Int. J. Aquat. Biol. (2025) 13(5): 26-43
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Enhancing shrimp health and immunity through diets supplemented with indigenous intestinal lactic acid bacteria: Implications for *Vibrio parahaemolyticus* infections

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Abstract: Lactic acid bacteria (LAB) have demonstrated significant potential as probiotic agents against various pathogens. The objective of this research was to investigate the potential of indigenous intestinal LAB in combating Acute Hepatopancreatic Necrosis Disease (AHPND) in *Litopenaeus vannamei*. The study ultimately identified the probiotic potential of *Enterococcus faecalis* isolated from shrimp intestines, focusing on its adaptability to varying salinity and pH levels, as well as its antibacterial efficacy. In this study, *L. vannamei* was fed freeze-dried *E. faecalis* combined with commercial feed to stimulate its immune system. Immune responses were assessed by measuring total hemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB), transglutaminase (TG) activity, lysozyme activity, and hemocyte phagocytosis. Additionally, specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), and survival rate (SR) were measured to assess the shrimp's growth performance. The experimental design included five treatments (0, 1, 2, 3, and 4 g of freeze-dried *E. faecalis* per kg of commercial feed), with each treatment consisting of four replicates. The results indicated that the T4 dosage was optimal for enhancing immune responses and growth performance while reducing mortality compared to other treatments. This research adds to the growing body of evidence supporting the use of *E. faecalis* as a probiotic in mitigating *Vibrio parahaemolyticus* infections.

Article history:
Received 4 April 2025
Accepted 28 August 2025
Available online 25 October 2025

Keywords:
AHPND
Enterococcus faecalis
Immune responses
Growth performances
Vibriosis

Introduction

Bacterial pathogens represent a significant economic threat and are among the major challenges to the sustainability of aquaculture globally (Hong et al., 2016; Restrepo et al., 2021). Currently, one of the most formidable emerging diseases affecting shrimp-producing countries is Acute Hepatopancreatic Necrosis Disease (AHPND). This disease has been identified as a significant cause of high mortality in shrimp, typically within 5 to 6 days (Santos et al., 2020; Lozano-Olvera et al., 2023). This severe disease is linked to the production of PirA/PirB toxins by specific strains of *Vibrio parahaemolyticus*, resulting in global economic losses exceeding $2.6 billion within the aquaculture industry (Beltran et al., 2023; Castellanos et al., 2023). In Indonesia, AHPND was first documented in 2011, with estimated financial losses in 2015 amounting to USD 26 million for *Litopenaeus vannamei* and USD 11 million for tiger shrimp (Shinn et al., 2018). The disease has been reported across various shrimp farms in multiple regions of Indonesia. The pathogenicity of *V. parahaemolyticus* strains causing AHPND in *L. vannamei* in Serang, Banten, Indonesia, has been further substantiated through histopathological studies (Saputra et al., 2023).

The use of antibiotics derived from chemical compounds to prevent shrimp diseases remains prevalent in the shrimp farming industry. However, there is increasing concern regarding the misuse and overuse of these drugs, as they can contribute to the development of antimicrobial resistance (AMR)

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DOI: https://doi.org/10.22034/ijab.v13i5.2487

(AftabUddin et al., 2017; Seethalakshmi et al., 2021). The excessive application of antibiotics in aquaculture has been associated with the emergence of antibiotic-resistant bacteria (Hossain et al., 2022). Prolonged antibiotic use has also been reported to have detrimental effects on both aquatic life and the environment (Bungau et al., 2021; Li et al., 2022). In response to these challenges, alternative approaches to antibiotic therapy are being actively explored. These alternatives include nanoparticles, biofilm-based vaccines, algal extracts, phytobiotics, probiotics, prebiotics, and symbiotics (Mishra et al., 2017). Probiotics have thus emerged as a promising alternative to antibiotics for treating and preventing diseases in cultured animals (Restrepo et al., 2021).

Probiotics are microorganisms that offer beneficial effects to their hosts, either by preventing colonization by pathogenic bacteria through antagonistic mechanisms or by enhancing animal health by stimulating the immune system (Hong et al., 2016). Several studies have demonstrated positive outcomes using lactic acid bacteria (LAB) isolated from food to combat *V. parahaemolyticus* infections in vitro (Subagiyo et al., 2017; Sorée et al., 2022). However, these studies have yielded limited and unsatisfactory results and are predominantly confined to scientific research settings. External probiotic products have been tested but have not been successful in addressing AHPND outbreaks. Notably, *Weissella cibaria* KY10, isolated from the digestive tract of healthy shrimp, has been shown to completely inhibit the growth of *V. parahaemolyticus* (Kanjan et al., 2022). Additionally, a strain of *Bacillus licheniformis* demonstrated significant in vitro antimicrobial activity against *V. parahaemolyticus* and effectively protected zebrafish against the pathogen in vivo (Peng et al., 2019). Boopathi et al. (2023) also noted that gut bacteria in shrimp play essential roles in host health and disease pathogenesis. These alternative methods aim to provide effective and environmentally safe solutions for disease management in shrimp aquaculture (Elias et al., 2023). Based on the above-mentioned problems, this study aimed to identify and characterize indigenous intestinal LAB from *L. vannamei* and determine whether the bacteria could prevent AHPND.

Materials and Methods

Indigenous bacteria from shrimp intestine isolation: *Litopenaeus vannamei* intestines from three samples were crushed, and 1 gram of each sample was added to 9 mL of physiological NaCl with five dilutions. Each bacterial dilution was performed on a pour plate containing deMan Rogosa Sharpe Agar (MRS-A) media, and the plates were incubated for 48 hours at 37°C (Li et al., 2020; Uyen et al., 2021). Macroscopic observations included colony color, colony shape, colony edge, colony centre, colony diameter, colony texture, and colony growth. Before further testing, the isolates obtained were rejuvenated first on NA media and incubated at room temperature to maintain the bacterial stock (Lambui and Jannah, 2017).

Bacterial biochemical tests: The tests followed the methods provided by Brown and Smith (2016) and Fallo and Sine (2016) with some modifications. The presence of the catalase enzyme was tested employing a 3% H_2O_2 solution. The isolates were collected with a sterile needle and placed on a preparation glass. Afterwards, a 3% H_2O_2 solution was applied to the bacteria, and positive catalase activity was indicated by foam formation on the colony. The urease test was performed by piercing the ose into the bottom third of the tube and incubating for 24 hours.

The MR-VP (Methyl Red Voges-Proskauer) test was conducted to determine the ability of bacteria to oxidize glucose, producing acid as the final product in high concentration (Sunatmo, 2007). One loop of bacterial culture was inoculated into 2.5 mL MR-VP liquid medium (in up to 2 test tubes) and incubated for 24 hours. After 24 hours of incubation, 3-4 drops of Red Methyl were added to the first test tube. A positive test was indicated by a change in the solution's color to red, indicating mixed-acid fermentation. In contrast, the second test tube added ten drops of solution A (α-naphthol) and ten drops of solution B (KOH). Furthermore, the test tube was vigorously shaken for 20-30 seconds, and a positive

test was characterized by the formation of a pink solution, indicating the presence of acid.

