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Identification, safety evaluation and probiotic potential of lactic acid bacteria isolated from wheat

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ABSTRACT

Lactic acid bacteria (LAB) are vital to the food and health industries, yet their diversity and probiotic potential on wheat surfaces remain underexplored. In this study, 199 Gram-positive and Catalase-negative bacterial strains were isolated and characterized from wheat grains and spikes, of which 45 were selected using RAPD-PCR and identified by 16S ribosomal DNA sequencing. The phylogenetic analysis of the 16S ribosomal DNA sequences grouped the strains into eight species: *Enterococcus faecium*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus mundtii*, *Enterococcus durans*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, and *Loigolactobacillus coryniformis*. The majority of *Enterococcus* strains exhibited hemolytic activity and were therefore excluded from further analysis. The remaining 17 strains, most of them belonging to *Lactobacillus* and *Pediococcus* genera, were sensitive to most antibiotics commonly used in clinical practice and were not able to degrade mucin. Principal component analyses (PCA) and heat map visualization identified *L. plantarum* (WB1.2) and *P. acidilactici* (WR6) as the most promising probiotic candidates due to their strong surface hydrophobicity, auto-aggregation capacity, and tolerance to simulated gastrointestinal conditions. Although further studies are needed, the results indicate that these strains possess probiotic and safety-related characteristics, positioning it as a promising candidate for use as starter cultures in food fermentations.

1. Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, facultative anaerobic microorganisms widely used in food fermentation and valued for their health-promoting effects, such as immune modulation and digestive support (Markowiak and Śliżewska, 2017). With the growing demand for natural ingredients in functional food, the exploration of novel LAB strains from underexplored ecological niches has gained significant attention. Plant surfaces, such as cereals and grains, harbor diverse microbial communities that adapt to specific environmental conditions, offering a potential reservoir for LAB with unique metabolic traits (Cichońska and Ziarno, 2022). Among these, wheat (*Triticum aestivum L.*), one of the most globally cultivated crops (Aung and Kim, 2023; Patra et al., 2023), may host LAB populations that remain under-characterized, despite their potential value for agricultural and food biotechnology applications.

The FAO/WHO definition of a probiotic as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Cordaillat-Simmons et al., 2020; Hill et al., 2014; Morelli and Capurso, 2012). Recent studies have highlighted the feasibility of isolating LAB from plant materials, such as fermented vegetables, fruits, and cereals (Gebre et al., 2023; Zheng et al., 2020). Many LAB strains isolated from various fermented products, including dairy, plant-based and meat products, have been shown to have health effects and probiotic potential, such as modulation of gut microbiota, enhancement of immune response, cholesterol-lowering effects, and antioxidative activities (Cristofori et al., 2021; Shori et al., 2022; Tian et al., 2022). However, limited research has focused on non-fermented, raw wheat grains and spikes as LAB sources. The microbial communities on wheat surfaces are influenced by environmental factors, agricultural practices, and plant physiology, potentially fostering LAB strains with stress tolerance or bioactive properties (Mukherjee et al., 2024). Traditional isolation methods, such as agar plating with selective media, remain effective for obtaining LAB colonies, but subsequent genetic

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characterization and safety assessments are critical to ensure suitability for human use (Yilmaz et al., 2025).

The isolation and identification of LAB are important aspects of microbiological research, particularly in the fields of food fermentation and probiotic development. In this study, we aimed to isolate, identify, and evaluate LAB strains from wheat grains and spikes. Wheat isolates were first characterized through morphological and Gram-staining analyses, and representative strains, selected by previous Random Amplified Polymorphic DNA (RAPD) characterization, were further subjected to 16S ribosomal DNA gene sequencing for species identification by phylogenetic analysis. The identified LAB isolates were assessed for safety through hemolytic activity, antibiotic susceptibility, and mucin degradation, as well as for probiotic potential based on their survival in simulated gastrointestinal conditions and adherence to intestinal cells. These evaluations aim to expand the understanding of wheat-associated LAB and support the selection of safe, functional strains for probiotic applications.

2. Material and methods

2.1. Raw materials, isolation, and phenotypic characterization

Sixteen wheat samples (*Triticum aestivum*, cultivar Tocayo), including nine from grains and seven from spikes, were collected from Castilla La Mancha and Castilla León regions in Spain. The isolation of LAB was carried out after the enrichment procedure (Fig. 1). Wheat samples (10 g) were enriched by adding 40 mL of modified de Man, Rogosa and Sharpe (mMRS) broth (Condalab, Pronadisa, Spain), containing 1 % lactose (Sigma-Aldrich, USA), 1 % maltose (Sigma-Aldrich, USA), 10 % yeast extract (Condalab, Pronadisa, Spain), and 0.1 g/L cycloheximide (Thermo Scientific, USA), and incubated at 37 °C for 4 d. Serial dilutions (10^{-3}–10^{-6}) were prepared using sterile peptone water 0.1 %, *w/v* (Oxoid, UK). Diluted samples were plated on Rogosa Agar Medium (MRS Agar, Condalab, Pronadisa, Spain). A total of 320 LAB colonies, 20 from each sample, were randomly selected as purified strains on MRS agar media. Gram-positive (by Gregersen's KOH methods) (Gregersen, 1978), and catalase-negative (determined by transferring fresh colonies from an agar medium to a glass slide and adding 5 % H_2O_2) colonies, were used to phenotypically characterize isolates. The purity was checked microscopically, and the cultures were maintained at −80 °C in 25 % glycerol stocks.

