

## Screening and Preliminary Characterization of Potential Probiotic Lactic Acid Bacteria from Vegetable Wastes

(Penyaringan dan Pencirian Awal Bakteria Asid Laktik Probiotik yang Berpotensi daripada Sisa Sayuran)

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### ABSTRACT

Southeast Asia generates over 8 million tons of vegetable waste annually, yet the microbial isolations from these wastes are rarely associated with potent probiotics. This study aimed to screen lactic acid bacteria (LAB) from fermented and non-fermented vegetable wastes for probiotic potential. LAB were isolated using serial dilutions on MRS agar supplemented with 1% CaCO<sub>3</sub>, yielding 17 isolates. Two isolates, FCU21 and FBS34, demonstrated notable probiotic potential by exhibiting antibacterial activity against *Aeromonas hydrophila* ( $6.3 \pm 0.08$  mm and  $6.2 \pm 0.04$  mm) and *Streptococcus agalactiae* ( $5.6 \pm 0.04$  mm and  $10.4 \pm 0.28$  mm). Initial identification based on morphological, phenotypic and biochemical characteristics showed Gram-positive, catalase-negative, non-spore-forming cocci. Genotypic analysis using 16S rRNA sequencing confirmed both isolates as *Enterococcus hirae*, with 99% similarity for FCU21 (from fermented cucumber) and 100% for FBS34 (from fermented mung bean sprouts), designated as *Enterococcus hirae* UPM01 and UPM02, respectively. Whole-genome shotgun sequencing showed distinct genomic features, including variations in base pairs, contigs and coding sequences. These findings highlight the promising potential of *Enterococcus hirae* strains as probiotics for aquaculture, warranting further investigation into their probiotic properties and applications.

Keywords: *Enterococcus hirae*; lactic acid bacteria; probiotic; vegetable waste

### ABSTRAK

Asia Tenggara menghasilkan lebih daripada 8 juta tan sisa sayuran setiap tahun, namun mikroorganisma yang dipencilkan daripada sisa ini jarang dikaitkan dengan probiotik yang berpotensi tinggi. Penyelidikan ini bertujuan untuk menilai bakteria asid laktik (LAB) daripada sisa sayuran yang ditapai dan tidak ditapai bagi penilaian potensi probiotik. LAB diasingkan menggunakan pencairan bersiri pada agar MRS yang ditambah dengan 1% CaCO<sub>3</sub>, menghasilkan 17 pencilan. Dua pencilan, FCU21 dan FBS34 menunjukkan potensi probiotik yang lebih baik dengan aktiviti antibakteria terhadap *Aeromonas hydrophila* ( $6.3 \pm 0.08$  mm dan  $6.2 \pm 0.04$  mm) dan *Streptococcus agalactiae* ( $5.6 \pm 0.04$  mm dan  $10.4 \pm 0.28$  mm). Pengenalpastian awal berdasarkan ciri morfologi, fenotip dan biokimia menunjukkan pencilan tersebut adalah kokus Gram-positif, katalase-negatif dan tidak membentuk spora. Analisis genotip menggunakan penjujukan 16s rRNA mengesahkan kedua-dua pencilan sebagai *Enterococcus hirae* dengan persamaan 99% untuk FCU (daripada timun yang ditapai) dan 100% untuk FBS4 (daripada taugoh kacang hijau yang ditapai), yang kemudiannya dinamakan sebagai *Enterococcus hirae* UPM01 dan UPM02. Penjujukan seluruh genom mendedahkan ciri genom yang berbeza, termasuk variasi dalam pasangan bes, kontig dan jujukan pengekodan. Penemuan ini menyerlahkan potensi besar strain *Enterococcus hirae* sebagai probiotik untuk akuakultur yang memerlukan kajian lanjut mengenai sifat dan aplikasi probiotiknya.

Kata kunci: Bacteria asid laktik; *Enterococcus hirae*; probiotik; sisa sayuran

## INTRODUCTION

The global surge in food production has led to a significant increase in agribusiness residues, including vegetable and fruit waste, which now constitute a major component of municipal solid waste worldwide. This rise has intensified social and environmental pressure to find meaningful ways to repurpose these by-products, as improper disposal contributes to environmental pollution, greenhouse gas emission and public hazards (Freitas et al. 2021; Sharma et al. 2022). Vegetable waste, comprising discarded post-consumption or processing residues, is increasingly recognized as a valuable resource rather than mere refuse. Recent studies highlight its potential as a rich source of bioactive compounds, including phytochemicals, antioxidants, antimicrobials, vitamins, and dietary fats, which possess beneficial technology and nutritional properties (‘Aqilah et al. 2023; Sharma et al. 2021). Traditionally used in animal feed, these wastes have shown positive effects on animal performance, while their composition that is rich in carbohydrates, proteins, fats, and organic compounds provides an ideal substrate for the growth of indigenous microorganisms, including potential probiotics (Jalal et al. 2023; Malenica, Kass & Bhat 2022).