The slide test or clumping factor was used to determine the presence of coagulase bonds. The slide test was done by placing a drop of ddH2O or sterile physiological NaCl on a glass slide, then suspending one ose of the culture. A drop of plasma was placed near the culture suspension, and the two were mixed with the ose, then shaken. A positive reaction occurs when a granular precipitate forms within 2-3 minutes (Dewi, 2013). A hemolysis test was performed on blood agar. A clear zone formed around the colonies on the media indicates that the microbes are pathogenic (Chin et al., 2000).

The indole test was carried out using a sterile ose, taking some colonies from the NA tilted and then inoculating them on indole media by stirring and then incubating at 37°C for 24 hours. After 24 hours, 1-2 drops of Kovac reagent were added. The glucose test was performed by inoculating the isolates into five 500 mL beakers containing sugar media (lactose, galactose, glucose, fructose, and mannitol) before adding phenol red to each medium. The samples were then placed in a test tube containing an inverted Durham tube and incubated at room temperature for 1 to 3 days, depending on the culture's growth. Positive results were indicated when gas bubbles were present in the Durham tube (Brown and Smith, 2016).

Molecular identification of lactic acid bacteria isolates by 16s rRNA: The genomic DNA of the isolate was extracted using the Quick-DNA Magbead Plus Kit (Zymo Research, D4081) provided by PT. Genetika Science Indonesia, Indonesia, according to the manufacturer's instructions. The strains were identified by molecular analysis, the genomic DNA was extracted, and 16S rDNA was amplified by primers 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACTT-3') (Matti et al., 2019; Zuo et al., 2019). Moreover, PCR using (2x) My Taq HS Red Mix (Bioline, BIO-25048) was performed in a total volume of 25 µl, containing 1 µl of DNA template, 12.5 µl of My Taq HS Red Mix (2X), 2 µl of each primer, and 9.5 µl of ddH_2O. The PCR program was 95°C for 3 min, followed by 35 cycles of 95°C for 15s, 52°C for 30s, and 72°C for 45 minutes. The final extension was 72°C for 2 minutes. Dissociation and melting curves of amplification products were performed at the end of each PCR to confirm that only a single PCR product was amplified and detected. The 2-ΔΔCT method was chosen for calculation. Finally, bi-directional sequencing was performed, and the overlapping sequence data produced were assembled to determine the gene sequence.

Bacteria growth curve: The bacterial growth curve analysis followed the García-Hernández et al. (2016) and Risna et al. (2022) method with some improvements. Ten microliters of bacterial isolates were inoculated into 10 mL of NB medium and incubated at 32°C. Bacterial growth was monitored every 3 hours for 48 hours using a UV-Vis spectrophotometer at 600 nm.

Determination of optimal growth at different pHs: To determine the optimal growth conditions for the lactic acid bacteria isolates at various pH levels, a single colony was subcultured in MRS broth from a 1% (v/v) fresh overnight culture. The pH was adjusted between 3, 5, 6, 7, and 9 using 1.0 M NaOH or 1.0 M HCl, and the cultures were incubated at 37°C for 24 hours. Bacterial growth was subsequently assessed by measuring the optical density at 600 nm using a spectrophotometer, with uninoculated broth serving as a control. This allowed for the evaluation of *Lactobacillus* isolate growth across different pH values (Yang et al., 2018; Prabhurajeshwar and Chandrakanth, 2019; Akoğlu, 2020).

Salinity tolerance test: The salinity tolerance of the isolated bacterial cultures was assessed using MRS broth with NaCl concentrations of 0, 30, 32, and 34 ppt based on the shrimp pond environment. Freshly prepared cultures were incubated at 37°C for 48 hours, with turbidity measurements taken at 24 and 48 hours. The negative control, consisting solely of the media, showed no bacterial growth (Arisandi et al., 2018; Tank et al., 2018; Khushboo et al., 2023).

Pathogen preparation and identification: The pathogen, *V. parahaemolyticus,* was isolated from infected *L. vannamei* and identified molecularly at

Table 1. First and nested Primer used in the current study.

Primer	Sequence (5' to 3')
AP4-F1	5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3'
AP4-R1	5'-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'
AP4-F2	5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3'
AP4-R2	5'- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'

Balai Besar Perikanan Budidaya Air Payau Jepara, Middle Java, Indonesia, following a method by the Organization for Animal Health (OIE), 2017, chapter 2.2.1. The PCR program was 95°C for 5 minutes for denaturation, followed by 30 cycles of 94°C for 30s, 60°C for 30s, and 70°C for 1 minute. The final extension was 72°C for 10 min. The study used the primers for the PCR method, including primers (first and nested) shown in Table 1. After PCR, amplicons are visualized by agarose gel electrophoresis.

Well diffusion agar assay: The antagonistic activity of potential probiotic strains was assessed against four strains of *V. parahaemolyticus* using the well diffusion agar assay (WDAA). The pathogenic bacteria were cultured in 10 mL of TSB containing 3% NaCl and incubated for 24 hours on TSA with 3% NaCl at 30°C. Colonies of *V. parahaemolyticus* from pure cultures were suspended in 10 mL of physiological medium and thoroughly mixed for 5 minutes. One millilitre of the suspension was spread onto TSA (Difco) plates. The potential probiotic strains were grown in 10 mL of Lactobacilli MRS broth (Difco Laboratories) for 24 hours, and 100 µL of the supernatant was added to wells in Muller-Hinton (MH) agar medium, which was then incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the clear inhibitory zones (mm) formed around the wells (Khouadja et al., 2017).

Experimental diets and shrimp preparation: The methodology followed by Hong et al. (2022) involved cultivating and incubating the lactic acid bacteria at 28°C for 48 hours on MRS agar. A single colony from a bacterial growth plate on MRS agar was transferred directly into 20 mL of sterile MRS broth before being incubated for 48 hours at 28°C in a shaking incubator (Biobase BJPX-ST10-L). After 48 hours, the bacterial solution was centrifuged at 3,500 rpm for 30 minutes, then rinsed twice with sterile PBS (0.02 M phosphate and 0.15 M NaCl). In the next step, the pellet containing cells was resuspended in PBS. The actual bacterial concentration was determined by total plate count (TPC) according to the Bacteriological Analytical Manual of the Food and Drug Administration (BAM-FDA) protocol (Maturin and Peeler, 2021). Moreover, the pellets were collected employing centrifugation at 3,500 rpm for 30 minutes, combined with 20% skimmed milk, and preserved at -70°C. The frozen bacterial sample was powdered using a freeze-dryer (Lyophilizer BK-FD10P) at -50°C and 0.01 MPa for 48 hours and then stored at 4ºC until use. Furthermore, the experimental diet was wholly constructed by combining the commercial shrimp feed with 5% fat content, 33% protein, 4% fiber, 12% water, and 14% ash and FLAB, which were arranged to various dosages, including 1 g kg^{-1} (T1), 2 g kg^{-1} (T2), 3 g kg^{-1} (T3), and 4 g kg^{-1} (T4) based on Gruber et al. (2023). Additionally, the FLAB and pellet feed were mixed with 0.1% Progol, a commercial binder. Furthermore, the feed was dried by exposure to air and subsequently placed into the feed container (Ekaputri et al., 2018; Purbomartono et al., 2023).

