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Research Article

Isolation, Screening, Characterization, and Identification of Potential Probiotic Lactic Acid Bacteria (LAB) from the Gut of Whiteleg Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Bacterial diseases represent a major constraint on the sustainability of shrimp aquaculture. Increasing antibiotic resistance necessitates eco-friendly alternatives. This study isolate and characterizes bacteriocin-producing Lactic Acid Bacteria (LAB) from the gut of healthy farmed *Litopenaeus vannamei* to evaluate their probiotic potential. Thirty presumptive LAB isolates were obtained from 50 shrimp samples. In vitro assessment included tolerance to simulated gastrointestinal conditions (pH 1.5 and 3% bile salts), safety (hemolysis and antibiotic susceptibility), and antimicrobial activity against four pathogens: *Vibrio parahaemolyticus, V. harveyi, V. alginolyticus,* and *V. vulnificus*. Isolate LV4 maintained viable counts of 6.72±0.13 Log CFU/mL at pH 1.5 and 5.17±0.48 Log CFU/mL at 3% bile salts. The neutralized cell-free supernatant of LV4 exhibited proteinaceous, broad-spectrum antimicrobial activity against all tested *Vibrio* pathogens. Isolate LV4 was non-hemolytic and sensitive to most clinically relevant antibiotics. Molecular identification using 16S rRNA gene sequencing identified LV4 as *Enterococcus faecalis*. This study confirms that the gut of *L. vannamei* is a valuable reservoir for robust LAB. Isolate *E. faecalis* LV4 is a strong candidate for further development as a probiotic to enhance shrimp health and disease resistance in aquaculture.

1. Introduction

Aquaculture, particularly the farming of whiteleg shrimp (*Litopenaeus vannamei*), is a critical component of global food production. However, the intensification of aquaculture practices has increased the susceptibility of farmed species to bacterial diseases, leading to significant economic losses. The industry has historically relied on antibiotics to control these pathogens. This widespread use has unfortunately contributed to the rise of antimicrobial resistance, a global threat to both animal and human health, prompting legislation and a search for safer, more sustainable alternatives (Vieco-Saiz *et al.* 2019; Pereira *et al.* 2022).

In response to these challenges, probiotics, particularly Lactic Acid Bacteria (LAB), have emerged as a promising eco-friendly strategy for disease management in aquaculture. LAB are considered safe microorganisms that can confer health benefits to the host by improving the intestinal microbial balance, enhancing the immune response, and increasing disease resistance (Ringø *et al.* 2018, Ringø *et al.* 2020a, 2020b). One of their primary modes of action is the production of various inhibitory compounds, including organic acids, hydrogen peroxide, and antimicrobial peptides, which can suppress the growth of pathogenic bacteria through direct antagonism and competitive exclusion (Vieco-Saiz *et al.* 2019).

Among the antimicrobial compounds produced by LAB, bacteriocins have garnered significant scientific interest. Bacteriocins are ribosomally synthesized

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proteinaceous peptides with potent antimicrobial activity, often against bacteria closely related to the producer strain as well as major food-borne and aquaculture pathogens (Alvarez-Sieiro *et al.* 2016). Unlike many conventional antibiotics, bacteriocins are biodegradable, effective at low concentrations, and often stable across wide ranges of pH and temperature, making them ideal candidates for use as biopreservatives and therapeutic agents (Wang *et al.* 2019). The production of these peptides provides a competitive advantage to the host bacteria and represents a key mechanism for their probiotic function (Poonam *et al.* 2025).

The gastrointestinal tracts of healthy aquatic animals are a rich, largely untapped reservoir of novel probiotic LAB with unique adaptive traits. While research has identified bacteriocin-producing LAB from various marine sources (Alonso *et al.* 2019), studies focusing specifically on commercially important species such as *L. vannamei* are crucial for developing host-specific solutions (Cai *et al.* 2022). Isolating beneficial bacteria from the gut of the target species ensures that the potential probiotics are well-adapted to the host's intestinal environment (Wanka *et al.* 2018). Several studies have successfully isolated bacteriocin-producing bacteria from shrimp, demonstrating potent activity against key aquaculture pathogens such as *Vibrio* spp. and *Aeromonas hydrophila* (Feliatra *et al.* 2018; Amin *et al.* 2020), underscoring the potential of the shrimp gut microbiota as a source of new biocontrol agents.

The genus *Enterococcus*, a common inhabitant of the gastrointestinal tract, produces a diverse array of bacteriocins, collectively known as enterocins. While some enterococci are associated with opportunistic infections, many strains, particularly those isolated from food and animal sources, have "Generally Recognized As Safe" (GRAS) status and are extensively studied for their probiotic and biopreservative potential (Hwanhlem *et al.* 2013). Although bacteriocins from *Enterococcus faecium* have been characterized in aquaculture contexts (Kumar *et al.* 2011), there is comparatively less research on bacteriocin-producing *Enterococcus faecalis* isolated from aquatic invertebrates. While *E. faecalis* has been isolated from sources ranging from freshwater fish to fermented foods (Shastry *et al.* 2020), its characterization from the gut of *L. vannamei* remains a novel area of investigation.

Therefore, this study was undertaken to isolate, characterize, and identify bacteriocin-producing LAB from the gut of healthy farmed Pacific white shrimp (*Litopenaeus vannamei*). The objective of the study was to isolate, screen, characterize, and identify the antimicrobial activity of its bacteriocin and to evaluate the isolate's potential as a probiotic for aquaculture application, offering a targeted approach to enhance shrimp health and promote sustainable production.