The fermentation of vegetable waste offers a promising pathway to produce probiotics, particularly lactic acid bacteria (LAB), which are known for their health-promoting properties, such as improved digestion, enhanced immunity and reduced inflammation (Cristofori et al. 2021; Wang, Zhang & Zhang 2021). Under anaerobic conditions, microbial fermentation breaks down complex carbohydrates into simpler compounds, yielding gases (methane), alcohols (ethanol), organic acids (lactic acid), and other metabolites (Deng, Liu & Wang 2020; Pérez-Díaz et al. 2013). LAB strains, including genera such as *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* are frequently isolated from fermented vegetable wastes and demonstrate probiotic potential, including tolerance to low pH and bile salts, antimicrobial activity and immunomodulatory effects (Singh, Shadan & Ma 2022). These findings underscore the viability of vegetable waste as a cost-effective and sustainable substrate for probiotic production, aligning with global efforts to reduce food waste and develop functional foods.

Given the growing interest in alternative sources of LAB, this study aims to isolate and identify LAB strains with probiotic potential. Previous research has demonstrated the feasibility of isolating LAB from vegetable waste, with strains exhibiting desirable probiotic properties such as antimicrobial activity and resilience to gastrointestinal conditions (Linares-Morales et al. 2021; Saadoun et al. 2021). By harnessing these unconventional sources, this study seeks to contribute to the development of innovative, sustainable solutions for waste valorisation and functional food production, with potential application in aquaculture. Further research in this area could pave the way for novel probiotic formulations, ultimately improving health outcomes and supporting global sustainable goals.

## MATERIALS AND METHODS

### SAMPLING OF VEGETABLE WASTES

Vegetable waste samples were collected from Selangor Wholesale Market, situated at an altitude of 145 feet above mean sea level, with geographical coordinates of 2°59'6"N and 101°40'15"E. A total of 30 waste samples, each weighing approximately 1 kg, were aseptically collected from 10 distinct sites, focusing on three types of vegetable waste: cucumber, white cabbage and mung bean sprouts. The samples were randomly selected, placed in sterile polybags and transported in an ice-cooled container to maintain their integrity during transit. Upon arrival at the laboratory, the samples were processed and analysed within 24 h of collection to ensure minimal degradation and preserve their microbiology and biochemical properties (Adane & Tsehayneh 2017).

### SAMPLE PREPARATION

Vegetable waste was washed with distilled water to remove impurities and divided into two groups: fermented and non-fermented samples. Non-fermented samples were cut, dried at 105 °C for 36 h to constant weight, ground into powder and stored in airtight containers in a cool and dry place. For fermented samples, the procedure followed Kaufmann and Schoneck (2007) and Mennes (1994) with some modifications. Briefly, 453 g of vegetable waste was mixed with 4.76 g of salt and wilted for 5-10 min. The mixture was then packed into a 5000 mL beaker, covered with 1000 mL of salt water (22.67 g of cooking salt added) and sealed with parafilm and aluminium foil to maintain anaerobic conditions. The samples were incubated at 25 °C for one week, dried at 105 °C for 48 h, ground into powder and stored as described above.

### DETERMINATION OF THE pH OF VEGETABLE WASTES

The vegetable waste was divided into two groups: fermented and non-fermented samples. For each sample, 100 g was homogenized with 100 mL of the 0.85% (w/v) sterile physiological saline using a lab-blender for 1 min (Lawal & Adedeji 2013). The homogenate was filtered through Whatman No. 1 filter paper, and the supernatant was collected. The pH of the supernatant was measured using a Hanna Instruments Checker® Portable pH Meter, calibrated with pH 4.0 and 7.0 (Cheng & Zhu 2005).

### DETERMINATION OF VIABLE BACTERIAL CELL COUNTS

The waste samples were homogenized and serially diluted ( $10^{-1}$  to  $10^{-8}$ ) in 85% (w/v) sterile physiological saline under aseptic conditions, following Ragavan and Das (2017) with slight modifications. From each dilution, 100  $\mu$ L was spread onto de Man Rogosa and Sharpe agar (MRS, Merck, pH  $5.7 \pm 0.2$ ) supplemented with 1%  $\text{CaCO}_3$  using a sterile bent rod. Plates were air-dried, sealed with parafilm and incubated at 37 °C for 24-48 h. Colony-forming units (CFU) were calculated using the formula:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{Total dilution}} = \frac{\text{Number of CFU}}{\text{mL}} \times \text{volume used}$$

Colonies with distinct morphologies were selected, sub-cultured for probiotic characterization and preserved on MRS slants at 4 °C.

#### PRIMARY IDENTIFICATION OF LABS

Fresh colonies were subjected to preliminary identification, including Gram staining, oxidase and catalase tests, endospore staining, motility (hanging drop method) and morphological observation (size, texture and colour) using phase contrast microscopy (Kopermsub & Yunchalard 2010). Isolates that were Gram-positive, non-spore-forming, catalase- and oxidase-utilization negative were classified as LAB (Coelho, Malcata & Silva 2022).

#### ANTAGONISTIC ASSAY

An antagonistic assay using the agar spot method with slight modifications was performed to screen isolates for inhibitory effects against *Aeromonas hydrophila* and *Streptococcus agalactiae*. Pathogens were cultured in Tryptone Soy Agar (TSA) broth at 37 °C for 24 h and streaked onto TSA plates. LAB isolates were grown in MRS broths, and their cell-free supernatants were obtained by centrifugation (Eppendorf, 5820 R) at 8000 rpm for 5 min. Ten microliters of supernatant were spotted onto TSA plates, which were then incubated at 37 °C for 24 h. To validate the assay, *E. faecium* 14203 was included as a positive control and sterile saline water as a negative control. The experiments were performed in triplicate, and isolates demonstrating the largest inhibition zones were selected as potential probiotics for further analysis (Rengpipat, Rueangruklikhit & Piyatiratitivorakul 2008).