2.2. Genotypic characterization, identification and sequence analysis

The method of DNA extraction from pure cultures by (Jeyaram et al., 2010) was used with some modifications. The extraction of genomic DNA from the LAB was carried out from 1 mL of the bacterial cultures after growth in MRS liquid media for 16 h at 37 °C. The cells were centrifuged at 10,000 rpm for 2 min (Hettich, Tuttlingen, Germany). Subsequently, the remaining bacterial pellet was washed twice with 1 mL of sterile 0.5 M NaCl and centrifuged at 10,000 rpm for 2 min. To produce cell lysis, the pellet was resuspended in a final concentration of 200 µL of TE-Buffer (10 mM Tris-Cl; 1 mM EDTA, pH 8.0.) and 2 µL of lysozyme (2 mg/mL). The samples were incubated at 37 °C for 30 min, then heated at 95 °C for 20 min and centrifuged at 10,000 rpm for 10 min. The supernatant was carefully pipetted out in a fresh tube for PCR analysis and stored at −20 °C until required.

DNA extracted from Gram-positive and catalase-negative isolates was used for RAPD-PCR assays. RAPD-PCR reactions were performed using the primers MCV (5'-AGTCAGCCAC-3′), PER1 (5'-AAGAGCCCGT-3′) and CORR1 (5'-TGCTCTGCCC-3′). Each reaction mixture (25 µL) contained 2.5 µL of 10× Buffer, 1.75 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL of 10 mM primer, 0.2 µL of 5 U/mL Taq DNA polymerase (Meridian Bioscience, Cincinnati, USA), 5 µL of DNA extraction supernatant and 13.55 µL of nuclease-free water to reach the final reaction volume. Amplifications were performed using a MultiGene thermal cycler (Labnet, Edison, USA) and the cycling program was started with an initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 30 °C for 1 min and extension at 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 10 min and the amplified product was stored at 4 °C. Amplification products were electrophoresed on 1.5 % (*w/v*) D-1 low electroosmotic agarose gels (Condalab, Pronadisa, Spain) and 10xNH4 buffer (Bioline, London, UK), stained with GelRed (EMD Millipore Corporation, Temecula, USA) and visualized under UV light. GeneRuler 100 bp Plus DNA Ladder (M-Medical S.r.l., Milan, Italy) and λ-DNA/Eco47I (*Ava*II) (Fermentas, Waltham, USA) were used as molecular weight markers.

2.3. Molecular identification and sequence analysis

RAPD-PCR profiles were obtained by agarose gel electrophoresis, and banding patterns were analyzed using GelJ software version 2.0

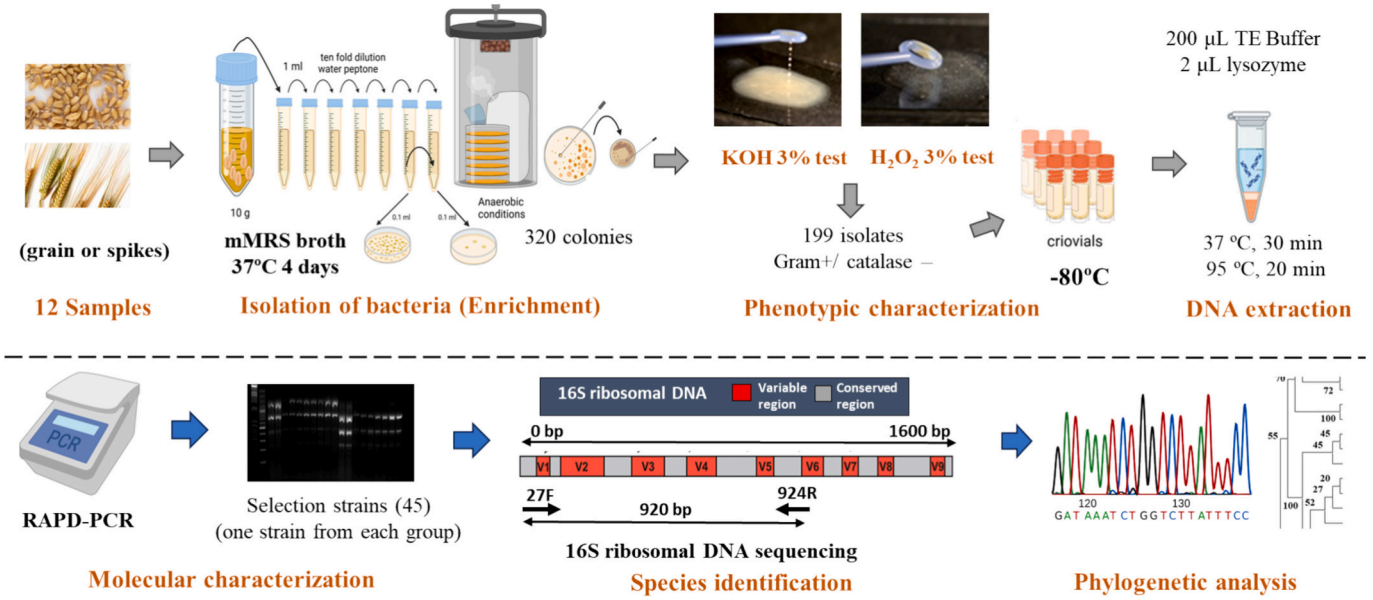

Fig. 1. Schema of isolation and identification of lactic acid bacteria (LAB).