2. Materials and Methods

2.1. Sample Collection and Isolation of Lactic Acid Bacteria

A total of 50 healthy whiteleg shrimp (15-25 g) were collected from five commercial earthen-bottom aquaculture ponds in Vinh Long province, Vietnam. Ten shrimp were randomly sampled from each pond to ensure environmental representation. Samples were transported alive in aerated containers and processed within two hours.

The gastrointestinal tract was dissected under sterile conditions. To account for inter-individual and environmental variation, gut samples were separated and processed according to their respective sampling sites. One gram from each composite sample was homogenized in 9 mL of tryptone salt broth. The homogenate was serially diluted, and 100 µL aliquots of the 10^{-6} and 10^{-7} dilutions were spread-plated onto De Man, Rogosa, and Sharpe (MRS) agar supplemented with 0.1% (w/v) $CaCO_3$. The plates were incubated aerobically at 30°C for 48 hours. Colonies exhibiting a distinct, clear halo, indicating acid production, were selected as presumptive LAB isolates (Ben Braïek *et al.* 2017). These isolates were purified by repeated streaking on fresh MRS agar plates and were preserved as stock cultures at -80°C in MRS broth containing 25% (v/v) glycerol for further analysis (Al Atya *et al.* 2015; Kang *et al.* 2020).

2.2. Phenotypic and Biochemical Characterization

Initially, 40 presumptive LAB isolates were characterized by Gram-staining, cellular morphology using an Olympus CX21 light microscope (Japan) at 1000x magnification, and motility testing. To confirm their identity as LAB, the isolates were then subjected to a series of key biochemical tests. These included tests for catalase, urease, citrate utilization, and the fermentation of glucose, lactose, and sucrose (Rahmawati *et al.* 2021). To ensure methodological validity, certified reference strains were used as controls for each biochemical assay. For the catalase test, *Staphylococcus aureus* ATCC 25923 (positive) and *Lactiplantibacillus plantarum* ATCC 14917 (negative) were used. Similarly, urease activity was validated using *Proteus mirabilis* ATCC 12453 (+) and *Escherichia coli* ATCC 25922 (-), while citrate utilization was evaluated with *Enterobacter*

aerogenes ATCC 13048 (+) and *E. coli* ATCC 25922 (-) (Kang *et al.* 2020).

Only the isolates that were Gram-positive, non-motile, and tested negative for catalase, urease, and citrate were selected as candidate probiotic strains. A total of 30 isolates fulfilled these criteria and were used for subsequent functional screening.

2.3. Screening for Antimicrobial Activity

The 30 selected LAB isolates were screened for the production of antimicrobial compounds against four significant aquaculture pathogens: *Vibrio parahaemolyticus, Vibrio harveyi, Vibrio alginolyticus*, and *Vibrio vulnificus*. These indicator strains were obtained from the culture collection of the School of Agriculture and Aquaculture, Tra Vinh University, Vietnam.

Antimicrobial activity was evaluated using the agar well diffusion method (Ogunbanwo *et al.* 2020). To prepare the cell-free supernatant (CFS), each LAB isolate was cultured in MRS broth for 18 hours, then centrifuged at 10,000 x g for 15 minutes at 4°C. To exclude inhibition from organic acids, the pH of the CFS was neutralized to 6.5 with 1 M NaOH. This resulting solution was designated the neutralized CFS (nCFS).

Indicator pathogen cultures were grown to an approximate density of 1.5×10^8 CFU/mL (0.5 McFarland standard), and 1 mL was used to seed 15 mL of semi-solid Mueller-Hinton Agar (MHA, 0.75% agar). After the seeded agar was poured into Petri dishes, wells (4 mm diameter) were aseptically punched and filled with 100 µL of the nCFS. As a negative control, wells were filled with sterile, neutralized MRS broth (Lin and Pan 2019). The plates were incubated at 30°C for 24 hours, after which the diameter of the inhibition zone was measured in millimeters (mm). Activity was classified as strong (>25 mm), moderate (13–25 mm), or weak (1–12 mm) (Rabaoui *et al.* 2023). All assays were performed in triplicate.

2.4. Safety Assessment: Hemolytic Activity and Antibiotic Susceptibility

2.4.1. Hemolytic activity

The hemolytic activity of the LAB isolates was evaluated as a preliminary safety indicator. Each isolate was streaked onto Columbia blood agar plates supplemented with 5% (v/v) sterile sheep blood. The plates were incubated at 30°C for 36 hours and then examined for zones of hemolysis around the colonies (Mohamad *et al.* 2020). *Staphylococcus aureus* ATCC 25923 was used as a positive control (β-hemolysis), and *Lactiplantibacillus plantarum* ATCC 14917 served as the negative (γ-hemolysis) control. The presence of a clear zone indicated β-hemolysis (lysis), a greenish zone indicated α-hemolysis (partial lysis), and no change indicated γ-hemolysis (non-hemolytic). This assay was performed in triplicate for each isolate.

2.4.2. Antibiotic Susceptibility Profile

The antibiotic susceptibility of the 30 isolates was determined using the Kirby-Bauer disk diffusion method. Each isolate was grown to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) and spread evenly onto the surface of MRS agar plates. Commercial antibiotic disks (Oxoid, UK) were placed onto the agar surface, consisting of: Doxycycline (30 µg), Ampicillin (10 µg), Tetracycline (30 µg), Kanamycin (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), and Chloramphenicol (30 µg).

Following incubation at 37°C for 24 hours, the diameters of the inhibition zones were measured. The isolates were classified as sensitive (≥20 mm), intermediate (15–19 mm), or resistant (≤14 mm) according to established interpretive criteria (Kathleen *et al.* 2016; Alebiosu *et al.* 2017). Quality control for the assay was performed using *Escherichia coli* ATCC 25922. Each test was conducted in triplicate.