#### SCANNING ELECTRON MICROSCOPIC ANALYSIS

The cell morphology of the selected bacteria isolates was examined using a scanning electron microscope (SEM) following the protocol of Pyar and Peh (2014). Samples were dehydrated sequentially in ethanol (Merck, Germany) at concentrations of 50%, 75%, 95% and 99.5% for 10 min each. Subsequently, 1 mL of hexamethyldisilane (Agar Scientific Limited, UK) was added for 10 min, removed, and air-dried at room temperature. The dried specimens were gold-coated and visualized under the scanning electron microscope (SEM-Hitachi SU8020).

#### IDENTIFICATION OF SELECTED LAB USING COMMERCIAL KIT

The biochemical identification and carbohydrate fermentation of *Lactococcus* isolates were performed using API 20 STREP (Bio Mérieux, France) following the

manufacturer's protocol (Bennani et al. 2017). Briefly, 2 mL bacterial suspensions were prepared from fresh cultures in sterile distilled water. The suspensions were aliquoted into the first half of the strip (VP to ADH), with 100 µL added to each cupule for VP to LAP and full suspension for the ADH test. For the second half (RIB to GLYG tests), the cupule was filled with a mixture of API GP Medium and bacterial suspension. Mineral oil was added to the underlined tests (ADH to GLYG) to create a convex meniscus, and the tray was sealed. Strips were incubated at 37 °C and read at 4 and 24 h. Fermentation profiles were interpreted using the APIWEB database (Bio Mérieux, France).

#### IDENTIFICATION OF SELECTED LAB USING 16S rRNA

Selected isolates were cultured in MRS broth at 37 °C for 24 h, and genomic DNA was extracted using the Bacterial DNA Extraction Kit (BioTeke Corporation, China). Cells were harvested, treated with lysozyme and Proteinase K, and DNA was purified via spin-column AC. The 16S rRNA gene was amplified using primers 27F and 1392R, with PCR products resolved by 1% agarose gel electrophoresis. Purification was performed using the FavorPrep Gel/PCR Purification Kit, and the PCR products were ligated into the pGEM-T easy vector. Transformation into *Escherichia coli* JM109 cells was conducted via heat shock, with successful transformation identified by blue-white screening on LB agar supplemented with ampicillin, X-Gal, and IPTG. Plasmid DNA was extracted, digested with EcoRI, and verified by gel electrophoresis. Sequencing of the 16SrRNA gene was performed by Macrogen Inc. (Seoul, Korea) using primers pAF and pHr. Forward and reverse sequencing were align using CLUSTAL W, and homology searches were conducted via NCBI BLAST. A phylogenetic tree was constructed using the Maximum Likelihood method in MEGA6.06, based on the Tamura-Nei model, with 20 reference strains of *Enterococcus* species for comparative analysis.

#### GENOME ANALYSIS

Selected isolates were cultured in MRS broth (Oxoid, UK) at 37 °C for 24 h, harvested by centrifugation at 3,000 rpm for 15 min, and washed thrice with 0.85% (w/v) sterile physiological saline. Genomic DNA was extracted from bacterial cell pellets, with DNA quantity and quality assessed using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis. For whole-genome sequencing, DNA libraries were prepared using the Nextera XT kit (Illumina, USA) and sequenced on the Illumina HiSeq platform (250 bp paired-end reads) by Novogene, China. Raw reads were trimmed to remove low-quality (Q score < 30) and short (<50 bp) sequences using Trimmomatic v0.39, followed by *de novo* assembly with the A5-miseq pipeline. Contigs (>1000 bp) were ordered against the *E. hirae* ATCC 9790 reference genome using progressiveMauve. Coding sequences (CDSs) were

predicted and annotated using the NCBI Prokaryotic Genome Annotation Pipeline and RAST. Assembly quality was evaluated with QUAST, and whole genome shotgun (WGS) data were deposited in the NCBI database.

#### STATISTICAL ANALYSIS

Results of the studies, which were performed in triplicate, are shown as the mean  $\pm$  standard deviation. The statistical package for social sciences (SPSS) version 21 was used to perform a one-way analysis of variance (ANOVA) and a Duncan multiple range test to separate the means. A significance level of  $p < 0.05$  was utilized to determine the significant results.

#### RESULTS AND DISCUSSIONS

##### pH VALUES OF THE VEGETABLE WASTE

The pH value of the fermented and non-fermented vegetable wastes is presented in Table 1. All samples exhibited acidic conditions with significant differences ( $p \leq 0.05$ ) between fermented and non-fermented groups. Among the fermented samples, cucumber had the lowest pH (3.56), followed by mung bean sprouts (3.57) and white cabbage (3.95). In contrast, non-fermented samples recorded higher pH values: cucumber (5.21), white cabbage (5.36) and mung bean sprouts (5.37). These align with Abebe (2017), who reported pH values of 4.4 - 5.5 in vegetable waste. Fermentation significantly reduced pH to 3.5-4.0, consistent with studies demonstrating that lactic acid fermentation produces organic acids, lowering pH to pH 3.0 - 4.0 (Khubber et al. 2022; Zhang et al. 2022). This acidic environment inhibits spoilage microorganisms (Zhao, Zhang & Zhang 2022) and creates a selective barrier against pathogenic bacteria, making it a promising source for screening of potential probiotics (McFeeters et al. 2013).