(University of La Rioja, Spain). Those exhibiting identical or highly similar profiles were grouped into the same molecular pattern. From each RAPD-PCR pattern, one representative strain was selected for species identification. These strains were then subjected to 16S ribosomal DNA sequencing using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') (Weisburg et al., 1991) and 924R (5'-CTT GTG CGG GCC CCC GTC AATTC-3') (Rudi et al., 1997). The reaction mixture (25 μL) contained 2.5 μL of 10× Buffer (containing 15 mM $MgCl_2$), 0.5 μL of 10 mM dNTP, 0.5 μL of both 10 mM primers, 0.2 μL of 5 U/mL Taq DNA polymerase (Meridian Bioscience, Cincinnati, USA), 5 μL of DNA and 15.8 μL of nuclease-free water. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 10 min and the amplified product was stored at 4 °C.

The PCR products were confirmed by agarose gel (1.5 %, *w/v*) electrophoresis, from the visualized DNA fragments, amplicons of approximately 920 bp were purified using the ExoSAP-IT Express PCR Product Cleanup rapid purification kit (Thermo Fisher Scientific, Vilnius, Lithuania), then sequenced using BigDye Terminator v3.3 Cycle Sequencing Kit (Thermo Fisher Scientific) and primers 27F and 924R. PCR products were sequenced on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, USA) in the Central Service for Experimental Research (SCSIE) of the University of Valencia.

To identify the bacterial isolates, the obtained 16S rDNA sequences were compared with reference sequences retrieved from the EMBL (European Molecular Biology Laboratory) sequence database. The reference strains used for alignment included *Enterococcus faecium* (LC035119.1, MN969372, OQ255845.1, KJ803879.1, OR544987.1), *Enterococcus durans* (MH111468.1), *Enterococcus mundtii* (MZ869176.1), *Lactobacillus acidophilus* (HM162411.1), *Lactobacillus delbrueckii* (OP108445.1), *Loigolactobacillus coryniformis* (MT597663.1), *Lacticaseibacillus casei* (KX057529.1), *Limosilactobacillus reuteri* (L23507.1), *Lactiplantibacillus plantarum* (MG383779.1), *Levilactobacillus brevis* (AB969780.1), *Pediococcus pentosaceus* (KY022733.1), *Pediococcus acidilactici* (LC274608.1), *Streptococcus thermophilus* (OR363387.1), *Escherichia coli* (AF511430.1), which were used to construct phylogenetic trees and determine the taxonomic identity of the isolates.

2.4. Phylogenetic analysis

To assign species identity, the obtained 16S ribosomal RNA sequences were first compared with reference sequences using the Basic Local Alignment Search Tool (BLASTn) from the National Center for Biotechnology Information (NCBI) database. Sequences showing ≥99 % similarity to known strains were considered preliminary species-level identifications. Sequences were aligned using the program MEGA X version 4.1 (Kumar et al., 2018). The genetic distances were calculated using the Jukes-Cantor model and the phylogenetic inference was obtained by the neighbor-joining (NJ) method (Saitou and Nei, 1987). The NJ tree and the statistical confidence of a particular group of sequences in the tree, evaluated by bootstrap test (1000 pseudoreplicates) (Hillis and Bull, 1993), were also performed using the MEGA X version 4.1 software (Kumar et al., 2018).

2.5. In vitro safety evaluation of LAB isolates

2.5.1. Hemolytic activity

Hemolytic activity evaluation was performed with the protocol developed by (Cui et al., 2018). The LAB isolates were cultured overnight in MRS broth at 37 °C before use. Then, 20 μL of each culture was inoculated on Columbia agar plates, containing 5 % (*w/v*) sheep blood (PA0004, Industrial Supplies Laboratory, SL, Barcelona, Spain) and incubated at 37 °C for 48 h. The appearance and color of the halos are indicative of the type of hemolytic activity: (i) a clear zone indicated β-hemolysis; (ii) a greenish zone indicated α-hemolysis; and (iii) the absence of a zone indicated γ-hemolysis. (Diana et al., 2015; Touret et al., 2018). Reference strains were purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). *Escherichia coli* CECT 5947 and *Lacticaseibacillus rhamnosus* CECT 288 were used as the positive and negative control, respectively. *E. coli* was firstly cultivated on Luria broth (LB) medium (Pronadisa, Spain). *L. rhamnosus* were cultivated under the same conditions described above.