2.5. Tolerance to Simulated Gastrointestinal Conditions

The ability of the 30 isolates to withstand gastrointestinal tract conditions was assessed by testing their tolerance to acidic pH and bile salts, following the protocol of Serrano-Nino *et al.* (2016).

For each assay, overnight cultures were harvested by centrifugation (8,000 rpm, 5 min, 4°C), washed twice with sterile distilled water, and resuspended in MRS broth to a standardized cell density of approximately 10^8 CFU/mL. For acid tolerance, 1 mL of the cell suspension was inoculated into 9 mL of MRS broth adjusted to pH 1.5 or pH 3.0 using 1 M HCl. For bile salt tolerance, 1 mL was inoculated into 9 mL of MRS broth supplemented with 1%, 2%, or 3% bile salts (w/v). A culture in standard MRS broth (pH 7.2 and 0% bile salts) served as the reference control for each respective experiment. After incubation at 37°C for 4 hours, viable cell counts (CFU/mL) were determined by plating serial dilutions on MRS agar. All experiments were performed in triplicate.

2.6. Molecular Identification and Phylogenetic Analysis

The isolate demonstrating the most promising probiotic characteristics (strong antimicrobial activity, favorable safety profile, and high GI tolerance) was selected for molecular identification via 16S rRNA gene sequencing. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA). The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Liu *et al.* 2020). The PCR amplification was performed in a Veriti thermocycler using GoTaq® Green Master Mix (Promega, USA) under the following conditions: an initial denaturation at 94°C for 3 min; followed by 30 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. A non-template control (NTC) using sterile water in place of DNA was included in each PCR run to check for contamination. The amplified product (~1,500 bp) was visualized on a 1% agarose gel, purified using a QIAquick PCR Purification Kit (Qiagen, Germany), and sent for sequencing at Next Gen Scientific Co., Ltd (Ho Chi Minh City, Vietnam).

2.7. Data and Sequence Analysis

All experimental assays were conducted in triplicate, and the results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 26.0). A one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test was used to determine significant differences between means, with p-values < 0.05 considered statistically significant.

The obtained 16S rRNA gene sequence was edited using BioEdit (v. 7.0) and identified by BLASTn against the NCBI GenBank database. A sequence identity of ≥99% was considered a match. A phylogenetic tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates in MEGA6 software to illustrate the evolutionary relationship of the isolate with closely related reference species (Ding *et al.* 2017).

3. Results

3.1. Isolation and Phenotypic Characterization of Potential Probiotics

From the 50 shrimp samples collected, a total of 40 presumptive Lactic Acid Bacteria (LAB) colonies were isolated from the gut of *L. vannamei* based on their distinct acid-producing halos on MRS agar. Following purification, these isolates were subjected to initial screening. All 40 isolates were confirmed to be Gram-positive, non-motile, and catalase-negative, which is consistent with the general characteristics of LAB. Based on their robust growth and a uniform biochemical profile (Table 1), 30 of these isolates were selected for comprehensive evaluation of their probiotic properties. The representative morphology of the isolates is illustrated in Figure 1, using the subsequent candidate strain LV4 as an example.

3.2. High Tolerance of Isolations to Simulated Gastrointestinal Conditions

A critical prerequisite for a probiotic is the ability to survive transit through the gastrointestinal tract. Therefore, the 30 selected isolates were assessed for their tolerance to low pH and high bile salt concentrations.

- Acid tolerance: The evaluated isolates exhibited variable resilience to acidic stress (Table 2). At pH 3.0, all 30 isolates maintained high viability, with no statistically significant reduction in viable counts compared to the control ($p<0.05$). This indicates that moderate acidity does not significantly impact the survival of these gut-derived strains. However, exposure to pH 1.5 simulating the extreme conditions of the gastric environment revealed significant strains-specific differences ($p<0.05$). Notably, isolates LV11, LV4, and LV18 demonstrated superior acid tolerance, maintaining viable counts of 6.93±0.13, 6.72±0.13, and 6.69±0.13 Log CFU/mL, respectively. Conversely, isolates LV7, LV9, and LV24 exhibited poor survival under these highly acidic conditions, leading to their exclusion from further probiotic characterization.
- Bile salt tolerance: The ability to withstand the antimicrobial action of bile salts in the small intestine is another essential probiotic trait. As shown in Table 3, the isolates displayed varied tolerance to bile salt concentrations of 1%, 2%, and 3%. While all isolates grew well in the absence of bile (0% control), their survival at the physiologically relevant concentration of 3% was a key differentiator. At this concentration, isolate LV18 demonstrated the most robust tolerance with a viable count of 5.41±0.68 Log CFU/mL, followed by LV21 (5.26±0.66 Log CFU/mL) and LV4 (5.17±0.48 Log CFU/mL). Conversely, isolates LV3, LV9, and LV23 exhibited poor survival, highlighting significant strain-specific differences in bile resistance.

3.3. Broad-spectrum Antimicrobial Activity against Enteric Pathogens

A key probiotic function is the inhibition of pathogenic bacteria. The antimicrobial capacity of the neutralized cell-free supernatant (nCFS) from each of the 30 isolates was tested against four major shrimp pathogens. The results of this screening are summarized in Table 4. A significant portion of the isolates demonstrated inhibitory activity. The effect was most pronounced against *V. parahaemolyticus*, with 24 isolates (80.0%) displaying antagonism, of which 16 produced moderate inhibition zones (13-25 mm). Strong activity was also observed against *V. vulnificus* (23 isolates, 76.7%), followed by *V. harveyi* (18 isolates, 60.0%) and *V. alginolyticus* (17 isolates, 56.7%).

