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RESEARCH ARTICLE

Antioxidant and probiotic potential of *Lacticaseibacillus paracasei* isolates from Malaysian water kefir grains

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Abstract

Water kefir is a traditional fermented beverage that comprises a microbially diverse community enriched in lactic acid bacteria (LAB). Previous studies have examined the predominant species recovered from each sample. However, functional strain-level variation among multiple LAB isolates within a single kefir grain source remains insufficiently explored. This study aimed to isolate and characterise *Lactobacillus* species from Malaysian water kefir grains and evaluate their antioxidant activity and probiotic potential. Bacterial isolates were initially characterized through colony morphology, Gram staining, and catalase testing. Antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays. Isolates demonstrating high levels of antioxidant activity were evaluated for probiotic characteristics, including acid and bile salt tolerance, adhesion to intestinal epithelial cells, and antibiotic susceptibility. Molecular identification was performed through 16S rRNA gene sequencing. Among fifteen isolates, isolates 2 and 5 exhibited significantly higher antioxidant activities ($p < 0.05$) with ABTS radical scavenging activities of 72.70 ± 3.38% and 77.74 ± 3.27%, and DPPH radical scavenging activities of 41.62 ± 1.18% and 48.07 ± 5.18%, respectively. Probiotic characterization demonstrated that isolate 2 exhibited higher bile tolerance and adhesion capacity with a 93.57 ± 1.31% survival rate under bile salt stress and 88.8 ± 6.3% adhesion rate to HT-29 cells. Molecular identification identified both isolates as *Lacticaseibacillus paracasei*, a species widely associated with probiotic functionality. Taken together, these findings highlight isolate 2 as a promising probiotic candidate with antioxidant potential, warranting further investigation to validate its functional efficacy and safety.

Keywords: antioxidant activity, *Lacticaseibacillus paracasei*, molecular identification, probiotics, water kefir

INTRODUCTION

The human gastrointestinal tract hosts a complex ecosystem of trillions of microorganisms collectively known as the gut microbiota, which plays a fundamental role in maintaining host homeostasis and overall health (Rinninella et al., 2023). This intricate microbial community, comprising an estimated 100 trillion symbiotic bacteria, significantly influences various physiological processes, including digestion, immune function, and metabolic regulation (Lynch et al., 2024; Uzbay, 2019). Lactic acid bacteria (LAB) benefit human health through their metabolic activities and support of key physiological functions (Mukherjee et al., 2024). These bacteria include *Lacticaseibacillus* and other related genera, have attracted increasing scientific interest in recent years. The genus *Lactobacillus* was reclassified in 2020 into multiple genera, including *Lacticaseibacillus*, to better reflect phylogenetic diversity (Zheng et al., 2020).

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Probiotics are defined by the World Health Organization as "live microorganisms that, when administered in sufficient amounts, provide health benefits to the host". They have emerged as potential agents for promoting gut health, enhancing immune responses, reducing cholesterol levels, and potentially preventing certain cancers (Huang et al., 2024; Hill et al., 2014; Salminen et al., 1999). The growing awareness has fuelled substantial market expansion, driven by rising consumer interest in the role of gut microbiota in maintaining health (Gao et al., 2024; Li et al., 2023). However, probiotic functionality is highly strain-specific. This highlights the need to explore new isolates from diverse sources, including naturally fermented foods, to identify novel probiotic candidates. Fermented beverages such as water kefir are characterised by a complex and ecologically stable microbial environment. This environment supports the viability and metabolic activity of LAB. Increasing evidence suggests that such naturally fermented products may offer functional benefits compared with commercial probiotic supplements (Cheng et al., 2024; Ibrahim et al., 2023; Garrote et al., 2010).

Water kefir produced via the fermentation of sucrose solutions with its grains represents a natural source of probiotic microorganisms (Lynch et al., 2021). This beverage is mildly acidic and naturally carbonated, resulting in a distinctive sensory profile. Water kefir grains, commonly referred to as "tibicos" or the "ginger-beer plant," have been reported in association with *Opuntia* ssp. cactus in Mexico (Pendón et al., 2022). Unlike milk kefir grains, which are traditionally linked to the Caucasus region, the geographical origin of water kefir remains less clearly defined (de Almeida et al., 2025; Rosa et al., 2017). Variations in environmental conditions, fermentation substrates, and regional practices may contribute to the microbial diversity observed in contemporary water kefir cultures.

The microbial composition of water kefir encompasses a diverse community of microorganisms, including lactic acid bacteria (LAB), acetic acid bacteria (AAB), and various yeast species, each contributing unique functional properties to the final product (Azizi et al., 2021). Among the LAB population, *Lactobacillus hilgardii*, *L. nagelii*, *L. casei*, and *L. paracasei* represent the most prevalent species in water kefir grains.