2.5.2. Antibiotic susceptibility

The antibiotic susceptibility was determined using standard disk-diffusion assays (Industrial Supplies Laboratory, SL, Barcelona, Spain). Overnight cultures in MRS were adjusted to an OD_{600} of 1.0. Autoclaved MRS agar medium was cooled to approximately 45 °C to keep it in a liquid state. The standardized bacterial suspension was added to the molten agar at a ratio of 1:100 (*v*/v), mixed thoroughly, and poured into sterile Petri dishes. The assay involved placing disks containing various antibiotics, such as chloramphenicol (30 μg/disc), gentamicin (10 μg/disc), clindamycin (2 μg/disc), erythromycin (15 μg/disc), vancomycin (30 μg/disc), streptomycin (10 μg/disc), penicillin (10 IU/disc), kanamycin (30 μg/disc), tetracycline (30 μg/disc), and ampicillin (10 μg/disc), onto the semi-solid agar surface before complete solidification. These antibiotics were selected based on their clinical and veterinary relevance, as well as their inclusion in safety assessments recommended by EFSA and other regulatory bodies for evaluating probiotic strains. After incubation at 37 °C for 48 h, the appearance of a clear inhibition zone around the discs was considered an indicator of susceptibility or sensitivity to the tested antibiotic (Gebre et al., 2023). Antibiotic susceptibility was interpreted according to the guidelines of the (Clinical and Laboratory Standards Institute (CLSI) M100, 2025), M100, 35th Edition and (The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2025), Breakpoint Tables for Bacteria, Version 15.0.

2.5.3. Mucin degradation

Mucin degradation assays were performed following a previously reported method (Kim et al., 2018) with minor modifications. LAB isolated strains were cultured in MRS broth w/o glucose (Liofilchem, Italy) supplemented with 0.5 % (*w/v*) mucin from the porcine stomach (Palex Medical, S.A., Barcelona, Spain), 1 % (w/v) mucin, 0.5 % (w/v) glucose and 1 % (w/v) glucose, strains were also cultured the unsupplemented MRS broth as control. After the inoculation, all samples were incubated in 15 mL centrifuge tubes under static conditions at 37 °C for a total of 48 h. The bacterial growth was assessed by measuring absorbance at 550 nm at 0, 5, 9, 24, 29, and 33 h using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany). The initial optical density value of the media was subtracted from the final value for each test sample. To further compare the growth differences among strains, the Exponential plateau model was fitted (eq. 1) with the fit $R^2 > 0.9$, the maximum OD_{550} and growth rate were obtained.

$$Y = Y_{max} - (Y_{max} - Y_0)^{-kx} \quad (1)$$

Where Y_{max} is the maximum OD_{550}, Y_0 is starting OD_{550}, k is rate constant, x is time, and Y is OD_{550}.

2.6. Potential probiotic characterization of LAB isolates

2.6.1. Resistance to simulated gastro-intestinal conditions

The tolerance of the strain to simulated gastrointestinal conditions was tested as described by (Zommara et al., 2023). LAB were cultured overnight in MRS broth until the OD_{600} reached 1.0, corresponding to approximately 10^8 CFU/mL. The cells were harvested by centrifugation at 10,000 ×*g* for 5 min at 4 °C and washed twice with sterile saline solution (0.9 % NaCl, *w/v*, pH 7.0). The resulting cell pellets were resuspended in artificial gastric fluid containing 0.9 % (*w/v*) NaCl and 3 mg/mL pepsin (Sigma-Aldrich, USA), adjusted to pH 3.0, and incubated at 37 °C for 3 h to simulate gastric conditions. After gastric treatment,

the LAB cells were centrifuged again and resuspended in artificial intestinal fluid, composed of 0.9 % (w/v) NaCl, 0.1 % (w/v) bile salts (Oxoid, UK), and 3 mg/mL pancreatin (Sigma-Aldrich, USA), with pH adjusted to 8.0, then incubated at 37 °C for an additional 4 h to simulate intestinal conditions. Surviving cells at 0 and 3 h of gastric condition, and at 4 h of intestinal condition were counted by plate counting using MRS agar medium. These times corresponded to the time the food remained in the stomach and small intestine, respectively. Survival was calculated using the formula (2).

$$\text{Survival rate}\,(\%) = \frac{\text{cell number}\,(\log\text{CFU}\bullet\text{mL}^{-1})\,\text{survived}}{\text{cell number}\,(\log\text{CFU}\bullet\text{mL}^{-1})\,\text{of initial inoculated}} \times 100 \quad (2)$$

2.6.2. Cell surface hydrophobicity

The hydrophobicity of LAB strains was determined by Chloroform extraction according to (Vlková et al., 2008) with minor modifications. Briefly, LAB was grown in MRS broth at 37 °C for 18 h, then overnight cultures were harvested by centrifugation at 6000 *g* for 15 min, washed twice in PBS, resuspended in 5 mL of the same buffer and the absorbance (OD_{600} nm) of the cell suspension was measured. Cell suspensions (3 mL) were mixed with 1 mL of chloroform, the solution was shaken for 2 min. It was left undisturbed for 30 min at 37 °C to ensure the complete separation of the two phases, then the top (water) phase was carefully collected, and the optical density was measured at 600 nm and recorded. Finally, the percentage of hydrophobicity was calculated using eq. (3).

$$\textit{Cell Surface Hydrophobicity}\,(\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (3)$$

Where A_t represents the absorbance of the aqueous phase at 600 nm wavelength after treatment with chloroform, and A_0 represents the absorbance at 600 nm before treatment with chloroform.