The white shrimp, *L. vannamei*, originated from a commercial shrimp farm in Trenggalek, East Java, Indonesia, and was raised at the Fishery Laboratory of the Department of Aquaculture at the University of Muhammadiyah Malang. Six hundred *L. vannamei* (0.42±0.07 g) were acclimatized with continuous aeration and fed commercial feed at 5% shrimp body weight per day for two weeks before treatment. Afterwards, twenty tanks were set up for five treatments, each containing 96 L of seawater (34 ppt salinity, 28 to 30°C, pH 7.8 to 8.2), before the shrimps were transferred for each treatment at a ratio of 30 animals/tank. The feeding trials were conducted for eight weeks with a daily regimen of around 5% of the

shrimp's weight. The shrimp were manually fed three times a day (at 9:00, 13:00, and 17:00), with the pellets being distributed gradually and proportionately to ensure that all the animals could uptake the treated feed (Hong et al., 2022; Hernández-Cabanyero et al., 2023). Additionally, the water quality parameters were monitored and regulated daily (at 09:00 and 16:00), including pH, temperature, salinity, and nitrogen compounds (Ulfa, 2018).

Sample collections and analyses

***Non-specific immune response analysis*:** One hundred microliters of hemolymph were transferred to a 1.5 mL Eppendorf tube containing 900 µl anticoagulant (0.34 g EDTA, 0.8 g sodium citrate, and 10 µl Tween 80 in 100 mL of distilled water, at pH 7.45 with the osmolality adjusted to 490 mOsm kg^{-1} with NaCl). The respiratory burst (RBs) was assessed using a modified approach from Chen et al. (2016), which used the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O^{2-}) generation. The RBs were calculated by dividing the amount of NBT decrease in 100 µl of hemolymph by the optical density measured at 620 nm.

Phenoloxidase (PO) activity was determined spectrophotometrically by reading the structure of dopachrome produced from L-dihydroxy-phenylalanine (L-DOPA) (Hernández-López et al., 1996; Wu et al., 2015; Gao et al., 2022). At 450 nm, an ELISA reader was used to measure optical density (OD), and results were expressed as the amount of dopachrome produced per 50 µL of hemolymph.

***Analysis of growth performance*:** The weight of each shrimp in every tank was measured individually to determine the growth performance and feed usage following the 8-week feeding trial. Following the feeding trial, the juvenile subjects underwent 24 hours of fasting, after which they were weighed and counted. The specified growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), and survival rate (SR) were determined using the conventional method, which accounts for each shrimp's weight and the number of surviving juveniles (Hong et al., 2022).

$$SGR = \frac{\text{ln of final weight (g)} - \text{ln of initial weight (g)}}{\text{number of days}} \times 100$$

$$WG = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{initial weight (g)}} \times 100$$

$$FCR = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}} \times 100$$

$$SR = \frac{\text{Final number of live shrimp}}{\text{Number of initial shrimp}} \times 100$$

Challenge test: The test followed the method provided by Hernández-Cabanyero et al. (2023) and Hong et al. (2022). A *V. parahaemolyticus* colony on TCBS agar was subcultured in 10 ml of Tryptone Soya Broth (Oxoid, Hampshire, UK) with 2% NaCl at 28°C for 24 hours to induce exponential growth. The broth culture was centrifuged at 3,000 revolutions per minute (rpm) for 30 minutes at 4ºC. The supernatant was separated, while the bacterial lysate was remixed in a salt solution (0.85% NaCl). This resulted in a concentration of roughly 10^9 colony-forming units (CFU) per millilitre for the challenge test. Following 60 days, shrimp (4 to 5 g) that had been given several experimental diets (15 shrimp per tank × 3 replication × 5 treatments = 225 shrimp per treatment) were immersed for 1 hour in water (20 ppt) containing the pathogen (10^8 cfu.mL^{-1}) before placed into the experimental tanks (96 L) at 27±1°C for seven days to record daily mortality.

Statistical Analysis: One-way analysis of variance (ANOVA) was used to analyze the experimental data. Multiple comparisons (Duncan test) were conducted to investigate significant differences among treatments using SPSS (version 17, USA). Results were displayed as the mean ± SD, with a significance level of $P<0.05$ for every test.

Results

Indigenous bacteria from the shrimp intestine: Morphological analysis indicated that only a single type of lactic acid bacteria grew in the MRS agar medium (Fig. 1). After 24 hours of incubation, the bacterial isolate exhibited a round (coccus) shape, white colour, and flat colony edges. The total population count of lactic acid bacteria ranged from 2.6×10^4 to 8.5×10^4 CFU mL^{-1}. Furthermore, the study found that the bacterial isolate formed coccus and *Staphylococcus* colonies (Fig. 2).

Figure 1. The isolated bacteria from the shrimp intestine were cultured in MRS media.

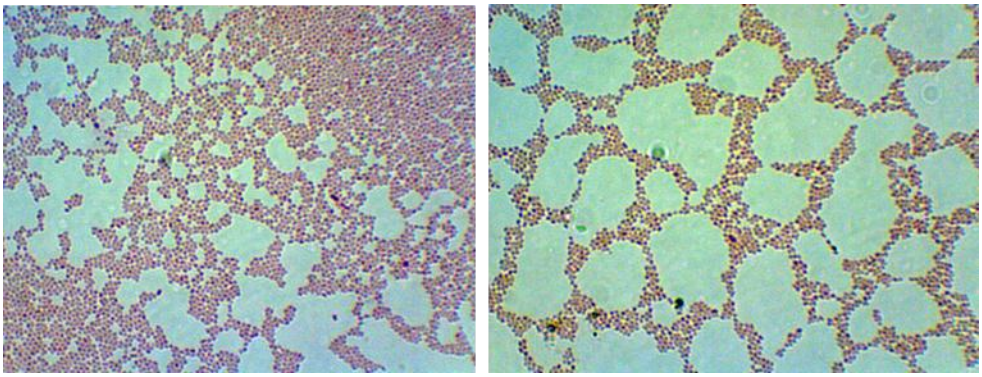

Figure 2. The appearance of isolated lactic bacteria under the microscope.

Bacterial biochemical tests: The identification process continued with biochemical characterization to predict the genus and categorize the *Staphylococcus* group, using 12 tests. According to these tests, 67% of the biochemical tests returned positive results, while the remaining were negative (Table 2). Consequently, based on morphological and biochemical testing results, along with identification using Bergey's Manual of Systematic Bacteriology, a single bacterial genus was identified in the isolates. The study identified the isolate as *Staphylococcus warneri*, a known human pathogen. Therefore, the analysis was further refined by sequencing the 16S rRNA gene to determine the molecular identity of the isolated bacteria.