From this initial screening, isolate LV4 was selected as the most promising candidate due to its consistent, broad-spectrum antagonism. The nCFS of LV4 exhibited moderate but significant inhibitory activity against all four pathogens tested. The largest zone of inhibition was observed against *V. parahaemolyticus* (18.5±0.5 mm), followed by *V. vulnificus* (18.0±0.4 mm), *V. alginolyticus* (17.1±0.7 mm), and *V. harveyi* (16.2±0.3 mm). Crucially, the negative control, consisting of sterile, neutralized MRS broth, showed no inhibition (0 mm) against any of the indicator strains, confirming that the observed activity was derived from the isolate.

Table 1. Morphological, physiological, and biochemical characteristics of the 30 selected LAB isolates

Characteristic	Observation/result
Colony morphology	Circular, convex, smooth, milky-white
Cellular morphology	Gram-positive (+), cocci or coccobacilli, typically in pairs or short chains, non-endospore-forming
Physiology	Non-motile (-)
Biochemical profile	Catalase (-), Urease (-), Citrate Utilization (-)
Carbohydrate fermentation	Glucose (+), Sucrose (+), Lactose (+)

All 30 selected isolates exhibited a uniform profile for the listed tests. (+) indicates a positive result; (−) indicates a negative result

3.4. Safety Assessment: Hemolytic Activity and Antibiotic Susceptibility

3.4.1. Hemolytic Activity

As a crucial safety prerequisite, all 30 isolates were screened for hemolytic activity. The results indicated that the majority of isolates (26 out of 30), including the high-performing candidate LV4, were non-hemolytic (γ-hemolysis) and showed no lytic activity on sheep blood agar. This result was consistent with the negative control, *L. plantarum* ATCC 14917. Four isolates displayed partial hemolysis (α-hemolysis) and were subsequently excluded from further consideration. No isolates exhibited complete lysis (β-hemolysis), in contrast to the positive control, *S. aureus* ATCC 25923, which showed clear (β-hemolysis).

3.4.2. Antibiotic Susceptibility Profile

An essential safety requirement for probiotics is the absence of acquired antibiotic resistance. Statistical

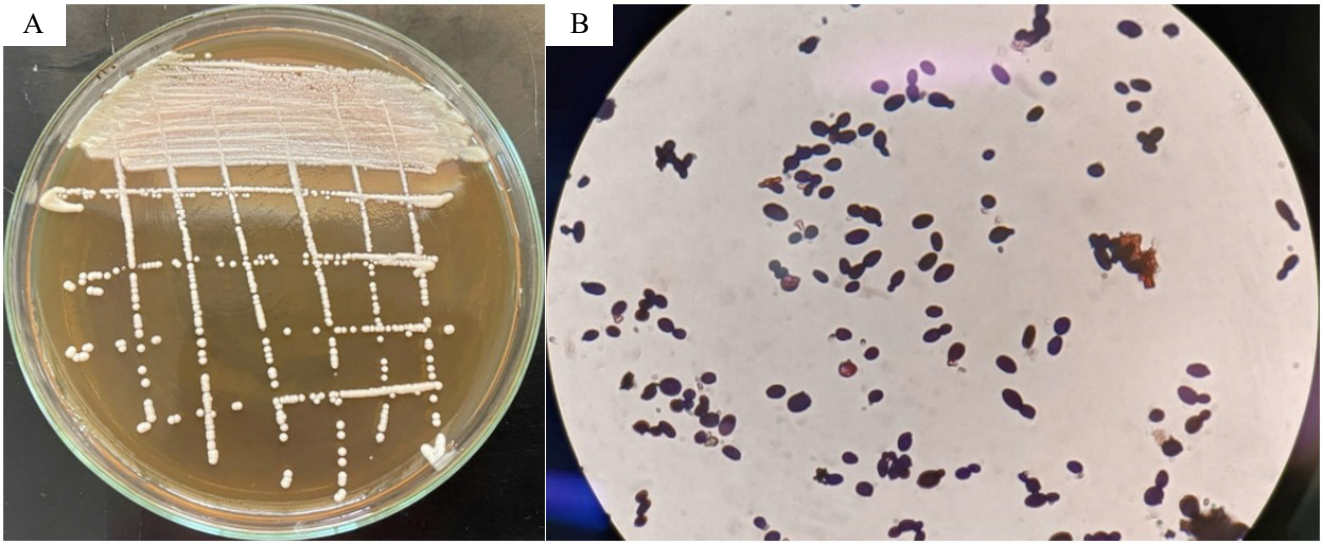

Figure 1. Representative morphology of isolate LV4. (A) Colony morphology on MRS Agar, (B) gram stain revealing Gram-positive, cocci-shaped cells

Table 2. Viability of selected LAB isolates after exposure to acidic conditions for 4 hours