Water kefir-derived LAB demonstrate a wide range of health-promoting properties, including anti-inflammatory, antioxidant, antimicrobial, and cholesterol-lowering effects (Talib et al., 2024; Georgalaki et al., 2021; Kumar et al., 2021b). These bioactivities have established water kefir as a probiotic beverage. Previous work demonstrated that predominant *Lactobacillus* species isolated from 10 Malaysian kefir samples displayed notable antioxidant capacity and probiotic potential (Talib et al., 2019). Probiotics with antioxidant activity have been widely investigated for their role in mitigating oxidative stress. Oxidative stress is implicated in the development of chronic diseases such as cancer, cardiovascular disease, diabetes, and age-related disorders (Silva et al., 2025; Yu et al., 2025; Seo, 2024). However, only one dominant isolate per sample was examined, leaving the broader diversity and functional characteristics of other *Lactobacillus* strains within a single kefir grain source unexplored. This knowledge gap is relevant considering the antioxidant capacity and probiotic properties of *Lactobacillus* species derived from Malaysian kefir.

To address this gap, the present study examined a single grain source reported to produce water kefir with high antioxidant activity (Kumar et al., 2021a). Therefore, this study aimed to examine the antioxidant and probiotic properties of *Lactobacillus* species isolated from Malaysian water kefir grains. Understanding the unique properties of these locally sourced species may offer valuable insights for the development of region-specific probiotic applications and potential therapeutic strategies.

MATERIALS AND METHODS

Water kefir grain preparation and activation

Water kefir grains were prepared and activated prior to the isolation of LAB. The grains were purchased from Kefir and Kombucha (Kuala Lumpur, Malaysia). Activation was carried out by cultivating 18 g of water kefir grains in 500 mL of 10% (w/v) jaggery solution, prepared by dissolving 50 g of jaggery (Kefir and Kombucha, Malaysia) in 500 mL of mineral water, following the protocol described by Kumar et al. (2021a) with minor modifications. Fermentation was conducted at room temperature (approximately 25–28 °C) for 24 hours under static conditions with limited oxygen exposure. Successful fermentation was indicated by grain flotation, gas bubble formation, and a reduction in pH of the fermentation medium.

Isolation and enumeration of lactic acid bacteria

Following fermentation, the fermented liquid was discarded, and the grains were thoroughly rinsed with mineral water to remove residual fermentation medium. Ten grams of activated water kefir grains were transferred into 90 mL of sterile 0.85% (w/v) sodium chloride solution at pH 7.2–7.4 (Merck, Darmstadt, Germany) and homogenised using a sterile hand-pull blender for 2 minutes to ensure uniform bacterial dispersion, as described by Talib et al. (2019). Serial ten-fold dilutions were prepared

using the same sterile sodium chloride solution as the diluent. Aliquots of 100 µL from the final three dilutions were spread-plated using de Man, Rogosa, and Sharpe (MRS) agar plates (Merck, Darmstadt, Germany) in triplicate. MRS medium is a selective culture medium widely used for the isolation and cultivation of lactic acid bacteria (LAB). The inoculated plates were incubated at 37 °C for 48 hours. Following incubation, colonies were enumerated and expressed as colony-forming units per millilitre (CFU/mL). Individual colonies exhibiting distinct morphological characteristics were selected from MRS agar plates and subcultured in 10 mL of MRS broth (Merck, Darmstadt, Germany), followed by incubation at 37 °C for 24 hours. To obtain pure cultures, 10 µL of each bacterial culture was re-streaked using fresh MRS agar plates using the quadrant streak method and incubated at 37 °C for 48 hours.

Preliminary characterization of bacterial isolates

Gram staining and morphological assessment

Gram staining was performed to differentiate bacterial cell wall characteristics and determine cellular morphology (Tripathi et al., 2025; Cappuccino & Welsh, 2018). The stained smear was examined under an inverted light microscope (Nikon, Japan) at 400× total magnification, which was sufficient to observe Gram reaction (purple staining) and general rod-shaped morphology of the isolates. Gram-positive, rod-shaped bacteria were selected for further analysis as potential *Lactobacillus* candidates.

Catalase activity testing

Catalase activity was assessed to differentiate LAB from other bacterial groups by determining the presence of the enzyme catalase (Reiner, 2010; Shangari & O'Brien, 2006). A small portion of the bacterial colony was transferred to a sterile microscope slide using a sterile inoculating loop. A single drop of 3% hydrogen peroxide solution (Sigma-Aldrich, USA) was added to the bacterial sample and gently mixed. The reaction was observed immediately, where effervescence (bubble formation) indicated positive catalase activity, while the absence of bubbles indicated negative catalase activity. Only catalase-negative isolates were retained for subsequent analysis, as this characteristic is typical of LAB.