Identification of bacteria using 16S rRNA gene sequencing: The PCR product from the indigenous lactic bacteria isolate showed a single band (Fig. 3), indicating that the sequences of both samples' 16S rRNA genes were effectively amplified at 1430 base pairs (Table 3). Moreover, Table 4 presents the analysis outcomes comparing the 16S rRNA gene sequences of the IBSI isolate with those available in the GenBank database. This information indicates that the isolates we have analyzed are predominantly identified as *Enterococcus faecalis*, a common and well-known species in the genus *Enterococcus*. The high sequence identity (100%) across all isolates suggests a strong match to these strains, which are well-characterized and documented in the NCBI database. The presence of different strain identifiers indicates slight variations within the species, though all fall under the *E. faecalis* classification.

This information (see Table 4 and Figure 4) indicates that the isolates we have analyzed are predominantly identified as *Enterococcus faecalis*, a common and well-known species in the genus *Enterococcus*. The high sequence identity (100%) across all isolates suggests a strong match to these strains, which are well-characterized and documented

Table 2. The bacterial isolate identification based on biochemical feature.

Test	Repetition 1	Repetition 2	Repetition 3	Repetition 4	Repetition 5
Gram	+	+	+	+	+
Catalase	+	+	+	+	+
Coagulase	-	-	-	-	-
Urease	+	+	+	+	+
Indole	-	-	-	-	-
Hemolysis	-	-	-	-	-
V-P	+	+	+	+	+
D-glucose	+	+	+	+	+
D-maltose	+	+	+	+	+
D-lactose	-	-	-	-	-
D-mannitol	+	+	+	+	+
D-fructose	+	+	+	+	+
Predicted species	*Staphylococcus warneri*	*Staphylococcus warneri*	*Staphylococcus warneri*	*Staphylococcus warneri*	*Staphylococcus warneri*

Table 3. Sequence assembly result – PCR products.

Sample Name	Sequences
The indigenous lactic bacteria isolate	**Sequence Assembly 1430bp.** 1 TGCAGTCGAA CGCTTCTTTC CTCCCGAGTG CTTGCACTCA ATTGGAAAGA GGAGTGGCGG 61 ACGGGTGAGT AACACGTGGG TAACCTACCC ATCAGAGGGG GATAACACTT GGAAACAGGT 121 GCTAATACCG CATAACAGTT TATGCCGCAT GGCATAAGAG TGAAAGGCGC TTTCGGGTGT 181 CGCTGATGGA TGGACCCGCG GTGCATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCC 241 ACGATGCATA GCCGACCTGA GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA 301 CTCCTACGGG AGGCAGCAGT AGGGAATCTT CGGCAATGGA CGAAAGTCTG ACCGAGCAAC 361 GCCGCGTGAG TGAAGAAGGT TTTCGGATCG TAAAACTCTG TTGTTAGAGA AGAACAAGGA 421 CGTTAGTAAC TGAACGTCCC CTGACGGTAT CTAACCAGAA AGCCACGGCT AACTACGTGC 481 CAGCAGCCGC GGTAATACGT AGGTGGCAAG CGTTGTCCGG ATTTATTGGG CGTAAAGCGA 541 GCGCAGGCGG TTTCTTAAGT CTGATGTGAA AGCCCCCGGC TCAACCGGGG AGGGTCATTG 601 GAAACTGGGA GACTTGAGTG CAGAAGAGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG 661 CGTAGATATA TGGAGGAACA CCAGTGGCGA AGGCGGCTCT CTGGTCTGTA ACTGACGCTG 721 AGGCTCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG 781 ATGAGTGCTA AGTGTTGGAG GGTTTCCGCC CTTCAGTGCT GCAGCAAACG CATTAAGCAC 841 TCCGCCTGGG GAGTACGACC GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA 901 AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT 961 CCTTTGACCA CTCTAGAGAT AGAGCTTTCC CTTCGGGGAC AAAGTGACAG GTGGTGCATG 1021 GTTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTTA 1081 TTGTTAGTTG CCATCATTTA GTTGGGCACT CTAGCGAGAC TGCCGGTGAC AAACCGGAGG 1141 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 1201 ATGGGAAGTA CAACGAGTCG CTAGACCGCG AGGTCATGCA AATCTCTTAA AGCTTCTCTC 1261 AGTTCGGATT GCAGGCTGCA ACTCGCCTGC ATGAAGCCGG AATCGCTAGT AATCGCGGAT 1321 CAGCACGCCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA 1381 GTTTGTAACA CCCGAAGTCG GTGAGGTAAC CTTTTTGGAG CCAGCCGCCT

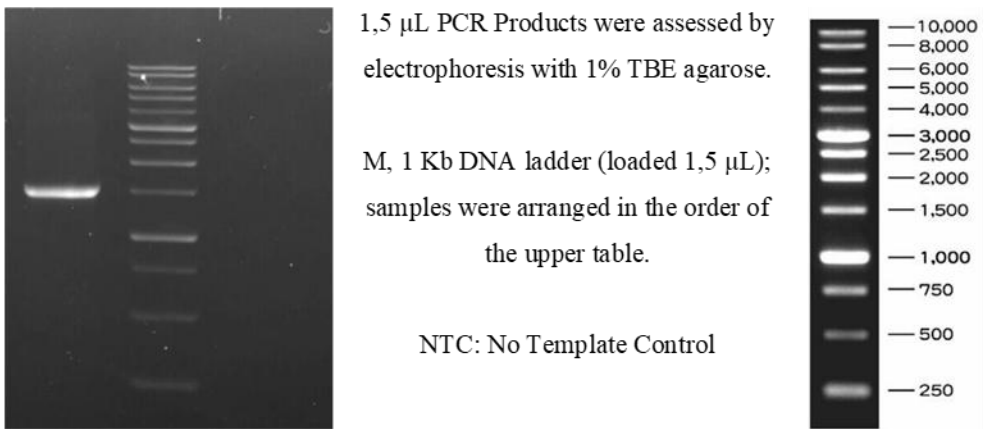

Figure 3. Gel Photo – PCR products

Table 4. Identification of indigenous lactic bacteria isolates by 16S rRNA gene sequencing.