Isolated ID	Viable bacteria isolates (Log CFU/mL ± SD)								
	pH 7.2			pH 3.0			pH 1.5		
LV1	8.21	±	0.17	6.95	±	0.42	5.69	±	0.40
LV2	8.33	±	1.19	8.40	±	0.27	5.62	±	0.22
LV3	7.44	±	0.14	7.54	±	0.16	5.54	±	0.11
LV4	8.39	±	1.01	7.98	±	0.82	6.72	±	0.13
LV5	9.23	±	0.89	8.83	±	0.34	5.84	±	0.51
LV6	7.86	±	0.63	6.97	±	0.32	4.65	±	0.68
LV7	7.23	±	0.97	6.52	±	0.54	3.62	±	0.28
LV8	7.45	±	0.49	5.87	±	0.26	5.69	±	0.32
LV9	7.20	±	1.02	6.38	±	0.17	3.48	±	1.23
LV10	8.85	±	0.89	8.29	±	0.36	5.35	±	0.22
LV11	9.08	±	0.34	8.10	±	0.26	6.93	±	0.13
LV12	7.96	±	0.95	6.88	±	0.33	6.32	±	0.16
LV13	8.59	±	0.27	7.30	±	0.17	5.97	±	0.54
LV14	9.06	±	0.18	7.19	±	0.68	6.48	±	0.70
LV15	9.30	±	1.35	8.96	±	0.27	5.31	±	0.21
LV16	8.41	±	0.13	6.87	±	0.24	6.33	±	0.12
LV17	8.30	±	0.28	7.94	±	0.73	6.04	±	0.22
LV18	8.76	±	0.39	7.60	±	0.25	6.69	±	0.13
LV19	8.91	±	0.75	8.22	±	0.30	6.48	±	0.39
LV20	8.21	±	0.51	6.09	±	0.26	5.93	±	0.13
LV21	8.07	±	0.78	7.52	±	0.17	5.70	±	0.13
LV22	8.84	±	0.53	8.45	±	0.34	5.58	±	0.22
LV23	7.73	±	0.29	7.11	±	0.17	5.67	±	0.11
LV24	7.39	±	1.09	6.66	±	0.43	3.33	±	0.96
LV25	9.50	±	0.91	7.58	±	0.51	5.64	±	0.25
LV26	8.68	±	0.05	7.24	±	0.25	6.50	±	0.13
LV27	9.49	±	0.68	8.98	±	0.28	5.55	±	0.24
LV28	8.33	±	0.63	7.70	±	1.52	5.98	±	0.73
LV29	8.28	±	0.79	7.06	±	0.33	6.41	±	0.17
LV30	8.78	±	0.47	8.24	±	0.30	6.11	±	0.17

Table 3. Viability of selected LAB isolates after exposure to bile salts for 4 hours

Isolated ID	Viable bacteria isolates (Log CFU/mL ± SD)											
	0% (Control)			1.00%			2.00%			3.00%		
LV1	8.21	±	0.17	5.82	±	0.58	4.72	±	0.52	4.61	±	0.72
LV2	8.33	±	1.19	6.07	±	0.65	4.78	±	0.60	5.08	±	0.65
LV3	7.44	±	0.14	5.64	±	0.60	1.53	±	0.26	1.32	±	0.26
LV4	8.39	±	1.01	6.63	±	0.71	5.21	±	0.75	5.17	±	0.48
LV5	9.23	±	0.89	5.96	±	0.63	5.25	±	0.57	4.97	±	0.64
LV6	7.86	±	0.63	5.96	±	0.63	5.18	±	0.56	4.97	±	0.64
LV7	7.23	±	0.97	6.53	±	0.68	5.55	±	0.68	3.71	±	0.43
LV8	7.45	±	0.49	5.78	±	0.62	4.80	±	0.62	4.42	±	0.49
LV9	7.20	±	1.02	5.58	±	0.60	4.60	±	0.60	2.18	±	0.30
LV10	8.85	±	0.89	5.82	±	0.61	4.94	±	0.54	4.83	±	0.61
LV11	9.08	±	0.34	6.24	±	0.66	5.16	±	0.57	4.83	±	0.71
LV12	7.96	±	0.95	5.95	±	0.27	5.12	±	0.55	4.63	±	0.35
LV13	8.59	±	0.27	4.72	±	0.52	3.95	±	0.45	3.40	±	0.25
LV14	9.06	±	0.18	5.68	±	0.55	5.25	±	0.57	4.70	±	0.55
LV15	9.30	±	1.35	5.76	±	0.62	4.54	±	0.50	4.28	±	0.13
LV16	8.41	±	0.13	5.77	±	0.62	4.67	±	0.52	4.11	±	0.35
LV17	8.30	±	0.28	6.00	±	0.64	4.54	±	0.50	4.30	±	0.37
LV18	8.76	±	0.39	6.40	±	0.68	5.54	±	0.38	5.41	±	0.68
LV19	8.91	±	0.75	6.64	±	0.70	4.12	±	0.46	4.66	±	0.31
LV20	8.21	±	0.51	6.49	±	0.69	5.69	±	0.61	4.84	±	0.62
LV21	8.07	±	0.78	6.25	±	0.66	5.54	±	0.59	5.26	±	0.66
LV22	8.84	±	0.53	5.86	±	0.62	5.16	±	0.56	4.87	±	0.62

Table 3. Continued

Isolated ID	Viable bacteria isolates (Log CFU/mL ± SD)											
	0% (Control)			1.00%			2.00%			3.00%		
LV23	7.73	±	0.29	6.05	±	0.65	1.99	±	0.27	1.61	±	0.34
LV24	7.39	±	1.09	6.02	±	0.64	5.12	±	0.55	5.04	±	0.64
LV25	9.50	±	0.91	5.84	±	0.62	4.92	±	0.54	4.85	±	0.63
LV26	8.68	±	0.05	6.64	±	0.70	5.24	±	0.57	4.99	±	0.60
LV27	9.49	±	0.68	5.43	±	0.59	4.77	±	0.52	4.45	±	0.59
LV28	8.33	±	0.63	5.42	±	0.58	5.17	±	0.56	4.43	±	0.58
LV29	8.28	±	0.79	6.44	±	0.68	4.68	±	0.52	4.11	±	0.70
LV30	8.78	±	0.47	5.82	±	0.64	4.23	±	0.47	4.17	±	0.58

Table 4. Sensitivity of bacteriocins produced by LAB isolates to treatment with proteolytic and lipolytic enzymes

