assay, because polar solvents were used.

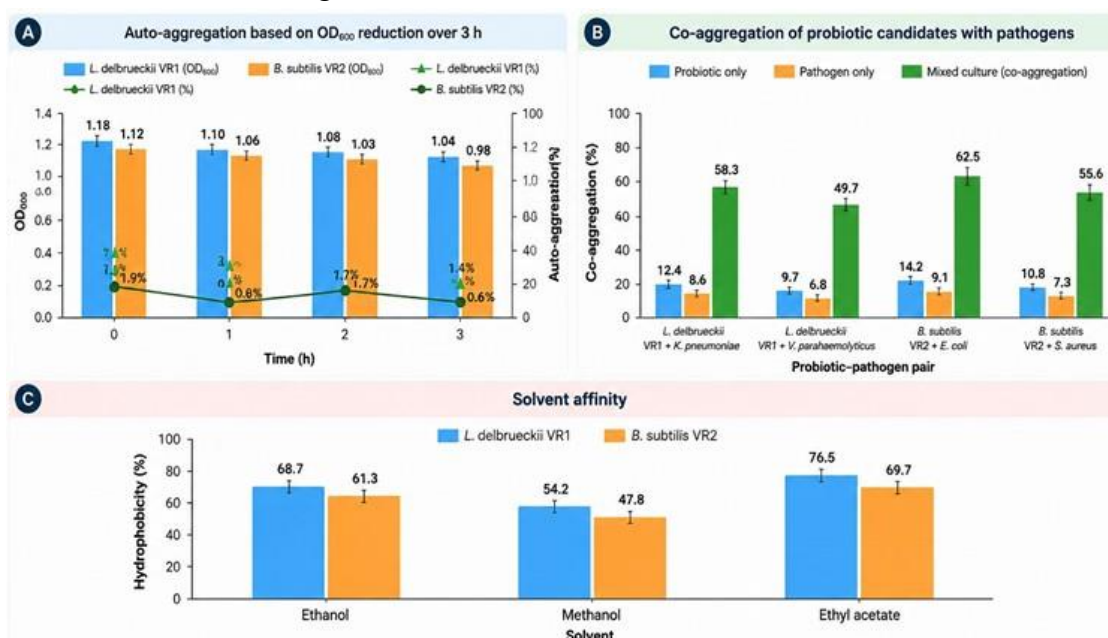


Figure 3: (A) Auto-aggregation of *L. delbrueckii* VR1 and *B. subtilis* VR2 over 3 h; OD₆₀₀ (bars) and percentages recalculated from the OD₆₀₀ values as $(A_0 - A_t)/A_0 \times 100$ (lines). (B) Co-aggregation of each probiotic-candidate-pathogen pair (VR1 with *K. pneumoniae* and *V. parahaemolyticus*; VR2 with *E. coli* and *S. aureus*) in probiotic-only, pathogen-only and mixed cultures. (C) Solvent affinity (adapted MATH/MATS assay) with ethanol, methanol and ethyl acetate. Data are means $\pm$ SD of three biological replicates.

Antibacterial, enzymatic and antioxidant activities

All six pathogens were inhibited by the cell-free supernatants of both isolates (Fig. 4 A, B). With unadjusted (acidic) supernatants, inhibition zones ranged from 9.8 mm (*V. parahaemolyticus*) to 12.7 mm (*S. Typhimurium*) for VR1 and from 10.6 mm (*V. parahaemolyticus*) to 14.1 mm (*S. Typhimurium*) for VR2. There were significant differences among groups (one-way ANOVA, $F=24.14$, $P=2.2 \times 10^{-7}$). After adjustment

to pH 7, zones were 1.7–4.0 mm smaller and ranged from 7.8 mm (*V. parahaemolyticus*) to 9.7 mm (*V. vulnificus*) for VR1 and from 8.9 mm (*V. parahaemolyticus*) to 11.2 mm (*V. vulnificus*) for VR2 (Fig. 4B); for *K. pneumoniae* the neutral-pH zones were 8.4 mm (VR1) and 9.6 mm (VR2). VR2 gave larger zones than VR1 against all six pathogens at both pH values. Extracellular enzyme activity was recorded as clearance zones (Fig. 4C). Lipolytic zones on coconut-oil agar were

12.6 mm (VR1) and 14.3 mm (VR2), and on canola-oil agar 10.8 mm and 12.1 mm. Amylolytic zones on starch agar were 8.7 mm and 10.5 mm. These are plate-based screening results only. In the DPPH assay (Fig. 4D), the absorbance at 517 nm was 0.78 for the reagent control (DPPH with PBS), 0.42 after treatment with VR1, 0.38 after treatment with VR2 and 0.25 for ascorbic acid (1 mg mL⁻¹,

positive control). These means correspond to scavenging activities of about 46.2% (VR1), 51.3% (VR2) and 67.9% (ascorbic acid). Both isolates therefore reduced DPPH absorbance, but by less than the positive control, and the difference between the two isolates was small.