Isolate	Access number on NCBI	Identify (%)	Suggested species
The indigenous lactic bacteria isolate	MN379680.1	100	*Enterococcus faecalis* strain TSGB2040
	MN379674.1	100	*Enterococcus faecalis* strain TSGB2051
	MN255730.1	100	*Enterococcus faecalis* strain TSGB1242
	MN255510.1	100	*Enterococcus faecalis* strain TSGB1211
	MN250309.1	100	*Enterococcus faecalis* strain TSGB1165
	LC484785.1	100	*Enterococcus* sp. CSCRZN6.1 gene
	LC484733.1	100	*Enterococcus* sp. CSCRZR7.2 gene
	MN208105.1	100	*Enterococcus faecalis* strain 380-a pink
	MK182799.1	100	*Enterococcus faecalis* strain YM0831
	MN400225.1	100	*Enterococcus faecalis* strain MG4562

Table 5. *Enterococcus faecalis* pH tolerance.

pH Level	Initial OD (600 nm)	Final OD (600 nm)	Growth Rate (ΔOD/Hour)	CFU/mL ($\times 10^8$)	Standard Deviation
3	0.10	0.12	0.0004	0.2	0.1
4	0.10	0.18	0.0013	0.4	0.1
5	0.10	0.33	0.0041	1.2	0.2
6	0.10	0.51	0.0080	2.1	0.3
7	0.10	0.72	0.0127	2.8	0.4
8	0.10	0.78	0.0143	2.9	0.4
9	0.10	0.50	0.0081	2.0	0.3

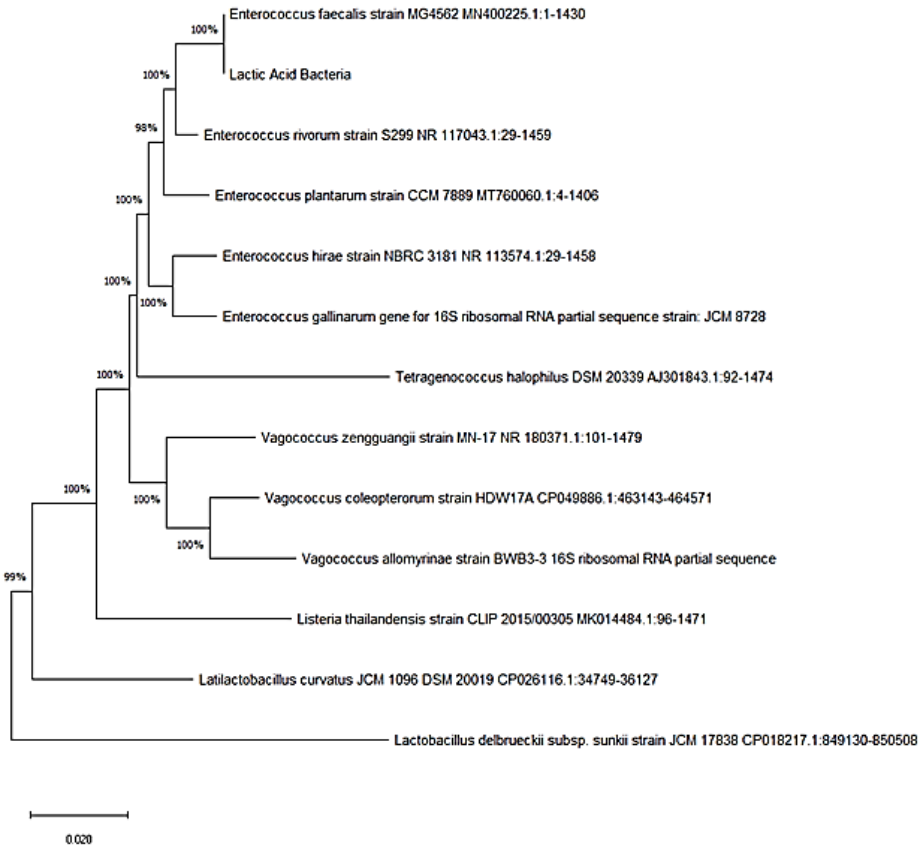

Figure 4. Cladogram based on 16S rRNA gene sequence of indigenous lactic bacteria isolates in a fragment of 1430 bp.

in the NCBI database. The presence of different strain identifiers indicates slight variations within the species, though all fall under the *E. faecalis* classification.

Bacterial growth curve: The present study revealed that Enterococcus faecalis can grow on MRS agar over 48 hours, as observed during the growth phases, including adaptation (lag), exponential (log),

Table 6. *Enterococcus faecalis* salinity tolerance.

Salinity (ppt)	Initial OD (600 nm)	Final OD (600 nm)	Growth Rate (ΔOD/Hour)	CFU/mL (×10⁷)	Standard Deviation
0	0.10	1.00	0.018	3.0	0.5
30	0.10	0.85	0.015	2.6	0.4
32	0.10	0.80	0.014	2.4	0.3
34	0.10	0.75	0.013	2.2	0.3

Table 7. *Vibrio parahaemolyticus* identification using PCR.

No	Name	Code	Result	Parameter	Method
1	Bacteria isolate	*Vibrio parahaemolitycus*	Positive	AHPND (Acute Hepatopancreas Disease)	OIE 2017, Chapter 2.2.1

Table 8. Growth performance of shrimp fed different levels of *Enterococcus faecalis*.

Parameter	Treatment				
	Control	T1	T2	T3	T4
Initial weight (g shrimp^{-1})	0.57±0.02^{a}	0.57±0.03^{a}	0.56±0.03^{a}	0.59±0.07^{a}	0.58±0.06^{a}
Final weight (g shrimp^{-1})	4.12±0.11^{a}	4.34±0.21^{ab}	4.41±0.14^{b}	4.89±0.25^{c}	5.17±0.27^{d}
Gain weight (%)	717.07±42.99^{a}	756.79±49.33^{ab}	782.62±42.79^{ab}	832.31±86.98^{bc}	885.96±72.90^{c}
Growth rate (g)	3.54±0.12^{a}	3.77±0.20^{ab}	3.85±0.14^{b}	4.31±0.24^{c}	4.59±0.24^{d}
Spesific growth rate (% day)	3.28±0.10^{a}	3.37±0.11^{ab}	3.43±0.09^{ab}	3.54±0.18^{bc}	3.64±0.14^{c}
Feed conversion ratio	1.44±0.08^{a}	1.33±0.05^{ab}	1.24±0.10^{b}	1.13±0.09^{c}	1.06±0.04^{d}
Survival rate (%)	73.33±2.36^{a}	74.00±2.79^{a}	80.00±2.36^{b}	81.33±1.83^{b}	86.00±3.65^{c}

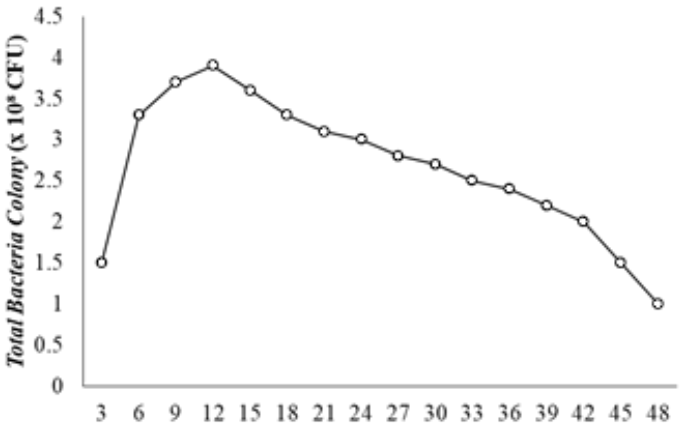

Figure 5. Growth curves of the IBSI for 48 hours in MRS media.

deceleration, stationary, and death (Fig. 5).