2.6.3. Cell auto-aggregation

Auto-aggregation capabilities were performed by the method of (Ben Taheur et al., 2016). LAB isolates were grown overnight at 37 °C in MRS-broth media. Then LAB culture was harvested by centrifugation at 6000 *g* for 10 min. Then, pellets were washed twice with PBS, re-suspended in the same buffer, and adjusted OD_{600} to 0.8 (A_0). The cell suspensions (4 mL) were vortexed for 10 s. After 5 h of incubation at 37 °C, the surface bacterial solution was taken and the absorbance at 600 nm was measured using a microplate reader. The auto-aggregation percentage was expressed as eq. (4).

$$\textit{Auto}-\textit{aggregation}\,(\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (4)$$

where A_t represents 5 h, the absorbance reading at 600 nm, and A_0 represents the absorbance at 0 h.

2.7. Statistical analysis

The data were represented as the mean ± standard deviation ($n = 3$), and the error bars in the figures represented the standard error. Data analysis and mapping were performed using GraphPad Prism 8 (Software, San Diego, USA) Software. Statistical analysis was carried out using ANOVA followed by post hoc Duncan's test by IBM SPSS Statistics19 (SPSS Inc., Chicago, Ill., U.S.A.). Lowercase letters within a specific concentration indicate significant differences ($p < 0.05$). Principal Component Analysis (PCA) was conducted using the scikit-learn library in Python. Before analysis, the data were standardized by z-score normalization (mean = 0, standard deviation = 1) to eliminate the influence of variable scale differences. Principal components with eigenvalues greater than 1 were retained, and their explained variance was assessed.

3. Results and discussion

3.1. Isolation, identification, and LAB distribution

This study aimed at characterizing LAB strains isolated from *T. aestivum* wheat samples collected in two important cereal-producing regions of Spain (Castilla-León and Castilla la Mancha). A total of 320 pure bacterial colonies were randomly obtained from the 16 wheat samples (20 colonies from each sample). Among them, 85 colonies were excluded as Gram-negative and 36 as catalase-positive, resulting in 199 isolates that were both Gram-positive and catalase-negative, which were selected as presumptive LAB strains for further analysis. They were subjected to RAPD-PCR analysis to assess their genetic diversity and to facilitate strain-level differentiation. Isolates exhibiting identical or highly similar RAPD patterns were clustered as the same strain, resulting in the identification of 45 unique LAB strains. Previous studies have shown that RAPD-PCR analysis is an appropriate molecular tool to differentiate LAB at the strain level (Albesharat et al., 2011; Jarocki et al., 2016).

Forty-five representative isolates, selected to reflect the genetic variability revealed by RAPD-PCR, were identified at the species level through phylogenetic analysis based on partial 16S rDNA gene sequences (Fig. 2, **Table 1**). The neighbor-joining tree (Fig. 2) also included seventeen reference sequences of LAB species retrieved from the EMBL database for comparative purposes. All 45 representative strains clustered into five distinct groups (Clusters I—V), each strongly supported by bootstrap values of 100 %, indicating high phylogenetic confidence. Cluster I included all *Enterococcus* strains, which could be subdivided into two groups. Group 1 (93 % bootstrap value) included a total of 24 strains identified as *Enterococcus faecium* (WB2.1, WB2.5, WB2.8, WB2.12, WB2.13, WB2.18, WB2.19, WG10, WG40, WD3, WTR26, W34, WTR4, WTR11, WB2.14, WB2.17, WB2.3, WB2.7, WTR18, WTR25, WTR19, WTR6 WD17 and WD1), and strains of *Enterococcus durans* (WTR20 and W28). For this group, there were two things to highlight. Firstly, the genetic divergences among isolates of *E. faecium* and *E. durans* were too low to confidently separate them into different species. In fact, in some sources, *E. durans* and *E. faecium* have been reported as the same species (Fox et al., 2017). Conversely, the phylogenetic tree revealed a high level of genetic divergence on the 16S sequences of *E. faecium* strains, which agrees with the different RAPD profiles shown by each one of the 24 *E. faecium* strains included in the sequence analysis. A high genetic diversity has already been reported by this frequently detected *Enterococcus* species in food samples (Daza Prieto et al., 2024). Group 2 (100 % bootstrap value) included strains identified as *E. mundtii* (WTS14, WTS15, WTS19 and WTS11).

Cluster II contained only strain WD13, which was identified as *Loigolactobacillus coryniformis*. Similarly, cluster III included only strain WG33, identified as *Lacticaseibacillus casei*. Cluster IV included two strains WB1.2 and WB3.1, identified as *Lactiplantibacillus plantarum*. Finally, cluster V included *Pediococcus* strains, which can be subdivided into two groups with high bootstrap values. The first (100 % bootstrap value) includes four strains (W17, WB1.5, WB1.4, and W9) identified as *Pediococcus pentosaceus*. The second group (95 % bootstrap value) included isolates identified as *Pediococcus acidilactici* (WC16, WR1, WR13, WR2, WR20, WTS17 and WR16). Genetic similarity between *P. pentosaceus* and *P. acidilactici* indicated their closer evolutionary relationship. Overall, sequence analysis enabled the identification of 8 different species (**Table 1**): *E. faecium* (24 strains), *P. acidilactici* (7 strains), *P. pentosaceus* (4 strains), *E. mundtii* (4 strains), *E. durans* (2 strains), *L. plantarum* (2 strains), *L. casei* (1 strain), *L. coryniformis* (1 strain).