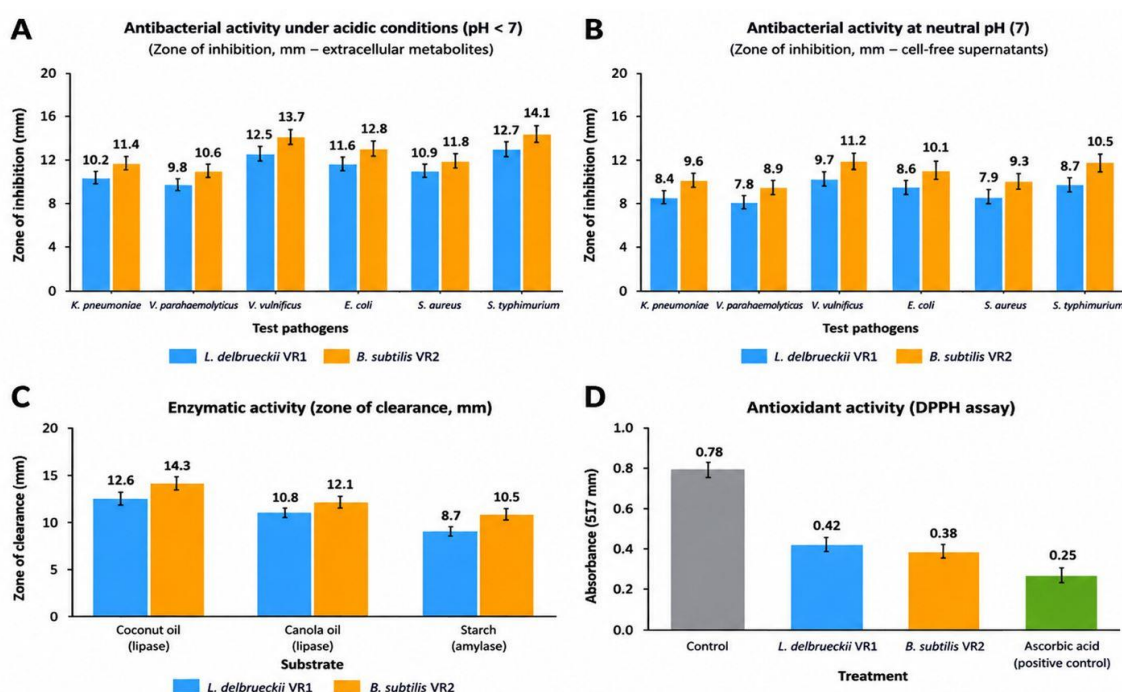
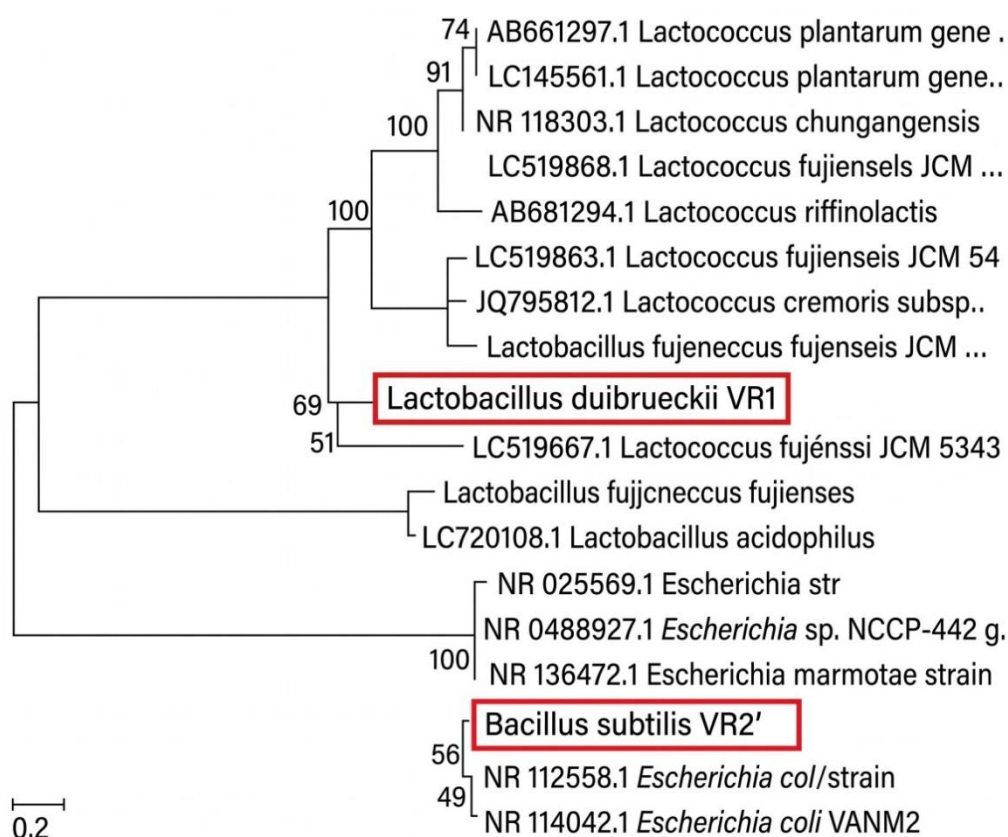