Indicator pathogen	Strong (>25 mm)	Moderate (13–25 mm)	Weak (1–12 mm)	Inactive	Total active isolates (%)
V. parahaemolyticus	0	16	8	6	24 (80.0 %)
V. harveyi	0	5	13	12	18 (60.0%)
V. alginolyticus	0	8	9	13	17 (56.7 %)
V. vulnificus	0	11	12	7	23 (76.7%)

analysis revealed significant differences in sensitivity levels among the 30 isolates ($p<0.05$) (Table 5). High rates of sensitivity among the isolates were observed for ampicillin (73.3%) and gentamicin (70.0%). However, notable resistance was also recorded for several antibiotics, including chloramphenicol (30.0%), gentamicin (26.7%), and ciprofloxacin (20.0%). This indicates the importance of strain-specific safety screening. The promising candidate isolate, LV4, demonstrated a favorable safety profile (Figure 2); it was sensitive to Doxycycline, ampicillin, tetracycline, kanamycin, gentamicin, and chloramphenicol, and showed intermediate sensitivity to erythromycin and ciprofloxacin.

Table 5. Antibiotic susceptibility profiles of the 30 LAB isolates

Antibiotic	Sensitive (S)	Intermediate (I)	Resistant (R)
Doxycycline (30 µg)	21 (70.0%)	5 (16.7%)	4 (13.3%)
Ampicillin (10 µg)	22 (73.3%)	4 (13.3%)	4 (13.3%)
Tetracycline (30 µg)	20 (66.7%)	5 (16.7%)	5 (16.7%)
Kanamycin (30 µg)	20 (66.7%)	5 (16.7%)	5 (16.7%)
Gentamicin (10 µg)	21 (70.0%)	1 (3.3%)	8 (26.7%)
Erythromycin (15 µg)	21 (70.0%)	4 (13.3%)	5 (16.7%)
Ciprofloxacin (5 µg)	17 (56.7%)	7 (23.3%)	6 (20.0%)
Chloramphenicol (30 µg)	15 (50.0%)	6 (20.0%)	9 (30.0%)

3.5. Molecular Identification of Isolate LV4 as *Enterococcus faecalis*

Based on its superior overall performance, specifically its high tolerance to acid and bile, potent antimicrobial activity, and favorable safety profile, isolate LV4 was selected for definitive molecular identification.

First, the 16S rRNA gene was successfully amplified by PCR, yielding an approximately 1,500 bp amplicon, as confirmed by agarose gel electrophoresis (Figure 3).

Subsequent Sanger sequencing and a BLASTn search against the NCBI GenBank database were performed. The results, detailed in Table 6, revealed that the 16S rRNA gene sequence of isolate LV4 shared 100% identity with *Enterococcus faecalis* strain NBRC 100480 (Accession: NR_113901.1).

To further confirm this identification, a phylogenetic analysis was conducted. The resulting neighbor-joining tree (Figure 4) shows that isolate LV4 clusters are definitively within the *Enterococcus faecalis* clade, supported by a high bootstrap value (99%). This combination of molecular evidence conclusively identifies isolate LV4 as a strain of *Enterococcus faecalis*. The sequence has been deposited in the GenBank database under accession number PX458018.

4. Discussion

The identification of *E. faecalis* LV4 as a probiotic candidate highlights the shrimp gut as a vital source for discovering host-adapted LAB. Morphological analysis (Figure 1) confirmed standard LAB traits, while molecular identification (Figure 3) and phylogenetic clustering (Figure 4) placed LV4 definitively within the *E. faecalis* clade. These findings align with research identifying native bacterial strains from the shrimp gut microbiota that exhibit strong antagonism against pathogens (Li *et al.* 2018; Knipe *et al.* 2021; Thompson *et al.* 2022; Amiin *et al.* 2023).

LV4 demonstrated remarkable resilience to gastric transit (Table 2). Survival in extreme acid is often attributed to F0F1-ATPase proton pumps (Nami *et al.* 2019; Han *et al.* 2021). The broad-spectrum inhibitory activity of LV4 against multiple relevant *Vibrio* species further underscores its potential as a strong biocontrol agent in shrimp aquaculture (Table 4).

4.1. The Shrimp Gut as a Reservoir for Host-adapted Probiotics

The successful isolation of 30 distinct LAB strains from the gut of healthy *L. vannamei* substantiates the intestinal tract of aquatic animals as a rich source for the discovery of novel, host-adapted probiotic candidates. As noted by Wanka *et al.* (2018) and Zuo *et al.* (2019), isolating beneficial bacteria directly from the target species is a strategic approach, as these microbes are already adapted to the host's unique physiological and environmental conditions. This adaptation is considered critical for enhancing their survival, colonization, and subsequent efficacy within the gut environment (Wanka *et al.* 2018; Zuo *et al.* 2019). Our findings align with a growing body of research that has identified native bacterial strains, including *Bacillus, Lactobacillus*, and other *Enterococcus* species from shrimp gut microbiota, exhibiting strong antagonistic properties against pathogenic vibrios (Knipe *et al.* 2021; Thompson *et al.* 2022; Amiin *et al.* 2023). The gut microbiota of shrimp is a dynamic ecosystem

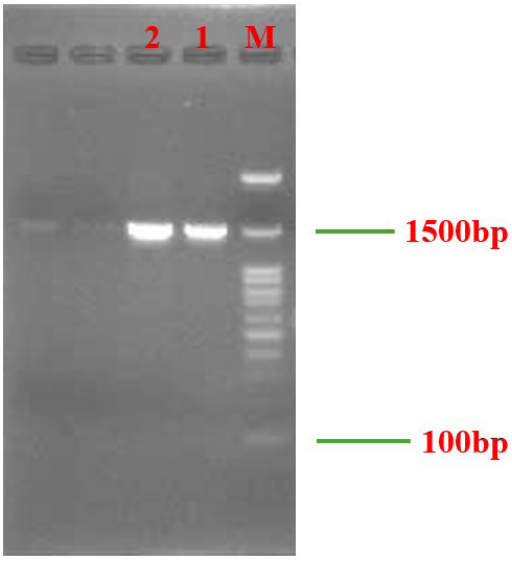

Figure 3. Agarose gel electrophoresis of the 16S rRNA gene amplified from isolate LV4. M: 100 bp DNA ladder; Lane 1: PCR product from isolate LV4 showing a clear band at approximately 1,500 bp