Table 1 also provided the total number of strains isolated and phenotypically identified as LAB (199) for each species considering that identical RAPD patterns should be considered as the same strain, while **Fig. 3** summarized the percentages of the total genera and species isolated. The predominant bacteria belonged to the genus *Enterococcus*

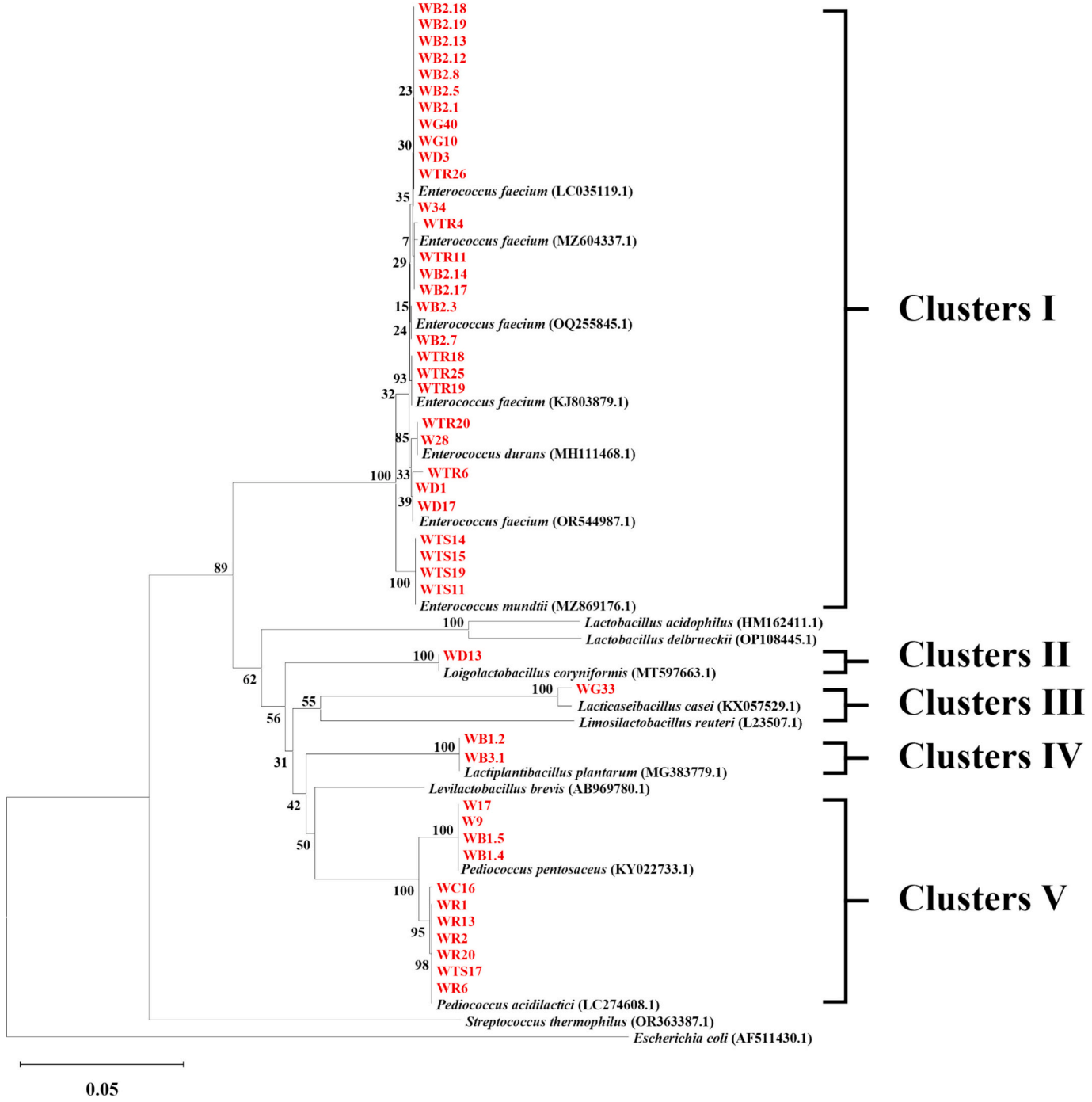

Fig. 2. Phylogenetic relationships of representative LAB isolates from the combined sequences of a 920 bp fragment of the 16S ribosomal DNA gene. Based on nucleotide divergences and estimated according to the Jukes-Cantor model. Node numbers are the frequency (in percent) with which a cluster appears according to the bootstrap test with 500 trials.

(60.8 %), followed by *Pediococcus* (31 %) and members of the reclassified *Lactobacillus* genus (8 %), now assigned to newly proposed genera (Zheng et al., 2020).