Antioxidant activity assessment

DPPH radical scavenging assay

The free radical scavenging activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay with Trolox (Sigma-Aldrich, USA) as the reference standard. The assay was performed according to the method described by Talib et al. (2019) with modifications for intact cells of bacterial cultures. A 24-hour bacterial culture was harvested by centrifugation at 6,000 × g for 10 minutes at 4°C, washed twice, and resuspended in sterile saline to a final concentration of 10^9 CFU/mL. An aliquot of 50 uL of the bacterial suspension was added to 250 uL of DPPH working solution and incubated at room temperature in the dark on an orbital shaker for 30 minutes. After incubation, the mixture was centrifuged at 6,000 rpm for 5 minutes to separate the cells, and the supernatant was collected. The absorbance of the supernatant was then measured at 517 nm using a microplate reader (Bio-Tek Instruments, USA). The percentage inhibition of DPPH radical was calculated using the formula:

$$\text{DPPH inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 represents the absorbance of the control and A_1 represents the absorbance of the sample.

ABTS radical scavenging assay

The antioxidant capacity of bacterial isolates was evaluated using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay, with ascorbic acid (Sigma-Aldrich, USA) serving as a positive control. The assay was conducted according to the method described by Moreira (2019) with modifications for intact cells of bacterial cultures. Bacterial cells were harvested using the same method described in the previous section. An aliquot of 10 uL of the bacterial suspension was added to 190 uL of ABTS working solution and incubated at room temperature in the dark on an orbital shaker for 30 minutes. After incubation, the mixture was centrifuged at 6,000 rpm for 5 minutes to pellet the cells, and the supernatant was collected. The absorbance of the supernatant was then measured at 734 nm using a microplate reader (Bio-Tek Instruments, USA). The percentage inhibition of ABTS radical was calculated using the formula:

$$\text{ABTS inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 represents the absorbance of the control and A_1 represents the absorbance of the sample.

Probiotic characteristics evaluation

Tolerance to low pH condition

The survival capacity of bacterial isolates under simulated gastric conditions was evaluated following the protocol described by Talib et al. (2019) with modifications. Bacterial cells were harvested using

the same method described in the previous section. The pellets were washed twice with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in 1 mL of PBS (10^9 CFU/mL) adjusted to pH 2.0 and 3.0 using 1 M hydrochloric acid (HCl) (Systerm, Malaysia). The pH values (2.0 and 3.0) were chosen to reflect the range of gastric acidity in the human stomach. A control sample was prepared using PBS at pH 7. The bacterial suspensions were incubated at 37 °C for 3 hours. After incubation, serial ten-fold dilutions were prepared, and 100 µL aliquots were spread-plated using MRS agar plates in triplicate. The plates were incubated at 37 °C for 48 hours, and the number of viable colonies was counted. The survival rate was calculated using the equation:

Survival rate (%) = [Final (CFU/mL)/Initial (CFU/mL)] ×100%

Tolerance to bile salt condition

Bile salt tolerance was evaluated using the method described by Talib et al. (2019) with modifications. Bacterial cells were harvested using the same method described in the previous section and resuspended in 1 mL of PBS (10^9 CFU/mL) containing 0.3% (w/v) and 0.5% (w/v) bile salts (Oxgall, Sigma-Aldrich, USA). Bile salts at concentrations of 0.3% (w/v) and 0.5% (w/v) were applied to simulate the physiological bile environment of the small intestine. A control sample was prepared using PBS without bile salts. The bacterial suspensions were incubated at 37 °C for 3 hours. After incubation, serial ten-fold dilutions were prepared, and 100 µL aliquots were spread-plated using MRS agar plates in triplicate. The plates were incubated at 37 °C for 48 hours, and the viable colonies were counted. The survival rate was calculated using the same formula as described for acid tolerance.