Figure 4: (A) Antibacterial activity of cell-free supernatants under acidic conditions (pH < 7) and (B) at pH 7, shown as inhibition zones (mm). (C) Lipase (coconut and canola oil) and amylase (starch) activity shown as clearance zones (mm). (D) DPPH absorbance at 517 nm for the reagent control, the two isolates and ascorbic acid (positive control). Values are means $\pm$ SD of three replicates.

Molecular identification and phylogenetic analysis

When primers 27F and 1492R were used, both isolates yielded near full-length (approximately 1,420 bp) 16S rRNA gene sequences. VR1 showed high similarity to *Lactobacillus delbrueckii* ATCC 9649^T, and VR2 showed $\geq 99.3\%$ identity to *Bacillus subtilis* reference sequences, so the

isolates were tentatively identified as *L. delbrueckii* (VR1) and *B. subtilis* (VR2). A neighbour-joining tree (Kimura 2-parameter model, 1,000 bootstrap replicates) including VR1, VR2 and related reference sequences is shown in Figure 5. Species-level assignment from 16S rRNA gene sequences alone should still be treated with caution, whatever the final topology.



Phylogenetic analysis based on 16S rRNA gene sequences showing the relationship of fish strains and natural isolates *Lactobacillus delbrueckii* VR1 and *Bacillus subtilis* VR2 with reference strains from CBI GenBank. The tree was constructed using the neighbour joining method with bootstrap values at the nodes. The isolates grouped closely with their respective species.

Figure 5: Neighbour-joining tree based on 16S rRNA gene sequences of the isolates tentatively identified as *L. delbrueckii* VR1 and *B. subtilis* VR2 and of reference strains from NCBI GenBank (Kimura 2-parameter model; bootstrap values from 1,000 replicates at nodes; scale bar, substitutions per site). Superscript T denotes type strains.

Discussion

The two isolates recovered from Nile tilapia in the Kaveri River showed a range of in vitro traits that are typically considered when prioritising microbial candidates for aquaculture, but the strength of evidence varied by assay. The acid, bile salt, NaCl and temperature assays were single-endpoint turbidity readings without viable counts, so they show OD responses and not tolerance; auto-aggregation was low, co-aggregation and inhibition zones were clearer, and the enzyme and antioxidant

assays were plate-based or single-endpoint screens. These results should therefore be viewed as preliminary in vitro screening data on potential probiotic candidates, which require in vivo testing; they do not demonstrate probiotic effects or safety.