Among *Enterococcus*, of the total isolated strains of LAB collected from wheat (199), 89 strains (44.7 %) were identified as *E. faecium,* making it the most frequently isolated species, 20 (10 %) as *E. durans* and 9 (4.52 %) as *E. mundtii*. Thus, *Enterococcus*, particularly *E. faecium* represented the majority of wheat-associated LAB, consistent with previous studies on wheat and other cereals (Salvucci et al., 2016). This was not surprising given that *Enterococcus* species exhibit remarkable adaptability to diverse environments (Bondi et al., 2020). In general, *Enterococcus* can be an opportunistic pathogen and lacks GRAS (Generally Regarded as Safe) status, their use as probiotics requires careful safety evaluation (Toc et al., 2022). Nevertheless, some *Enterococcus* species including *E. faecium* and *E. durans* offer potential health benefits like cholesterol reduction, antimicrobial compound production, adhesion to gut cell strains and have been proposed as probiotics (Dikbaş et al., 2024; Kanak et al., 2023; Li et al., 2018). In fact, specific strains of *E. faecium* and *E. durans* have recently been incorporated into food production due to their probiotic and fermentation properties (Liu et al., 2023; Çınar Acar et al., 2025; Tarique et al., 2024).

Concerning the *Pediococcus* genus, *P. acidilactici* (40 strains, 20.1 %)

Table 1
Genotypic identification results and hemolytic activity of LAB isolates.

Sequence results	Source of isolates	Cultivar	Geographical origin	Isolate codes	No. of isolates (RAPD)	Accession number	Hemolysis
Enterococcus durans	*T.aestivum* grain	Tocayo	Castilla-La Mancha	W28	19	PV159099	γ
	T.aestivum grain	Tocayo	Castilla-León	WTR20	1	PV159100	β
Enterococcus faecium	*T.aestivum* grain	Tocayo	Castilla-La Mancha	W34	1	PV159105	β
	T.aestivum grain	Tocayo	Castilla-La Mancha	WD1	1	PV159106	β
	T.aestivum grain	Tocayo	Castilla-La Mancha	WD17	4	PV159108	β
	T.aestivum grain	Tocayo	Castilla-La Mancha	WD3	9	PV159107	γ
	T.aestivum grain	Tocayo	Castilla-La Mancha	WG10	15	PV159110	β
	T.aestivum grain	Tocayo	Castilla-La Mancha	WG40	12	PV159111	β
	T.aestivum grain	Tocayo	Castilla-León	WTR11	9	PV159124	β
	T.aestivum grain	Tocayo	Castilla-León	WTR18	2	PV159125	β
	T.aestivum grain	Tocayo	Castilla-León	WTR19	1	PV159126	β
	T.aestivum grain	Tocayo	Castilla-León	WTR25	1	PV159127	β
	T.aestivum grain	Tocayo	Castilla-León	WTR26	9	PV159128	β
	T.aestivum grain	Tocayo	Castilla-León	WTR4	5	PV159123	β
	T.aestivum grain	Tocayo	Castilla-León	WTR6	2	PV159109	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.1	2	PV159112	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.12	3	PV159117	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.13	1	PV159118	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.14	3	PV159120	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.17	2	PV159121	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.18	1	PV159119	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.19	2	PV159122	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.3	1	PV159113	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.5	3	PV159114	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.7	1	PV159115	β
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB2.8	3	PV159116	β
Enterococcus mundtii	*T.aestivum* grain	Tocayo	Castilla-León	WTS11	1	PV159101	β
	T.aestivum grain	Tocayo	Castilla-León	WTS14	4	PV159102	β
	T.aestivum grain	Tocayo	Castilla-León	WTS15	2	PV159103	β
	T.aestivum grain	Tocayo	Castilla-León	WTS19	2	PV159104	β
Lacticaseibacillus casei	*T.aestivum* grain	Tocayo	Castilla-La Mancha	WG33	6	PV159086	γ
Lactiplantibacillus plantarum	*T.aestivum* spike	Tocayo	Castilla-La Mancha	WB1.2	3	PV159084	γ
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB3.1	1	PV159085	γ
Loigolactobacillus coryniformis	*T.aestivum* grain	Tocayo	Castilla-La Mancha	WD13	6	PV159087	γ
Pediococcus acidilactici	*T.aestivum* grain	Tocayo	Castilla-La Mancha	WC16	19	PV159092	γ
	T.aestivum grain	Tocayo	Castilla-León	WR1	3	PV159093	γ
	T.aestivum grain	Tocayo	Castilla-León	WR13	12	PV159096	γ
	T.aestivum grain	Tocayo	Castilla-León	WR2	2	PV159094	γ
	T.aestivum grain	Tocayo	Castilla-León	WR20	2	PV159097	γ
	T.aestivum grain	Tocayo	Castilla-León	WR6	1	PV159095	γ
	T.aestivum grain	Tocayo	Castilla-León	WTS17	1	PV159098	γ
Pediococcus pentosaceus	*T.aestivum* grain	Tocayo	Castilla-La Mancha	W17	10	PV159089	γ
	T.aestivum grain	Tocayo	Castilla-La Mancha	W9	9	PV159088	γ
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB1.4	1	PV159090	γ
	T.aestivum spike	Tocayo	Castilla-La Mancha	WB1.5	1	PV159091	γ

Identification of representative LAB isolates was based on partial 16S rRNA gene sequencing. Species names were assigned using sequence alignment against reference strains from the EMBL/NCBI database. Hemolytic activity was assessed on 5 % sheep blood agar plates after 48 h incubation at 37 °C.α-hemolysis: green-hued zones around colonies; β-hemolysis: a clear zone of hydrolysis around the colonies; γ-hemolysis: no zone around colonies. All experiments were performed in duplicate.