Adhesion capacity to intestinal epithelial cells

The adhesion capability of bacterial isolates to intestinal epithelial cells was assessed using HT-29 human colorectal adenocarcinoma cells, following the protocol described by Talib et al. (2019) with modifications. A monolayer of HT-29 cells was cultured in six-well tissue culture plates at a density of 5×10^5 cells/mL in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C in 5% CO_2 atmosphere for 24 hours until reaching 90-100% confluence. Before the adhesion assay, the cell monolayer was washed twice with sterile PBS (pH 7.2-7.4). Bacterial cells were harvested using the same method described in the previous section and resuspended in antibiotic-free RPMI-1640 medium to achieve a concentration of 10^9 CFU/mL. One milliliter of bacterial suspension was added to each well containing the HT-29 cell monolayer, corresponding to an estimated bacteria-to-cell ratio (MOI) of 2000:1. The plates were then incubated at 37 °C in 5% CO_2 for 2 hours to allow bacterial adhesion. Following incubation, the monolayer was washed twice with sterile PBS to remove non-adherent bacteria. The adhesion ability was assessed by serial dilution and plating on MRS agar in triplicate to enumerate viable bacteria. Plates were incubated at 37 °C for 48 hours, and adherent bacteria were enumerated. The adhesion percentage was calculated using the formula:

Adhesion (%) = [Adhered bacteria (CFU/mL)/Total added bacteria (CFU/mL)] × 100%

Antibiotic susceptibility testing

Antibiotic susceptibility was determined using the Kirby-Bauer disc diffusion method (Wanger & Chávez, 2021). Five antimicrobial agents (Oxoid, Hampshire, UK) were tested: vancomycin (30 µg), gentamicin (10 µg), ampicillin (10 µg), tetracycline (30 µg), and penicillin (10 IU). MRS agar was used as LAB do not grow optimally on Mueller-Hinton agar, in accordance with previous LAB susceptibility studies. A 24-hour bacterial culture was adjusted to approximately 10^8 CFU/mL prior to being uniformly spread onto MRS agar plates, and the discs were aseptically placed on the inoculated agar surface with adequate spacing to prevent overlapping inhibition zones. The plates were then incubated at 37 °C for 48 hours. Following incubation, the diameter of inhibition zones was measured in millimetres using a ruler and compared to CLSI interpretive criteria (CLSI, 2023). Isolates were interpreted as susceptible, intermediate, or resistant based on established breakpoints. The absence of an inhibition zone indicated bacterial resistance to the tested antibiotic.

Lactic acid bacteria identification with 16S rRNA sequence analyses

Bacterial genomic DNA was extracted from pure cultures using the Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. A 24-hour bacterial culture of the selected isolate were centrifuged for 10 minutes at 5,000 × g. The obtained genomic DNA was quantified using a Nanodrop spectrophotometer (Implen, Germany). PCR amplification of the 16S rRNA gene was performed using universal bacterial primers. The primer sequences were as follows: forward primer 5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-TACCGCGGCTGCTGG CAC-3'. The expected amplicon size was

approximately 500 bp. PCR products were verified by agarose gel electrophoresis. The PCR reaction mixture (50 µL total volume) consisted of 25 µL of PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µM of each primer, 1 µg of genomic DNA template, and sterile water to achieve the final volume. PCR amplification was performed using a SelectCycler IISBT9600 thermal cycler (Select BioProducts, USA) with the following parameters: initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 1 minute. A final extension step was performed at 72 °C for 10 minutes, followed by cooling to 4 °C. PCR products were confirmed on 1.5% agarose gel electrophoresis and sent for Sanger sequencing. The resulting nucleotide sequences were analysed and compared against the National Center for Biotechnology Information (NCBI) database using BLAST search (rRNA/ITS databases: 16S rRNA sequences) to determine species identity based on the highest sequence similarity match (≥97%).

Statistical analysis

All experiments were performed in biological triplicates, and data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using IBM SPSS Statistics version 31.0 (IBM Corp., Armonk, NY, USA). Differences among groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Isolation and preliminary characterization of lactic acid bacteria (LAB) from water kefir grains

A total of 15 bacterial isolates were recovered from the Malaysian water kefir grains. The isolates were differentiated based on colony characteristics, including size, colour, form, margin, elevation, and texture. Following preliminary screening, eight isolates (Isolates 1, 2, 3, 4, 5, 6, 7, and 8) were identified as Gram-positive, catalase-negative bacilli and were considered lactic acid bacteria (LAB) candidates. As shown in Table 1, these isolates exhibited generally similar morphological features, with minor variations observed in colony size, margin, elevation, and texture. These phenotypic characteristics are consistent with lactic acid bacteria, supporting their selection for subsequent functional screening.