##### TOTAL VIABLE BACTERIA COUNT

Table 2 presents the viable bacteria cell count of the fermented and non-fermented vegetable wastes.

Fermented bean sprouts significantly ( $p \leq 0.05$ ) exhibited the highest bacterial counts ( $11.24 \times 10^9 \pm 109.61$ ), followed by fermented cucumber ( $9.52 \times 10^9 \pm 40.50$ ) and fermented white cabbage ( $3.46 \times 10^9 \pm 100.71$ ). In contrast, non-fermented samples showed significantly lower counts: mung bean sprouts ( $1.01 \times 10^5 \pm 56.15$ ), cucumber ( $1.23 \times 10^5 \pm 22.50$ ) and white cabbage ( $0.35 \times 10^4 \pm 10.02$ ). The high bacterial count ( $\sim 10^9$  CFU/mL) in fermented samples is consistent with previous studies reporting microbial populations ranging from  $10^1$  to  $10^9$  CFU/g in the fermented vegetables (Xiong et al. 2022).

Lactic acid bacteria (LAB) are typically dominant in fermented foods (Khubber et al. 2022). The nutrient-rich composition of vegetable waste, including carbohydrates, proteins, fats, and a range of organic and inorganic compounds, provides an ideal environment for microbial proliferation (Assouhoun et al. 2013). Non-fermented samples exhibited lower bacterial counts ( $\sim 10^4$ - $10^5$  CFU/mL), below spoilage thresholds, as reported by González-Arenzana et al. (2020), but within the range ( $10^5$ - $10^9$  CFU/g) observed in raw vegetables (Abdullahi & Abdulkareem 2010), indicating their potential to support microbial growth under suitable conditions.

##### ISOLATION OF LAB

A total of 17 colonies were obtained and designated based on their origin: CU(n) from non-fermented cucumber, BS(n) from non-fermented mung bean sprouts, FCU(n) from fermented cucumber, FMB(n) from fermented mung bean sprouts and FCA(n) from fermented cabbage. All isolates were Gram-positive, non-motile and non-spore-forming cocci, except BS21 and FCU6, which were rod-shaped. Most isolates were catalase-negative, except for CU10 and FCU17, and oxidase-negative except for FCU26 and FBS32 (Table 3). These findings align with previous studies that reported LAB isolation from cassava wastewater (Silva Tomoto et al. 2022), fermented kitchen refuse (Gao et al. 2020; Luo, Lim & Pradhan 2024), fish processing waste (Hanol Bektaş, Ucar & Giray 2020; Johnson et al. 2022) and agricultural waste (Wu et al. 2021; Zhang et al. 2021). This study successfully isolated 17 LAB

TABLE 1. pH value of fermented and non-fermented vegetable wastes

Samples	pH value
Non-fermented white cabbage	5.36 $\pm$ 0.17 <sup>b</sup>
Non-fermented cucumber	5.21 $\pm$ 0.10 <sup>b</sup>
Non-fermented mung bean sprouts	5.37 $\pm$ 3.97 <sup>b</sup>
Fermented white cabbage	3.95 $\pm$ 0.07 <sup>a</sup>
Fermented cucumber	3.56 $\pm$ 0.06 <sup>a</sup>
Fermented mung bean sprouts	3.57 $\pm$ 0.07 <sup>a</sup>

According to the Duncan test, the means with the same letter in columns are not significant different ( $p > 0.05$ ). Results are means of three values  $\pm$  SD (standard deviation)

TABLE 2. Viable bacteria cell counts of fermented and non-fermented vegetable wastes

Samples	CFU/mL
Non-fermented white cabbage	$0.35 \times 10^4 \pm 10.02^a$
Non-fermented cucumber	$1.01 \times 10^5 \pm 22.50^b$
Non-fermented mung bean sprouts	$1.24 \times 10^5 \pm 56.15^b$
Fermented white cabbage	$3.46 \times 10^9 \pm 100.71^c$
Fermented cucumber	$9.52 \times 10^9 \pm 40.50^d$
Fermented mung bean sprouts	$11.24 \times 10^9 \pm 109.61^e$

According to the Duncan test, the means with the same letter in rows are not significant different ( $p > 0.05$ ). Results are means of three values  $\pm$  SD (standard deviation)

TABLE 3. Morphological and biochemical tests of bacteria isolated from vegetable waste

No.	Isolates code number	Gram staining	Morphology	Motility	Endospore	Oxidase	Catalase
1.	CU8	Positive	Cocci	Non-motile	-	-	-
2.	CU10	Positive	Cocci	Non-motile	-	-	+
3.	BS21	Positive	Rod	Non-motile	-	-	-
4.	BS23	Positive	Cocci	Non-motile	-	-	-
5.	BS30	Positive	Cocci	Non-motile	-	-	-
6.	FCU6	Positive	Rod	Non-motile	-	-	-
7.	FCU17	Positive	Cocci	Non-motile	-	-	+
8.	FCU21	Positive	Cocci	Non-motile	-	-	-
9.	FCU26	Positive	Cocci	Non-motile	-	+	-
10.	FCU29	Positive	Cocci	Non-motile	-	-	-
11.	FBS4	Positive	Cocci	Non-motile	-	-	-
12.	FBS31	Positive	Cocci	Non-motile	-	-	-
13.	FBS32	Positive	Cocci	Non-motile	-	+	-
14.	FBS34	Positive	Cocci	Non-motile	-	-	-
15.	FCA16	Positive	Cocci	Non-motile	-	-	-
16.	FCA36	Positive	Cocci	Non-motile	-	-	-
17.	FCA37	Positive	Cocci	Non-motile	-	-	-

+: Positive reaction, -: Negative reaction

strains from both fermented and non-fermented vegetable wastes (*Vigna radiata*, *Cucumis sativus*, and *Brassica oleracea*), in agreement with prior findings on LAB from fermented vegetables and foods (Garcia-Gonzalez et al. 2020; Kumar, Sharma & Singh 2022).