Determination of optimal growth at different pHs: The growth of *E. faecalis* across pH levels shows a preference for slightly alkaline conditions, with optimal growth at pH 8, where CFU peaked at 2.9×10^{8}. Growth increases from acidic (pH 3.0) to neutral (pH 7.0), indicating enhanced cell viability; however, it declines beyond pH 8 due to potential stress caused by extreme alkalinity. These findings highlight *E. faecalis*'s adaptability to various pH conditions and its potential use as a probiotic, where stable pH levels are crucial for efficacy (Table 5).

Salinity tolerance: The study of *E. faecalis* growth across varying salinity levels indicates a robust tolerance to marine-like conditions, with growth maintained even at 34 ppt. The highest growth was observed under non-saline conditions, with optical density (OD) and colony-forming units (CFU/mL) decreasing progressively as salinity increased to 30, 32, and 34 ppt (Table 6).

Pathogen identification: Table 7 and Figure 6 show that the DNA of the bacterial pathogen samples isolated from *L. vannamei* hepatopancreas is similar to that of *V. parahaemolyticus*. As a result, the bacteria were reliably used as a negative control group during the study. The DNA of the bacteria had 230 base pairs, precisely the same as the DNA of the *V. parahaemolyticus* product stored in Balai Besar Perikanan Budidaya Air Payau Jepara, Middle Java, Indonesia.

Well diffusion agar assay: The study aimed to determine whether *E. faecalis* influenced *V. parahaemolyticus*. This was demonstrated by the Minimum Inhibitory Concentration (MIC), which showed a significant difference among replicates ($P<0.05$). Based on Figure 7, *E. faecalis* could inhibit *V. parahaemolyticus* continually after nine repetitions. The MIC data revealed that the IBSI could resist

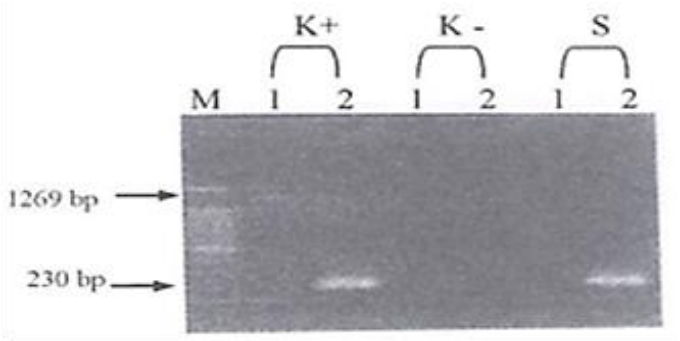

Figure 6. Comparison of DNA sample and DNA control using gel electrophoresis (M: Marker (100 bp DNA ladder), K-: Negative control, K+: Positive control (AHPND), fragment size step 1: 1,269 bp, fragment size step 2: 230 bp, and S: Isolate bacteria).

V. parahaemolyticus, with a range of 5.7 to 6.4 mm.

Effect of *E. faecalis* on the growth performances of *L. vannamei*: The data presented in Table 8 clearly demonstrate the positive effects of various treatments (T1, T2, T3, and T4) on the growth and survival metrics of shrimp, compared to the control group. At the start of the experiment, the initial weights of the shrimp were statistically similar across all groups, with no significant differences, ensuring that any observed changes in growth and survival can be attributed to the treatments applied. Over the course of the study, shrimp in the treatment groups exhibited significant improvements in final weight, weight gain percentage, growth rate, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate. Notably, the T4 treatment was the most effective, resulting in the highest final weight (5.17±0.27 g), the greatest weight gain percentage (885.96±72.90%), and the highest growth rate (4.59±0.24 g).

Additionally, shrimp in the T4 group achieved the highest specific growth rate (3.64±0.14% per day) and the most efficient feed conversion ratio (1.06±0.04), indicating optimal feed utilization. Moreover, survival rates were markedly improved in the treatment groups, with T4 again leading with an 86.00±3.65% survival rate, compared to 73.33±2.36% in the control. The results confirm that these differences are significant, underscoring the efficacy of the T4 treatment in enhancing both growth performance and survival in shrimp. These findings suggest that T4 treatment could be highly beneficial for aquaculture practices aimed at maximizing shrimp production.

Effect of *E. faecalis* on the hemocyte immune responses: The data reveal that the treatments (T1, T2, T3, and T4) significantly enhanced various immune parameters in shrimp compared to the control (Table 9). The total hemocyte count (THC) increased significantly in all treatment groups compared to the control ($P<0.05$), indicating enhanced immune cell production. Transglutaminase activity, crucial for clotting and wound healing, was also elevated, particularly in the T3 group, suggesting improved cellular defence mechanisms. Respiratory burst activity, reflecting the production of reactive oxygen species (ROS), was significantly higher in the treated groups, indicating a stronger oxidative response to pathogens.

Lysozyme activity, essential for bacterial cell wall degradation, revealed that all treatments showed more promising outcomes than the control, further emphasizing the enhanced immune function. Prophenoloxidase activity, vital for melanization, was most pronounced in T4 (0.72±0.01), highlighting its role in pathogen defence. Lastly, phagocytosis activity, a critical immune process, was significantly elevated in T1, T3, and T4, indicating that these treatments effectively enhanced the shrimp's ability to engulf and neutralize pathogens. Overall, the treatments, particularly T3 and T4, demonstrated a robust capacity to enhance shrimp immune competence, offering valuable insights for improving shrimp health and disease resistance in aquaculture.

Challenge test: Feeding a diet supplemented with *E. faecalis* significantly impacted the cumulative mortality rate of the shrimp, independent of the *E. faecalis* dose used in the feed. It is important to note that the isolate was administered at its LD50. The mortality data indicate that the control group (T0) experienced the highest cumulative mortality (61.33% by Day 7), highlighting the steady increase in mortality over time in the absence of intervention. Among the treatments, T4 was the most effective, resulting in the lowest cumulative mortality (20.00% by Day 7), followed by T3 (26.67%), T2 (28.00%), and T1 (34.67%). Each treatment significantly reduced mortality compared to the control, with T4

Table 9. Immune parameters of shrimp in response to feeding different experimental diets consisting of different levels of *Enterococcus faecalis*.