Table 6. Top BLASTn hit for the 16S rRNA gene sequence for isolate LV4

Subject description	Max score	Query cover	E-value	Percent identity	Accession number
Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA, partial sequence	1624	1624	0.0	100.00 %	NR_113901.1

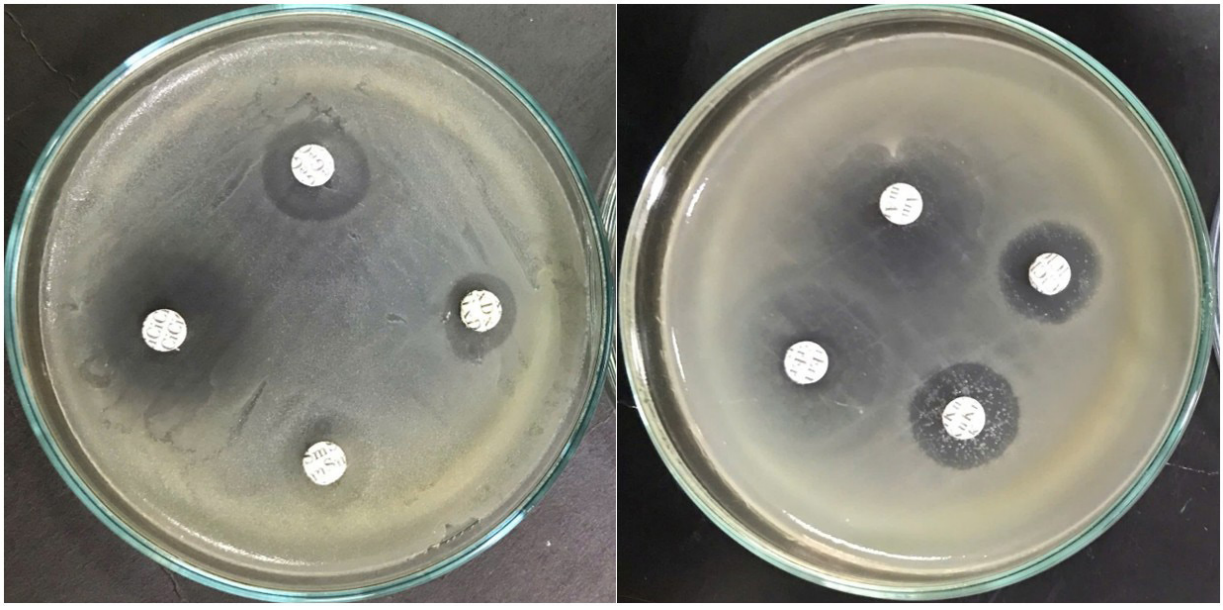

Figure 2. Representative antibiotic susceptibility test for isolate LV4. Zones of inhibition indicate sensitivity to Doxycycline, Ampicillin, Tetracycline, Kanamycin, Gentamicin, and Chloramphenicol. Intermediate sensitivity is shown for Erythromycin and Ciprofloxacin

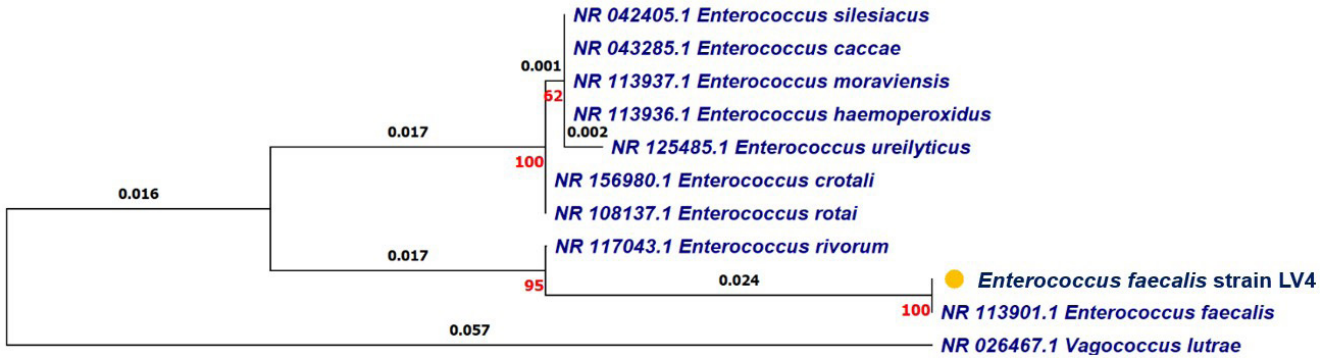

Figure 4. Phylogenetic tree constructed using the Neighbor-Joining method, illustrating the evolutionary relationship of isolate LV4. The analysis was based on 16S rRNA gene sequences and involved related *Enterococcus* species. Bootstrap values (from 1,000 replicates) are indicated at the nodes. The scale bar represents the number of nucleotide substitutions per site

crucial for nutrition, immunity, and disease resistance (Li *et al.* 2018). The identification of *E. faecalis* LV4 in this context is particularly noteworthy. It extends this body of evidence by presenting a strain of a species less commonly reported as a high-performing probiotic from this specific host, highlighting that the indigenous microbiota of *L. vannamei* remains a valuable and underexplored resource for developing targeted, effective probiotics.