#### SELECTION OF ISOLATED PROBIOTIC BY ANTAGONISTIC ASSAY

To screen potential probiotics, an antagonism test against aquatic pathogens (*Aeromonas hydrophila* and *Streptococcus agalactiae*) was conducted. Of 17 isolates, only two, FCU21 (fermented cucumber) and FBS34 (fermented mung bean sprouts), showed clear inhibition zones (Figure 1). FCU21 and FBS34 exhibited inhibition

zones of  $6.3 \pm 0.08$  mm and  $6.2 \pm 0.04$  mm, respectively, against *A. hydrophila* (Table 4). Against *S. agalactiae*, FCU21 showed a smaller zone of  $5.6 \pm 0.04$  mm, while FBS34 demonstrated significantly greater inhibition at  $10.4 \pm 0.28$  mm. These values were markedly higher than the negative control (sterile MRS agar, 0.0 mm) and demonstrated measurable antagonistic activity, although lower than the inhibition observed with the positive control (*E. faecium* 14203,  $12.5 \pm 0.3$  mm and  $15.2 \pm 0.4$  mm). The results highlight strain-specific activity, with FBS34 showing enhanced inhibition against *S. agalactiae*. LAB with antimicrobial properties have been widely reported (Khubber et al. 2022; Kumar, Sharma & Singh 2020), and the antagonism activity in this study may be attributed to bacteriocin-like substances, organic acids, hydrogen

peroxide or other antimicrobial metabolites (Liu, Wang & Zhang 2023; Zhao, Zhang & Zhang 2022). These findings align with previous studies demonstrating LAB efficacy against similar aquatic pathogens (Johar, Al-Musharafi & Al-Sabahi 2024; Thi, Nguyen & Le 2023), supporting their potential as probiotic alternatives to antibiotics in aquaculture and animal feed. Accordingly, both isolates were selected for further characterization.

#### MORPHOLOGY OF SELECTED ISOLATES

Microscopic examination showed that both isolates appeared as Gram-positive and cocci-shaped bacteria (Figure 2). Further characterization using electron microscopy images (SEM-Hitachi SU8020 Model) confirms the cellular morphology of the isolates (Figure 3). Based on this observation, both isolates were selected for subsequent detailed experimental analyses.

#### IDENTIFICATION OF SELECTED ISOLATES

FCU21 and FBS34 were preliminarily identified using the API 20 STREP kit, with *E. faecium* 14203 as the reference strain (Pelinescu et al. 2009). The strains differed in their reactions to  $\beta$ -galactosidase, alkaline phosphatase and leucine amino peptidase. Compared to the reference, both isolates exhibited distinct reactions to glycogen, Hippurate hydrolysis, and  $\beta$ -galactosidase, and failed to grow on raffinose (Table 4). Based on API 20 STREP, FCU21, and FBS34 were identified as *E. faecium* with 65% and 69% similarity, respectively.

However, molecular identification via 16s rRNA and whole-genome shotgun sequencing showed both strains to be *Enterococcus hirae*, highlighting discrepancies between biochemical and molecular methods. The partial 16S rRNA sequences of the selected isolates were compared with GenBank entries using BLAST (NCBI), showing 99 to 100% identity to *E. hirae*, contradicting the biochemical

identification results (Table 5). Consequently, the strains were designated as *E. hirae* UPM01 (FCU21) and *E. hirae* UPM02 (FBS34). Phylogenetic analysis constructed using library data confirms their classification, with the isolates clustering closely with *E. hirae* (Figure 4). Strains with similar sequences were grouped together, indicating a close evolutionary relationship. The phylogenetic tree reliably identified the isolates as *E. hirae*.

Draft genome analysis of *E. hirae* UPM01 and *E. hirae* UPM02, annotated against *E. hirae* ATCC 9790, showed distinct genomic features (Table 6). *E. hirae* UPM01 comprises 59 contigs (2,720,863 bp), 2,649 coding sequences (CDS), 53 tRNAs and 7 rRNAs, while *E. hirae* UPM02 contained 31 contigs (2,700,533 bp), 2,549 CDS, 61 tRNAs and 3 rRNAs. Both strains had a GC content of 37%, with the largest contigs at 524,829 bp and an N50 value of 234,365 bp. KEGG analysis highlighted enrichment in 'Lipid metabolism' and 'Carbohydrate metabolism' genes. Additionally, both strains harboured two gene clusters for secondary metabolite production, encoding a putative class II lantipeptide and a terpene. A single antimicrobial resistance (AMR) gene, *AAC* ( $\delta'$ ), conferring aminoglycoside resistance through antibiotic inactivation, was identified. The whole-genome shotgun sequences were deposited in the GenBank database under accession numbers PSQK00000000 (UPM01) and PSQL00000000 (UPM02).