Parameter	Treatment				
	Control	T1	T2	T3	T4
Total hemocyte count ($\times 10^5$ cells ml^{-1})	18.33±1.32^a	27.88±5.39^b	36.6±4.96^b	38.66±3.81^b	39.73±8.40^b
Transglutaminase (OD 450)	0.45±0.07^a	0.60±0.03^{bc}	0.55±0.02^b	0.62±0.05^c	0.58±0.03^{bc}
Respiratory burst (OD 620)	0.30±0.05^a	0.47±0.08^b	0.44±0.06^b	0.52±0.06^b	0.49±0.05^b
Lysozyme (OD 450)	0.50±0.06^a	0.65±0.04^b	0.62±0.04^b	0.67±0.03^b	0.63±0.04^b
Prophenoloxidase (OD 450)	0.55±0.01^a	0.68±0.03^b	0.69±0.02^b	0.70±0.02^{bc}	0.72±0.01^c
Phagocytosis Activity (%)	36.60±2.19^a	53.40±2.07^c	48.60±1.67^b	53.20±0.84^c	51.40±1.82^c

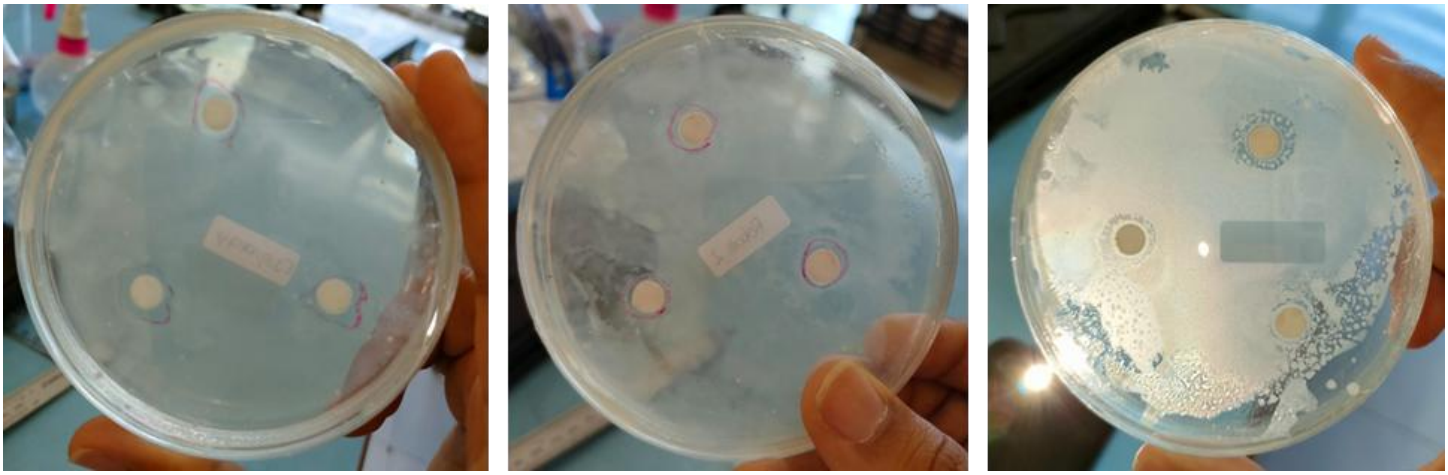

Figure 7. The antimicrobial activity of *Enterococcus faecalis* against *Vibrio parahaemolyticus* was estimated using the agar-disc diffusion method.

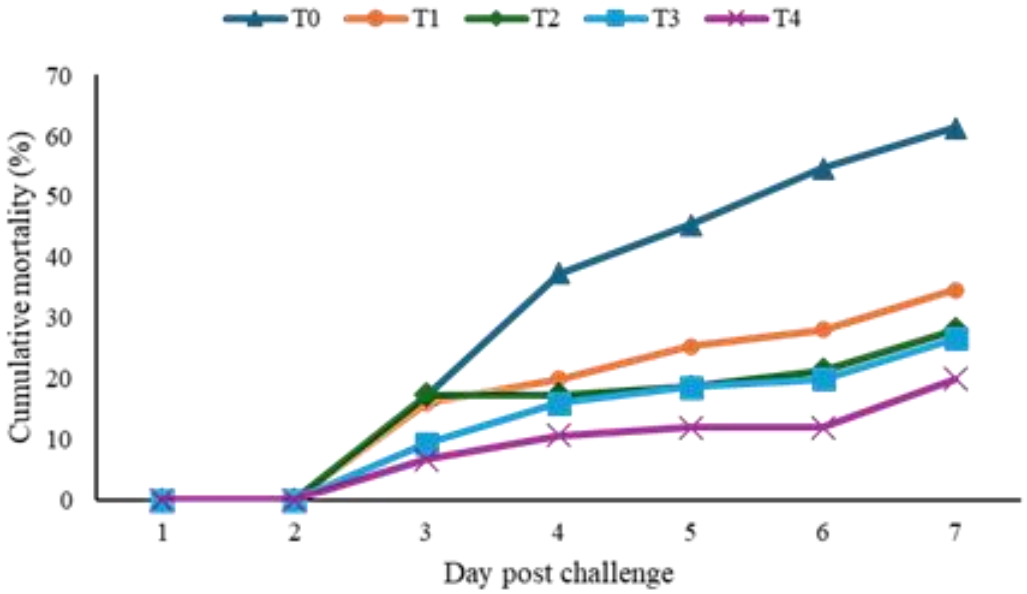

Figure 8. The effect of feeding diets supplemented with *Enterococcus faecalis* on the cumulative mortality rate of the shrimp.

providing the most significant protective effect. The data suggest that these treatments offer varying degrees of protection, with T4 being the most effective in mitigating mortality over the 7 days (Fig. 9).

Discussions

The isolate as the potential probiotic of shrimp: The results suggest that the isolates analysed are *E. faecalis*, a widely recognised species within the *Enterococcus* genus. They have been extensively

studied for their probiotic potential, demonstrating various beneficial properties and antibacterial efficacy against *V. parahaemolyticus* in the well-diffusion agar assay. Research has shown that *E. faecalis* strains can be effective against pathogens such as *E. coli* (Chandran et al., 2022). Similarly, *E. faecalis* MG5206, isolated from kimchi, showed no antibiotic resistance or virulence factors and demonstrated high gastrointestinal viability, further supporting its probiotic potential (Kim et al., 2022). Moreover, *E. faecalis* exhibits strong tolerance to marine-like conditions, maintaining growth even at 34 ppt salinity. The highest growth was in non-saline conditions, with optical density (OD) and colony-forming units (CFU/mL) decreasing as salinity increased. These results align with those of García-Solache and Rice (2019), who found that Enterococcus species can adapt to various saline environments. However, growth rates decline as salinity exceeds optimal freshwater conditions.

Enterococcus faecalis exhibits optimal growth over a wide pH range (from acidic to alkaline), consistent with studies by Mubarak and Soraya (2018) and Weckwerth et al. (2013), which report similar patterns in *Enterococcus* species. Beyond pH 8, growth declines, reflecting the findings of Weckwerth et al. (2013) on reduced viability in extreme alkaline conditions. These results highlight *E. faecalis's* adaptability across a wide pH range, which is crucial for probiotic formulations and industrial applications that require a stable pH. As a result, the present study demonstrates the significant probiotic potential of *E. faecalis*, due to its antibacterial efficacy against pathogens such as *V. parahaemolyticus*. Its adaptability to marine salinity and a wide pH range enhances its suitability for a range of applications.