4.2. Exceptional Resilience to Gastrointestinal Stresses

A fundamental prerequisite for any effective orally administered probiotic is the ability to survive the harsh conditions of the gastrointestinal tract during transit (Han *et al.* 2021). Our study revealed significant strain-specific variation in tolerance to low pH and high bile salt concentrations, a finding consistent with the broader literature (Nami *et al.* 2019). Isolate LV4 demonstrated remarkable resilience, maintaining high viability (6.72 Log CFU/mL) after exposure to a highly acidic environment (pH 1.5) and a high concentration of bile salts (5.17 Log CFU/mL at 3%). This dual resistance is a critical determinant of probiotic potential, as it ensures that a sufficient number of viable cells can reach the intestine to colonize and exert their beneficial effects (Braïek *et al.* 2018).

The robust tolerance of LV4 suggests the presence of highly efficient, intrinsic stress-response mechanisms. Mechanistically, as suggested by Nami *et al.* (2019) and Han *et al.* (2021), survival in extreme acid is often attributed to the activity of F0F1-ATPase proton pumps, which actively export protons to maintain intracellular pH homeostasis. Simultaneously, tolerance to bile is frequently linked to the enzymatic activity of bile salt hydrolases (BSH), which detoxify conjugated bile salts (Nami *et al.* 2019; Han *et al.* 2021). The high survival rates of LV4 strongly imply that it possesses these mechanisms or functionally similar ones.

The performance of LV4 is notably robust when compared to other strains reported in the literature. For instance, while Sriwulan *et al.* (2019) identified an *Enterococcus avium* strain from shrimp that also tolerated pH 1.5, its bile tolerance was confirmed at only 0.3% bile salts, a tenfold lower concentration than the 3% navigated by LV4. Similarly, a study by Gu *et al.* (2025) on *Pediococcus acidilactici* and *Enterococcus lactis* from shrimp reported survival at much milder acidic conditions (pH 5.0 and 6.0) and lower bile salt concentrations (0.03% and 0.9%). The ability of LV4 to withstand both extreme acidity and a high, physiologically relevant bile concentration places it in a select group of highly durable candidates, making it exceptionally well-suited to function effectively in the challenging gut environment of shrimp.

4.3. Broad-spectrum Antimicrobial Activity against Aquaculture Pathogens

A primary mechanism by which probiotics enhance host health is of inhibiting pathogenic microorganisms. The neutralized cell-free supernatant of *E. faecalis* LV4 displayed moderate, broad-spectrum antimicrobial activity against four major shrimp pathogens: *V. parahaemolyticus, V. harveyi, V. alginolyticus*, and *V. vulnificus*. Crucially, the complete inactivation of this inhibitory effect by trypsin treatment confirmed that the antimicrobial agent is proteinaceous, a hallmark characteristic of a bacteriocin. The production of such compounds provides a significant competitive advantage, allowing the probiotic to suppress pathogens, modulate the gut microbiota, and protect the host from infection (Kumar *et al.* 2011).

This antagonistic capability is particularly valuable in aquaculture, where *Vibrio* species cause devastating disease outbreaks. Our results, with inhibition zones ranging from 16.2 mm to 18.5 mm (as shown in Table 4), are highly encouraging and compare favorably with other findings. For example, these zones are comparable to those reported by Ghomrassi *et al.* (2016), who found that *E. faecium* isolates from fish produced zones of 14–17 mm against *Vibrio* spp. They also appear more potent than the Lactobacillus strains isolated by Tank *et al.* 2018), which showed weaker inhibition zones of 11–13 mm against *V. parahaemolyticus*. Our results are consistent with previous studies demonstrating the potential of enterocins, bacteriocins produced by *E. faecalis* and *E. faecium,* to control aquatic pathogens (Ghomrassi *et al.* 2016; Khushi *et al.* 2022). The ability of LV4 to inhibit a wide range of relevant pathogens underscores its potential as a powerful biocontrol agent.

4.4. Favorable Safety Profile of *Enterococcus faecalis* LV4

The identification of isolate LV4 as *Enterococcus faecalis* necessitates a careful evaluation of its safety, as this species has a dual reputation as both a beneficial commensal and an opportunistic pathogen. A strain-specific safety assessment is therefore paramount.

Our phenotypic screening revealed that LV4 is non-hemolytic (γ-hemolysis), a crucial safety indicator that rules out the potential to lyse red blood cells. Most importantly, the antibiotic susceptibility profile of LV4 was highly favorable. The isolation was sensitive to a majority of the clinically relevant antibiotics tested, including ampicillin, tetracycline, and gentamicin. This favorable profile is a critical finding, as it suggests the absence of transferable antibiotic resistance genes is a major concern for enterococci isolated from clinical settings (Pandova *et al.* 2023) and clearly distinguishes LV4 from virulent, multidrug-resistant strains. Thus, the combination of non-hemolytic activity and a clean antibiogram provides a solid preliminary foundation for the safe development of *E. faecalis* LV4 for aquaculture.

In conclusion, this study identifies *Enterococcus faecalis* LV4, isolated from the gut of *L. vannamei*, as a superior probiotic candidate. The *E. faecalis* isolates demonstrated strong tolerance to acidic pH and bile salts variations, along with good antimicrobial activity. These characteristics suggest that the isolates possess several desirable probiotic traits. However, safety assessment, including screening for virulence genes and antibiotic resistance determinants, is essential before considering their application as probiotic strains. Future work should prioritize whole-genome sequencing and in vivo trials to evaluate the efficacy of LV4 in improving shrimp growth and immune response.

Conflict of Interest

The authors declare that there is no conflict of interest.

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