Antioxidant activity of selected isolates

The antioxidant potential of selected isolates was evaluated using DPPH and ABTS free radical scavenging assays. Variation in scavenging activity was observed across the isolates, as summarised in Table 2. Isolate 5 exhibited the highest ABTS inhibition value at 77.74 ± 3.27%, followed by isolate 2 at 72.70 ± 3.38%, both of which were lower than the positive control (L-ascorbic acid). In the DPPH assay, isolate 5 demonstrated the highest activity (48.07 ± 5.18%), while isolate 2 showed 41.62 ± 1.18%, both lower than the positive control (Trolox). Statistical analysis indicated that both isolates 2 and 5 showed significantly higher antioxidant activities than the remaining isolates ($p < 0.05$). These two isolates were therefore selected for further probiotic evaluation based on their higher antioxidant activity.

Table 1. Morphological characteristics of isolates that are Gram-positive and catalase-negative bacilli

Isolate	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
Size (mm)	1	2	1	<0.5	2	<0.5	1.5	1
Colour	Opaque white	Shiny white	Opaque white	White	White	White	White	White
Form	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Undulate	Entire	Entire	Entire
Elevation	Convex	Flat	Flat	Convex	Flat	Flat	Flat	Umbonate
Texture	Viscid	Viscid	Viscid	Viscid	Brittle	Viscid	Viscid	Viscid

Table 2. Antioxidant analysis of isolates against ABTS and DPPH assays

Sample	ABTS Decolorization%	DPPH Decolorization%
L-Ascorbic Acid (0.25 mg/mL)	86.26 ± 0.95	-
Trolox (0.25 mg/mL)	-	83.13 ± 0.84
1	45.52 ± 2.54[a]	38.39 ± 1.71[a]
2	72.70 ± 3.38[b]	41.62 ± 1.18[ab]
3	14.74 ± 3.75[d]	21.92 ± 5.88[c]
4	71.52 ± 3.36[b]	38.92 ± 1.49[a]
5	77.74 ± 3.27[b]	48.07 ± 5.18[b]
6	55.27 ±1.60[c]	36.62 ± 0.97[a]
7	50.81 ± 1.58[ac]	34.62 ± 0.77[a]
8	45.85 ± 2.49[a]	22.96 ± 1.48[c]

The data presented are representative as mean ± standard deviation (SD) (n=3). Significant differences the same column were determined using one-way ANOVA according to Tukey HSD multiple comparison test ($p < 0.05$) and are indicated by different letters.

Evaluation of probiotic characteristics

Gastrointestinal tolerance (acid and bile conditions)

Survival under simulated gastrointestinal conditions was assessed under acidic (pH 2.0 and pH 3.0) and bile salt (0.3% and 0.5% ox gall) conditions. As shown in Figure 1, isolate 2 exhibited significantly higher survival than isolate 5 under all tested conditions ($p < 0.05$). At pH 3.0, isolate 2 maintained a high survival rate (92.86 ± 1.31%), whereas isolate 5 showed lower survival (78.57 ± 5.90%). Under more acidic conditions (pH 2.0), survival rates declined markedly, with only isolate 2 remaining viable. Under bile salt exposure, isolate 2 demonstrated strong tolerance, maintaining survival rates above 93% at both 0.3% and 0.5% bile concentrations. In contrast, isolate 5 showed significantly lower survival (53.63 ± 1.65% and 50.3 ± 1.90%, respectively). These results indicate that isolate 2 possesses greater tolerance to simulated gastrointestinal conditions, suggesting enhanced potential for survival during transit.

Adhesion capacity to intestinal epithelial cells

In addition to survival under gastrointestinal conditions, adhesion to intestinal epithelial cells was assessed using HT-29 cells. Isolate 2 exhibited a high adhesion rate (88.8 ± 6.30%), whereas isolate 5 showed significantly lower adhesion (15.90 ± 7.50%) ($p < 0.05$), as shown in Figure 2. This difference highlights strain-dependent variation in epithelial adherence and suggests a greater potential for interaction with the intestinal epithelium by isolate 2.

Antibiotic susceptibility testing

Antibiotic susceptibility profiles of the selected isolates were determined using the disc diffusion method against five clinically relevant antibiotics, as summarised in Table 3. Both isolates showed no inhibition to vancomycin and gentamicin, indicating resistance to both antibiotics under the conditions tested. In comparison, both isolates showed measurable inhibition zones against ampicillin (22–28 mm), tetracycline (31–35 mm), and penicillin (35–44 mm), indicating susceptibility based on established interpretative criteria. Although minor variations in inhibition zone diameters were observed between isolates, both isolates demonstrated comparable susceptibility profiles for the antibiotics tested.

Identification of selected isolates by 16S rRNA gene sequencing analysis

Species identification was confirmed through sequencing of the 16S rRNA gene. BLAST analysis of consensus sequences revealed ≥99% identity to *Lacticaseibacillus paracasei* reference sequences in the GenBank database, with E-values of 0.0 for both isolates, as shown in Table 4. Despite being classified as *L. paracasei* at the species level, the isolates corresponded to different strain entries in the database, suggesting the presence of intraspecific genetic diversity.