The two isolates were recovered on a single medium, MRS agar, from a pooled sample of three fish. They should be regarded as selected culturable isolates and not as prominent members of the tilapia gut community, which would have to be characterised by

culture-independent sequencing. That VR2, a spore-forming rod, grew on MRS agar is not surprising, since MRS is not selective for *Bacillus*, but it also shows that the isolation strategy did not target this genus. Adding a *Bacillus*-appropriate medium such as nutrient agar would give a more complete picture of the culturable community. The species names also remain provisional: the high 16S rRNA gene sequence similarity within the *B. subtilis* group and among subspecies of *L. delbrueckii* limits resolution, and genome-based comparison is needed to confirm species-level affiliation, together with deposition of the isolates in a recognised culture collection.

The absence of haemolysis is generally treated as a minimum safety criterion when screening probiotic candidates (Li *et al.*, 2020), and neither isolate produced a haemolytic zone; this does not, however, establish safety. Transferable resistance or virulence-associated genes could be carried by strains of both groups; therefore, susceptibility testing versus an appropriate CLSI or EUCAST standard is a prerequisite, ahead of any development of these isolates as feed additives.

Tolerance to acid, bile salts and salinity is an established selection criterion for potential probiotic candidates (Ayyash *et al.*, 2021; Kusada *et al.*, 2021), but the present assays did not measure tolerance directly. OD readings in PBS, peptone water or TSB can reflect cell density, cell lysis, aggregation or precipitation of medium

components, and the uninoculated controls themselves varied from 0.20 to 0.70 with pH (Fig. 2A). Consequently, readings of the isolates above the control (VR2 pH 1.5) need to be verified through viable counts (CFU mL⁻¹) at both start and end of exposure. A bile-free control was omitted from the bile salt assay and no concentration-dependent trend was observed; the NaCl range tested (0–1.0%) was also low, and the temperature series was short. The highest VR1 value (37°C) was above the 24–32°C water temperatures at the sampling site, and the highest VR2 value (4°C) is not consistent with its lower values at 15 and 25°C. Neither result indicates a growth optimum or relevance to the gut of a poikilothermic host.

Auto-aggregation, co-aggregation and cell surface affinity are cell-association traits that are often used as indirect indicators of adhesion to the intestinal surface (Govindaraj *et al.*, 2021; Jin *et al.*, 2021; Taha *et al.*, 2023). Auto-aggregation of both isolates following 3 h was only 11.9% (VR1) and 12.5% (VR2) and lower at earlier times, so the data do not indicate a strong propensity to self-aggregate, and values from other studies cannot be directly compared, as incubation times, OD standardisation and calculations differ. Co-aggregation of each isolate with its paired pathogens (VR1 with *K. pneumoniae* and *V. parahaemolyticus*; VR2 with *E. coli* and *S. aureus*) in mixed culture (49.7–62.5%) was about 4.4–5.2 times the higher of the corresponding single-culture control values (6.8–14.2%), which is directionally consistent

with reports of co-aggregation between probiotic candidates and pathogens (Juntarachot *et al.*, 2023; Kardooni *et al.*, 2023). This shows cell association under the assay conditions and not competitive exclusion or colonisation. Special attention should be paid to the solvent-affinity values (47.8–76.5%). Ethanol and methanol are miscible with water, and ethyl acetate is only partially soluble in it; therefore, the mixtures did not tend to produce the two distinct phases seen in a hydrocarbon assay, so that a decrease in OD can be due to dilution, cell lysis or precipitation, as well as partitioning. Thus, these values cannot be compared with hydrophobicity values obtained using hydrocarbons (Mathipa-Mdakane and Thantsha 2022; Xing *et al.*, 2023), while later studies with a hydrocarbon such as hexadecane or xylene are needed on classical MATH. All six pathogens were inhibited by the cell-free supernatants of both isolates (Fig. 4A and 4B), and for each pathogen at both pH values, zones induced by VR2 were larger than from VR1. Neutralising the supernatant reduced the zones by 1.7–4.0 mm, which suggests that acidity contributed to the inhibition. Zones at pH 7 remained sizeable (7.8–11.2 mm), however, so acidity alone does not appear to account for the activity. The inhibitory compounds were not identified: organic acids were not measured, and hydrogen peroxide and other neutral-stable compounds were not excluded. No bacteriocin production test was performed, and no purification or fractionation of the supernatants was done; thus, the activity cannot be

attributed to bacteriocins or any specific compound. Antimicrobial compounds have been reported from fish-gut *Bacillus* (Seerangaraja *et al.*, 2017; Vijayaram *et al.*, 2016, 2017), but whether VR1 or VR2 produces any such compound was not shown here. The pathogens tested included *Vibrio* species associated with fish and others associated mainly with other hosts; activity against tilapia pathogens such as *Streptococcus iniae* (Büyükdeveci *et al.*, 2023) was not tested.