Comparative analysis showed that *E. hirae* UPM01 and *E. hirae* UPM02, despite sharing the same species designation, represent distinct strains with unique genomic features. For instance, *E. hirae* INF E1, isolated from cultured milk, differed in contig number (22), total base pairs (2,807,725 bp), and GC content (36.8%) (Porcellato, Østlie & Skeie 2014). The presence of secondary metabolite gene clusters in *E. hirae* UPM01 and *E. hirae* UPM02, similar to those in *E. hirae* INF E1, underscores their metabolic potential. Metabolic modelling of these

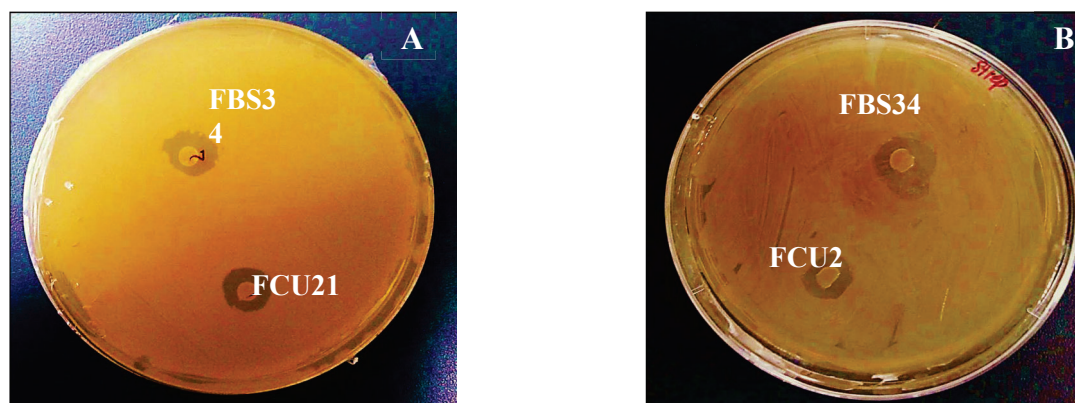
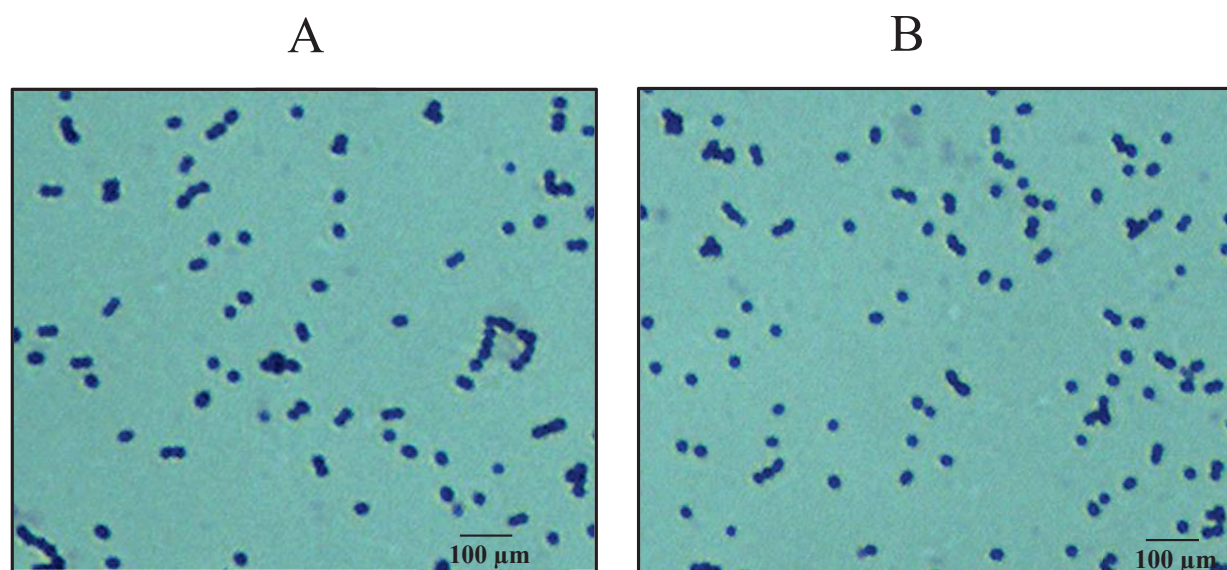
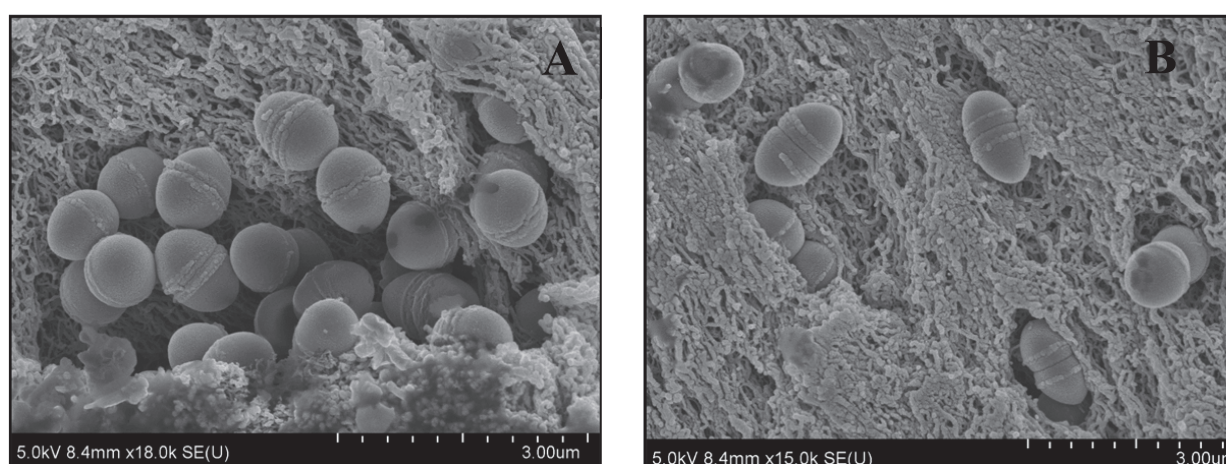