DISCUSSION

This study isolated 15 lactic acid bacteria (LAB) from Malaysian water kefir grains, with two selected isolates demonstrating antioxidant activity and probiotic-related characteristics. Preliminary identification confirmed that the isolates were Gram-positive, catalase-negative bacilli, consistent with typical lactic acid bacteria characteristics. Species-level identification using 16S rRNA sequencing assigned both isolates to *Lacticaseibacillus paracasei*, although they corresponded to different strain entries in the GenBank database, suggesting possible intraspecific variation.

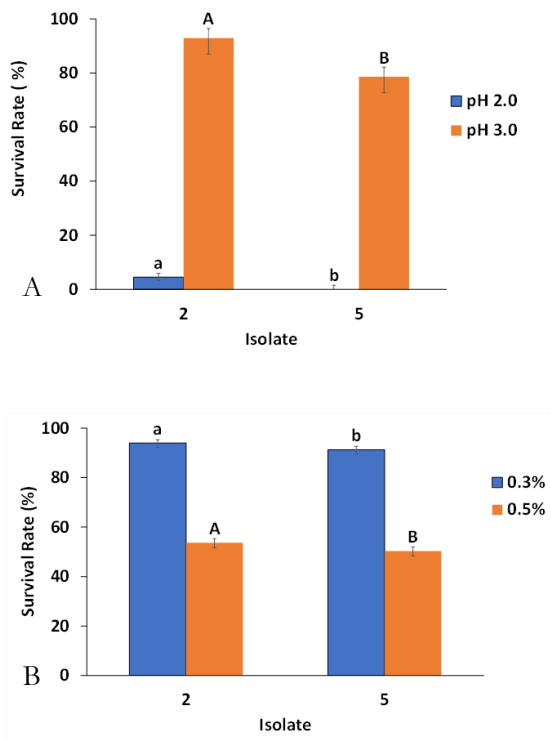

Figure 1. The survival rate of isolated *Lactobacillus* spp. from water kefir grain samples after 3 hours. A. Survival under acidic conditions (pH 2.0 and pH 3.0). B. Survival in 0.3% and 0.5% bile salt conditions. All data are expressed as mean ± standard deviation (SD, n=3). Statistical significance was determined using one-way ANOVA with Tukey's HSD multiple comparison test ($p < 0.05$). a–b Different lowercase letters in superscript on the bar graph indicate significant differences ($p < 0.05$) for pH 2 and 0.3% bile salt. A–B Different uppercase letter in superscript on the bar graph indicates a significant difference ($p < 0.05$) for pH 3 and 0.5% bile salt.

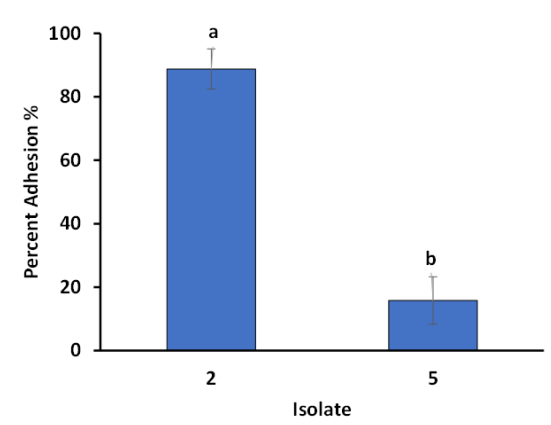

Figure 2. Percentage of adhered isolated *Lactobacillus* spp. from water kefir grain samples to the HT-29 cell line. Data are expressed as mean ± standard deviation (SD, n=3). Statistical significance was determined using one-way ANOVA with Tukey's HSD multiple comparison test ($p < 0.05$). Different superscript letters (a, b) indicate significant differences.

Table 3. Antibiotic susceptibility results against selected *Lactobacillus* sp. isolates. The susceptibility profiles of the selected isolates were determined against a panel of antibiotics to assess their resistance and sensitivity patterns.

Isolate	Zone of Inhibition (mm)				
	VA	CN	AMP	TE	P
2	-	-	23.0 ± 2.6	35.0 ± 1.7	37.0 ± 1.7
5	-	-	28.0 ± 2.0	33.33 ± 1.2	44.0 ± 4.0

Note: Vancomycin (VA), gentamicin (CN), ampicillin (AMP), tetracycline (TE) and penicillin (P). Values are expressed as mean ± SD of triplicates. Resistant (–); moderately susceptible (10–20 mm), susceptible (21–30 mm), and very susceptible (>31 mm).