Effect of *E. faecalis* on the growth performances and survival rate: The results of the current study are consistent with findings from other research exploring the role of probiotics in shrimp aquaculture, further validating the efficacy of these treatments in enhancing growth performance and survival rates. Probiotics, beneficial microorganisms that positively influence the gut microbiota, have been increasingly recognized for their potential to improve the health and productivity of aquaculture species (Alvanou et al., 2023; Ntakirutimana et al., 2023; Torres-Maravilla et al., 2024). The significant improvements observed in the T4 group, particularly in final weight, growth rates, and survival, can be attributed to probiotics' ability to enhance nutrient absorption, boost immune responses, and maintain overall gut health. Several studies have reported similar benefits of probiotics in shrimp. For instance, Wu et al. (2024) found that administering specific probiotic strains significantly increased final body weight and specific growth rate in *L. vannamei*, as observed in the present study. Chen et al. (2021) reported improvements in feed conversion ratio (FCR) with probiotic supplementation, enhancing feed efficiency by modulating gut microbiota and increasing digestive enzyme activity. These findings align with the improved FCR observed in the T4 group, where feed utilization was optimized, leading to superior growth outcomes.

Furthermore, the enhanced survival rates in the T4 group are supported by research showing that *E. faecalis* can improve shrimp's resistance to pathogens. Proespraiwong et al. (2023) demonstrated that probiotics reduced mortality rates in shrimp by strengthening their immune systems and increasing the production of antimicrobial peptides. Similarly, Fuandila et al. (2020) and Nimrat et al. (2020) reported that probiotic supplementation increased survival rates in shrimp challenged with pathogenic bacteria, further underscoring the protective effects of probiotics observed in this study. In summary, the significant improvements in growth performance, feed efficiency, and survival rates in the T4 group corroborate the findings of multiple studies, highlighting the potential of probiotics as a powerful tool in shrimp aquaculture. The consistent results across studies suggest that integrating probiotics into aquaculture practices can lead to more sustainable, profitable shrimp farming operations, making probiotics a valuable component of modern aquaculture strategies.

Effect of *E. faecalis* on the immune responses: Total

hemocyte count (THC) can fluctuate due to various factors, including environmental stressors, infections, immunostimulants, and endocrine activities (Qyli et al., 2020; Mengal et al., 2023). Hemocytes play a crucial role in various physiological and pathological processes, including antigen recognition, phagocytosis, encapsulation, nodule formation, and the release of humoral factors (Adegoke et al., 2023; Stanley et al., 2023). The results of the current study indicated that all treatment groups (T1, T2, T3, and T4) significantly enhanced various immune parameters in shrimp. These findings are consistent with previous research on immune modulation in shrimp through dietary interventions (Gruber et al., 2023; Hernández-Cabanyero et al., 2023; Proespraiwong et al., 2023). The total hemocyte count (THC), a critical indicator of the immune response, exhibited substantial increases across all treatment groups, with the most pronounced effect observed in the T4 group. This enhancement in hemocyte production aligns with the findings of Hong et al. (2022), who reported that dietary supplementation with probiotics significantly elevated THC levels in *L. vannamei*, thereby improving immune function.

Transglutaminase activity, essential for clotting and wound healing, was also elevated, especially in T3, suggesting enhanced cellular defense mechanisms. This supports the findings of Fernandes et al. (2021), who observed that transglutaminase activity in shrimp was significantly enhanced by specific dietary additives, thereby improving wound healing and enhancing pathogen defense. Furthermore, investigations on TG gene silencing in white shrimp have revealed that TG activity is essential for immune defense mechanisms, affecting phagocytic activity, clearance efficiency, and carbohydrate metabolism, highlighting the critical role of TG in shrimp immunity (Chang et al., 2016).

The cellular immune system of shrimp comprises phagocytes, also known as hemocytes, which can kill bacteria by producing superoxide anions and other reactive oxygen species (ROS) (Iunes et al., 2021). The observed increase in respiratory burst activity, particularly in T3, indicates a stronger oxidative response to pathogens and aligns with the study by Ji et al. (2011), which reported that enhanced respiratory burst activity was associated with improved shrimp resistance to bacterial infections. Moreover, Monier et al. (2023) demonstrated that the application of *Bacillus* species probiotics increased the respiratory burst activity of whiteleg shrimp, indicating an enhancement of innate immunity. These findings suggest that probiotics play a crucial role in enhancing the respiratory burst of shrimp, a vital component of their immune system, ultimately improving their ability to combat infections and overall health.

Lysozyme activity, crucial for bacterial cell wall degradation (Ragland and Criss, 2017; Skrzyniarz et al., 2023), was higher in all treatments than in the control group, further emphasizing the improved immune defense observed in this study. This finding is corroborated by the research of Huang et al. (2024), who found that dietary probiotics significantly enhanced lysozyme activity in shrimp, resulting in improved disease resistance. The application of *Bacillus* species probiotics in water significantly increased lysozyme activity in whiteleg shrimp, thereby improving innate immunity and disease resistance against *Fusarium solani* infections (Monier et al., 2023). These findings collectively highlight the crucial role of dietary probiotics in enhancing lysozyme activity and bolstering disease resistance in shrimp.

Prophenoloxidase activity, vital for the melanization process (Wang et al., 2022; Jin et al., 2023), was most pronounced in T4, which is in agreement with the work of Huang et al. (2022), who demonstrated that dietary interventions could significantly enhance prophenoloxidase activity, thereby improving the shrimp's ability to combat pathogens. Comparable studies, such as those by Lee et al. (2024) and Proespraiwong et al. (2023), have reported similar findings: various immunostimulants significantly increase prophenoloxidase levels, thereby enhancing the immune defenses of aquatic organisms. These results align with previous research, which demonstrates that increased prophenoloxidase activity correlates with improved resistance to

pathogens and overall health in shrimp. Thus, the data support the efficacy of the treatments in bolstering the shrimp's immune system, with T4 being the most effective.

Challenge test: The study's findings show that the treatments significantly reduced mortality compared to the control, with Treatment 4 (T4) being the most effective. This finding aligns with other research, such as that of Khushi et al. (2022), which reported that *E. faecalis* notably decreased mortality in giant freshwater prawns infected with *Vibrio* species. Similarly, Swain et al. (2009) and Ai et al. (2022) reported that probiotics, such as *Enterococcus*, improve host resistance to pathogens, which likely explains the enhanced survival in T4. The dose-response pattern observed here aligns with findings by Hong et al. (2022), suggesting that higher doses or more effective formulations may lead to better survival outcomes. Overall, these results highlight the potential of probiotics, particularly *E. faecalis*, in reducing mortality in aquaculture, underscoring the strong protective effect of T4.

Conclusion

The data suggest that *E. faecalis* is a versatile species with significant probiotic potential, as it thrives across diverse environmental conditions. The results demonstrated that *E. faecalis* improved the innate immunity of *L. vannamei* by increasing THC, TG, RBs, phagocytosis, and lysozyme activities. Moreover, the probiotic also enhanced shrimp growth performance during the study. The treatments significantly reduced mortality compared to the control, with T4 being the most effective. These findings highlight T4's superior protective effect over the 7 days.

Acknowledgments: The authors are grateful to the Laboratory of Aquaculture, Department of Aquaculture, University of Muhammadiyah Malang, Indonesia**.** The Faculty of Agriculture and Animal Sciences and the Student Creativity Program Fund supported this research.

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