FIGURE 1. Selection of potential probiotics based on the antagonism test against *A. hydrophila* (A) and *S. agalactiae* (B). Only two isolates (FCU21 and FBS34) are capable of producing inhibition zones against aquatic pathogens

TABLE 4. The diameter of inhibition zones produced by selected LAB

Isolates	Aquatic pathogens	
	<i>A. hydrophila</i>	<i>S. agalactiae</i>
FCU21	$6.3 \pm 0.08^b$ mm	$5.6 \pm 0.04^c$ mm
FBS34	$6.2 \pm 0.04^b$ mm	$10.4 \pm 0.28^b$ mm
<i>E. faecium</i> 14203 (positive control)	$12.5 \pm 0.3^a$ mm	$15.2 \pm 0.4^a$ mm
Sterile saline water (negative control)	$0.0 \pm 0.00^c$ mm	$0.0 \pm 0.00^d$ mm

According to the Duncan test, the means with the same letter in rows are not significant different ( $p > 0.05$ ), (n = 3)

FIGURE 2. Gram staining of isolates FCU21 (A) and FBS34 (B) showed Gram-positive and cocci shape bacteria at  $100 \times$  magnification (scale =  $100 \mu\text{m}$ )FIGURE 3. Scanning electron microscope micrograph (SEM) of isolates FCU21 (A) and FBS34 (B) (scale =  $3 \mu\text{m}$ )

isolates is crucial for elucidating their adaptability to the isolation environment and identifying potential probiotic traits. Comparative genomic studies of *E. hirae* strains may further aid in developing novel probiotics.

The limitations of biochemical identification, such as the API 20 STREP kit, were evident in this study. While useful for differentiating cocci species through carbohydrate fermentation profiles (Bennani et al. 2017), the kit often misidentified *Enterococcus* species, particularly nonclinical strains (Facklam & Teixeira 2003; Loong

et al. 2020; Velasco et al. 2004). For example, Robredo et al. (2000) reported misclassification of *E. faecium* as *E. durans* and *E. casseliflavus*. Such inaccuracies likely stem from subjective visual interpretation and the kit's bias toward clinical isolates, limiting its reliability for environmental or agricultural strains (Jackson, Fedorka-Cray & Barrett 2004). Molecular techniques, such as 16s rRNA sequencing, offer more precise and rapid identification (Gerace et al. 2022; Muhamad Rizal et al. 2020), emphasizing the need for integrated approaches to validate strain identification (Abdelsalam et al. 2023).

TABLE 4. Identification of *Enterococcus* spp. isolated from fermented vegetable wastes by API 20 STREP

Biochemical tests	Isolates		
	FCU21	FBS34	<i>E. faecium</i> 14203
Arginine dihydrolase	+	+	+
Glycogen	-	-	+
Voges – Proskauer	+	+	NS
Hippurate hydrolysis	-	-	+
Esculin hydrolysis	+	+	+
$\alpha$ -Galactosidase	-	-	+
Pyrrolidonylarylamidase	+	+	+
$\beta$ -Glucuronidase	-	-	-
$\beta$ -Galactosidase	-	+	+
Alkaline phosphatase	+	-	NS
Leucine amino peptidase	-	+	+
Ribose	+	+	+
Lactose	+	+	+
Trehalose	+	+	+
Mannitol	+	+	+
Inulin	-	-	-
Sorbitol	-	-	-
Raffinose	+	+	-
Amidon	-	-	NS
Arabinose	-	-	-

+: Positive reaction, -: Negative reaction; NS: Not Stated. FCU21, *Enterococcus faecium*; FBS34, *Enterococcus faecium*

TABLE 5. The similarity of 16S rRNA sequence of two isolates compared with strains from database

Isolates	E-value	Strains homology	Accession No.	% Similarity
FCU21 (UPM01)	0.0	<i>Enterococcus hirae</i> strain R 16S ribosomal RNA gene, partial sequence	NR 037082.1	99
FBS34 (UPM02)	0.0	<i>Enterococcus hirae</i> strain R 16S ribosomal RNA gene, partial sequence	NR 037082.1	100