Table 4. Identification of selected isolates from Malaysian water kefir grains by Sanger sequencing analysis of the 16S rRNA gene using the GenBank database.

Isolates	NCBI Accession number	Identification from Sanger 16S rRNA gene sequencing	Query coverage	E-value	Identity (%) with GenBank database using BLASTn
2	NR_025880.1	*Lacticaseibacillus paracasei* strain R094	99%	0.0	100%
5	NR_025880.1	*Lacticaseibacillus paracasei* subsp. tolerans strain NBRC 15906	96%	0.0	99%

The occurrence of *Lacticaseibacillus spp*. in water kefir grains has been widely reported across different geographical regions, including Mexico, Malaysia, and Belgium (Romero-Luna et al., 2020; Talib et al., 2019; Verce et al., 2019). However, the microbial composition of water kefir grains may vary depending on geographical origin and fermentation substrate (Arrieta-Echeverri et al., 2023; Zannini et al., 2022). Environmental conditions and processing factors have also been reported to influence microbial diversity (Abdi et al., 2025; Gamba et al., 2019). Despite minor differences in colony characteristics such as size and elevation, morphological features alone were insufficient to distinguish isolates reliably. This limitation is consistent with previous findings that phenotypic traits provide only preliminary identification and require confirmation through molecular approaches. Such variability is not unexpected, given the known diversity of microbial populations in water kefir systems (Garmendia et al., 2025).

The antioxidant activities observed in the present study are consistent with recent reports on LAB isolated from fermented foods. *Lactobacillus* strains have been reported to exhibit ABTS -radical scavenging activities of approximately 40–90% and DPPH-scavenging activities of 30–80%, depending on strain characteristics and assay conditions (Qayyum et al., 2024; Ghiasi et al., 2023; Liu et al., 2022). In particular, *Lactobacillus* species isolated from fermented vegetables and dairy products have been reported to demonstrate moderate to high DPPH scavenging activity, typically within the range of 30–60% for intact cells (Ghiasi et al., 2023; Liu et al., 2022). In comparison, several isolates in the present study, particularly Isolates 2 and 5, showed comparable or higher antioxidant activity, indicating relatively strong antioxidant potential. The observed variability among isolates further supports the strain-dependent nature of antioxidant activity, consistent with the principle that probiotic functional properties must be evaluated at the individual strain level (Binda et al., 2020). Based on their consistently higher and statistically significant antioxidant activities across both ABTS and DPPH assays, Isolates 2 and 5 were selected for further probiotic characterisation.

Survival under acidic and bile conditions is essential for probiotics to function effectively in the gastrointestinal tract. In this study, between the two selected isolates, isolate 2 demonstrated strong acid resistance, maintaining 92.86 ± 1.31% viability at pH 3.0 and remaining the only strain to survive at pH 2.0, whereas Isolate 5 showed lower survival of 78.57 ± 5.90%. Acid survival at pH 3.0 is commonly used as a benchmark for probiotic screening, as it approximates gastric conditions. The performance of isolate 2 falls within the upper range reported for *Lactobacillus* species, supporting its potential as a probiotic candidate. Differences in acid tolerance among isolates originating from the same water kefir grain source are consistent with the strain-specific nature of probiotic functional properties described earlier in this study, with similar variability reported among *Lactobacillus spp.* (Zommara et al., 2023). This difference may be attributed to strain-specific adaptive mechanisms, such as variations in membrane integrity, bile salt hydrolase activity, or stress response systems that enhance survival under gastrointestinal conditions.

Bile tolerance results also favoured isolate 2, which retained high survival at both 0.3% and 0.5% bile concentrations. These findings are comparable to those reported for several *L. paracasei* strains, such as *L. paracasei* L2 and *L. paracasei* subsp. paracasei LBC 81, which demonstrates good survival under similar bile conditions (M'hamed et al., 2022; Vogado et al., 2020). The high survival observed for isolate 2 indicates tolerance to bile-induced stress, whereas the lower survival of isolate 5 suggests reduced tolerance under these conditions.

Adhesion to intestinal epithelial cells and antibiotic susceptibility profiles are key characteristics for probiotic evaluation. Adhesion to the intestinal epithelium supports interaction with the host, protection against pathogens, and modulation of host responses. Isolate 2 showed strong adhesion to HT-29 cells (88.8 ± 6.3%), while isolate 5 showed much lower adherence (15.8%± 7.5%). Comparable

adhesion has been reported for *L. paracasei* strains MG4272 and MG4577, as well as other LAB such as *L. fermentum*, which demonstrate high adhesion in HT-29 cell models (Suebwongsa et al., 2024; Lee et al., 2023).