On lipid and starch media, both isolates produced clear zones, which indicates extracellular lipase and amylase activity under plate conditions. Enzyme-producing bacteria have been isolated from the digestive tract of fish (Bairagi *et al.*, 2002; Ray *et al.*, 2012), but plate zones are semi-quantitative screening results, and this study did not test whether VR1 or VR2 produce these enzymes in the fish gut or whether they would affect digestion or feed utilisation. In the DPPH assay the isolates reduced absorbance, and scavenging activity (about 46% and 51%) was lower than that of ascorbic acid (67.9%). This comparison is not like for like, because whole-cell suspensions at about 10^8 CFU mL⁻¹ were compared with a pure compound at 1 mg mL⁻¹. Peptides rich in hydrophobic amino acids have been linked to the antioxidant activity of probiotic strains (Varsha *et al.*, 2021; Sakoui *et al.*, 2022), but no such compounds were investigated here, and the DPPH result does not show protection of the fish against oxidative stress.

The principal shortcomings of this work are that the data were generated from pooled samples across three fish, isolated from a single medium, expressed as OD readings in non-growth media without corresponding viable counts, the absence of an appropriate bile-free control, an adapted solvent-affinity assay and identification using only 16S rRNA gene sequences and no in vivo work. Future working priorities include genome sequencing for taxonomic confirmation and antimicrobial-resistance and virulence gene screening, viable counts under acid, bile, and salt exposure, classical MATH with hydrocarbons, inhibitory compound characterisation utilised along with a *Bacillus*-appropriate isolation medium alongside MRS agar and controlled feeding & challenge trials in Nile tilapia.

Conclusion

In this study, two culturable bacterial isolates recovered from the gut of *Oreochromis niloticus* were characterised in vitro as preliminary data. Both were Gram-positive rods, designated VR1 and VR2, and were tentatively identified as *Lactobacillus delbrueckii* VR1 and *Bacillus subtilis* VR2. In the assays used for initial screening, they showed OD responses under acid, bile salt, NaCl and temperature challenge, low auto-aggregation (up to 12.5%), higher co-aggregation with the pathogens tested, solvent-affinity values from an adapted assay, inhibition of pathogens by cell-free supernatants, lipase and amylase

activity on plates, and DPPH radical-scavenging activity. The OD-based readings were single-endpoint turbidity measurements without viable counts and are not evidence of growth, survival or tolerance.

Overall, the data describe some in vitro characteristics of interest in *L. delbrueckii* VR1 and *B. subtilis* VR2 but do not demonstrate probiotic efficacy or safety in Nile tilapia. This is a preliminary screening study. Further work should include genome-based taxonomic confirmation, deposition of the isolates in a recognised culture collection, antimicrobial-resistance and virulence screening, viable-count-based tolerance assays, and controlled in vivo feeding and challenge trials in *O. niloticus* in which growth performance, feed utilisation, gut persistence, immune responses and resistance to pathogen challenge can be measured.

Accordingly, *L. delbrueckii* VR1 and *B. subtilis* VR2 should be regarded only as potential probiotic candidates under evaluation. Until their taxonomic identity, antimicrobial-resistance profile, virulence potential, genetic safety and efficacy in vivo have been established, no recommendation for use in aquaculture can be made.

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Competing interests

The authors declare no competing interests.

Authors contribution

SV: conceptualisation, data curation, funding acquisition, investigation, project administration, resources, supervision, formal analysis and writing of the original draft. RT, MG and RK: writing, review and editing. All authors read and approved the final manuscript.

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Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The 16S rRNA gene sequences have been submitted to GenBank/INSDC (submission IDs SUB16250843 and SUB16252060).

Ethics approval

Fish were obtained in compliance with local fisheries regulations of Tamil Nadu, India, from routine artisanal fishery harvests at the Kaveri River collection site (Section 2.1), not from retail markets. No experimental procedures were performed on live vertebrate animals in the laboratory; formal Institutional Animal Ethics Committee (IAEC) approval was therefore not required under the applicable guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA) for

isolation of gut bacteria from such specimens. We can provide a written exemption statement on request.

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