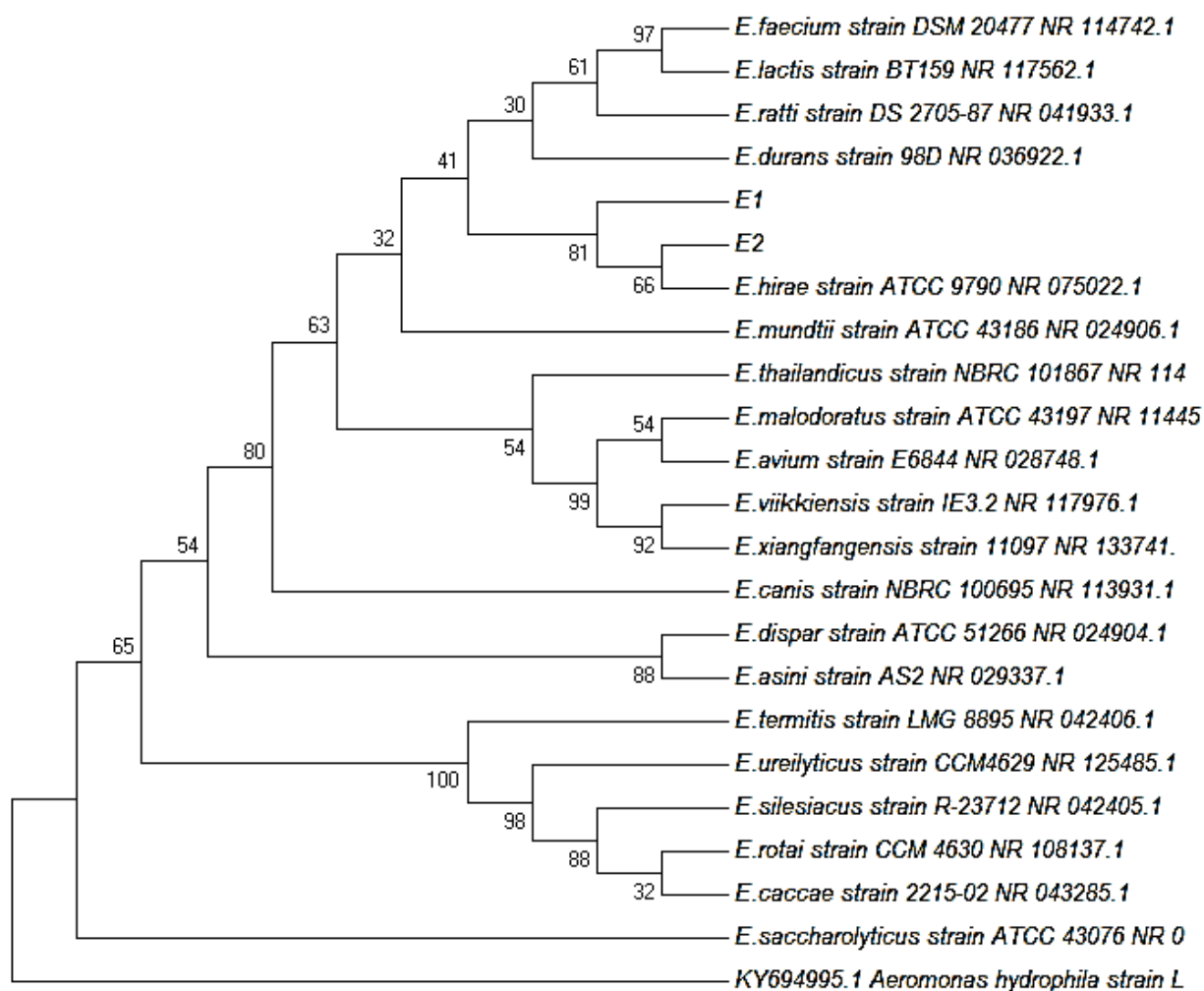


FIGURE 4. Phylogenetic tree analysis of nucleotide sequences of 16S rRNA genes of E1 UPM01 (E1) and UPM02 (E2) with 20 other known species

TABLE 6. Genome analysis of *E. hirae* UPM01 and *E. hirae* strains UPM02

Isolates/Analysis	<i>E. hirae</i> UPM01	<i>E. hirae</i> UPM02
Number of contigs	59	31
Total base pair	2,720,863 bp	2,700,533 bp
Largest contigs	524,829 bp	524,829 bp
Total number of coding sequences, CDS	2,649	2,594
N50	234,365 bp	234,365 bp
GC content, %	37	37
Protein-coding genes	53 tRNAs 7 rRNAs	61 tRNAs 3 rRNAs
KEGG database	Lipid and carbohydrate metabolism	Lipid and carbohydrate metabolism
Secondary metabolites	II lantipeptide and a terpene	II lantipeptide and a terpene
Antimicrobial resistance (AMR) gene	Aminoglycoside antibiotic	Aminoglycoside antibiotic

## CONCLUSION

Identifying microorganisms in vegetable waste offers critical insights into their potential as probiotic sources. Exploring these non-traditional materials for probiotics represents a promising strategy for waste valorization, aligning with the 'waste-to-wealth' concept. This approach supports sustainable advancements of agriculture and food industries, particularly in Southeast Asia, and lays the foundation for developing functional foods or dietary supplements from waste-derived probiotics. Although research on the probiotic potential of vegetable waste is still emerging, compiling foundation data is essential to guide future studies. Such investigation could drive innovative solutions for enhancing human health while addressing the global food waste challenge, fostering a circular economy.

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