Variability in adhesion capacity among these isolates highlights the strain-dependent nature of probiotic functional properties, even among isolates originating from the same water kefir grain source. Probiotic activity does not depend on long-term colonisation of the intestinal tract, as short-term adhesion or close association with the epithelial surface may be sufficient to allow bacterial components or metabolites to interact with the host (Gorreja & Walker, 2022). Taken together, the strong adhesion observed for isolate 2, together with its tolerance to acidic and bile conditions, supports its further probiotic characterisation, whereas the limited adhesion observed for isolate 5 may reduce its potential to establish effective host interactions.

The antibiotic susceptibility profiles of the isolates are consistent with previous reports for *L. paracasei* strains considered as probiotic candidates, which are generally susceptible to β-lactam antibiotics such as ampicillin and penicillin, as well as tetracycline (Lee et al., 2023; Anisimova et al., 2022). Resistance to vancomycin among lactobacilli is well documented and regarded as intrinsic, arising from the absence of the D-Ala-D-Ala binding target in the cell wall (Seifabadi & Baserisalehi, 2021). While susceptibility to gentamicin has been reported in some *Lactobacillus* species, resistance has also been observed in others, highlighting variability in antibiotic susceptibility among lactobacilli (Dang et al., 2023; Singhal et al., 2020). This variability reflects strain-dependent differences and may be influenced by ecological origin and genetic background. However, antibiotic susceptibility was assessed using the disc diffusion method, which provides only a preliminary indication of resistance profiles (Hossain, 2024). Determination of minimum inhibitory concentrations (MIC) would provide a more precise evaluation of antibiotic susceptibility and is recommended for future studies (Gajic et al., 2022). In addition, no genotypic analysis was performed to detect antibiotic resistance determinants, which limits the ability to distinguish between intrinsic and acquired resistance mechanisms, an important consideration in probiotic safety evaluation (Anisimova et al., 2022).The differences in taxonomic resolution among the isolates reflect the known limitations of 16S rRNA gene sequencing in distinguishing closely related taxa within the *Lacticaseibacillus paracasei–casei* complex (Laref & Belkheir, 2022; Torres-Miranda et al., 2022). Species in this group share highly similar 16S rRNA gene sequences, often exceeding 97% sequence identity, which limits the ability of this marker to reliably distinguish closely related species and strains (Olivier et al., 2023). Although both isolates showed high similarity to *Lacticaseibacillus paracasei* reference sequences in the GenBank database, they corresponded to different strain entries, indicating possible intraspecific variation.

Despite their close phylogenetic relatedness, the isolates showed clear differences in antioxidant activity and probiotic-associated functional characteristics, as observed in this study. This finding is consistent with previous reports indicating that probiotic functional properties are highly strain-dependent and cannot be inferred solely from species-level identification (Kelidkazeran et al., 2025; Bengoa et al., 2021). Variability in metabolic activity, stress tolerance, adhesion capacity, and bioactive compound production has been widely documented among strains belonging to the same *Lacticaseibacillus* species.

Overall, these results indicate that 16S rRNA gene sequencing provides a widely used approach for initial taxonomic assignment. However, it is insufficient by itself to predict functional performance among closely related isolates. The combination of molecular identification with phenotypic and functional characterisation is therefore essential when evaluating and selecting potential probiotic candidates for further application (Kelidkazeran et al., 2025; Shah et al., 2024).

The present study focused on in vitro evaluations, providing a foundational understanding of the antioxidant and probiotic-associated properties of the selected isolate. While these results may not fully capture performance under physiological conditions, they offer a valuable guide for designing subsequent in vivo studies to further validate their functional potential (Rudzka et al., 2025; Anjum et al., 2022).

CONCLUSION

In conclusion, this study isolated lactic acid bacteria from Malaysian water kefir grains and evaluated their antioxidant activity and probiotic potential. Among the recovered isolates, functional variability was observed, with isolates 2 and 5 exhibiting strong radical scavenging activity in both ABTS and DPPH assays. Probiotic characterisation further demonstrated that isolate 2 possessed higher tolerance to acidic and bile conditions, as well as stronger adhesion to intestinal epithelial cells, than isolate 5. These findings highlight the strain-dependent nature of antioxidants and probiotic-associated properties among water kefir-derived

isolates. Collectively, isolate 2 emerged as the most promising candidate for further investigation as a functional probiotic strain derived from Malaysian water kefir. Further studies, including molecular characterisation beyond 16S rRNA sequencing and in vivo validation, are required to confirm its functional potential and safety profile.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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