was the most frequently isolated species, followed by *P. pentosaceus* (21 strains, 10.55 %). It is interesting to note the high frequency of isolation of *P. acidilactici*, which was the second most frequently isolated species after *E. faecium* in the present study. Previous studies have reported *Pediococcus* species as the dominant LAB in cereals and fermented cereals (Atter et al., 2024; Gizachew et al., 2023; Mamhoud et al., 2016; Osimani et al., 2015). However, *Pediococcus* species, particularly *P. pentosaceus* and *P. acidilactici,* are widely recognized as probiotics in the food industry (Blumberg et al., 2025; Li et al., 2025; Megur et al., 2025; Wang et al., 2025).

Finally, LAB strains from the former *Lactobacillus* genus were isolated at lower frequencies compared to *Enterococcus* and *Pediococcus*. Six strains were identified as L. *casei* (3 %), six as L. *coryniformis* (3 %), and four as L. *plantarum* (2 %). This finding aligns with previous studies reporting that *Lactobacillus* are detected at very low percentages in wheat samples compared to enterococci (Alfonzo et al., 2017). The genus *Lactobacillus* was reclassified in 2020 as part of a broader reorganization of lactic acid bacteria, leading to the creation of 23 new genera within the family *Lactobacillaceae*, to better reflect phylogenetic relationships among more than 300 species (Zheng et al., 2020), some of which were identified in the present study (*Lactiplantibacillus*, *Lacticaseibacillus,* and *Loigolactobacillus*). These *Lactobacillus* species are highly recognized for their remarkable probiotic qualities (Shah et al., 2024). Moreover, fermentation by *Lactobacillus* species is widely applied in food production due to its nutritional value and associated health benefits (He et al., 2024; Sharma et al., 2023).

3.2. *In vitro* safety evaluation

3.2.1. *Hemolytic activity*

The hemolytic activity assay for LAB is a crucial method for evaluating their safety, particularly in the application of probiotics and fermented foods (Koirala and Anal, 2021). Bacteria with hemolytic activity have the potential to degrade intestinal epithelial cells, leading to changes in the intestinal mucosal barrier and facilitating the invasion of pathogens and other harmful substances (Di Vincenzo et al., 2024). Thus, we analyzed the hemolytic activity of the 45 LAB strains identified by sequence analysis using blood agar plates. As shown in **Table 1**, all *Enterococcus* strains except *E. durans* (W28) and *E. faecium* (WD3) showed β-hemolysis. The β-hemolytic activity observed in most *Enterococcus* strains suggests potential virulence traits, which align with their classification as opportunistic pathogens (Krawczyk et al., 2021).

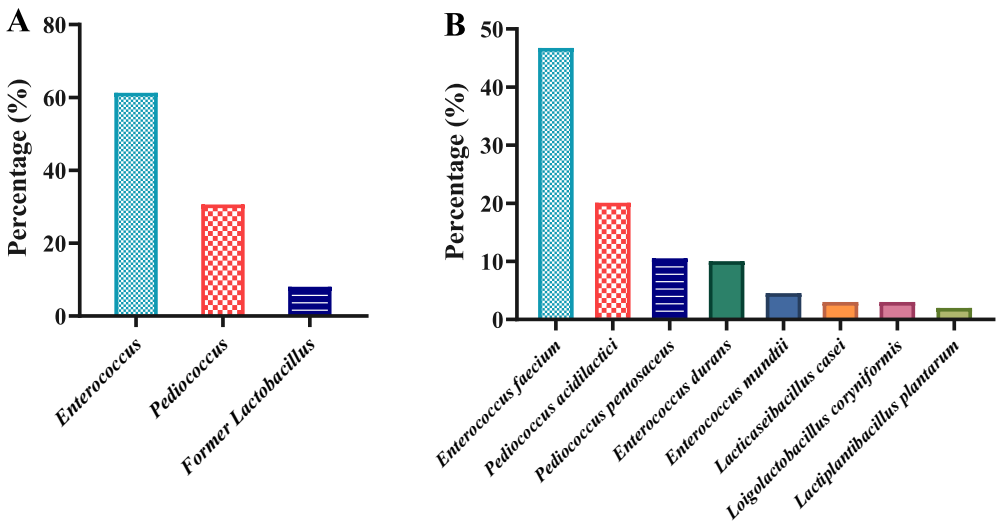

Fig. 3. Relative abundance of different LAB species isolated from wheat samples based on 16S rRNA gene sequencing. (A) Distribution of LAB at the genus level, showing the proportions of *Enterococcus*, *Pediococcus*, and former *Lactobacillus*. (B) Species-level distribution of the identified LAB isolates. Taxonomic classification follows the recent reorganization of the *Lactobacillus* genus into 23 novel genera within the *Lactobacillaceae* family.

Nevertheless, neither the production of a yellow color nor green halos, which have indicated hemolysis (γ-hemolysis) were noticed in all the 17 remaining strains belonging to species of L. *plantarum*, *L. casei*, *L. coryniformis*, *P. pentosaceus, and P. acidilactici* (**Table 1**).

3.2.2. *Antibiotic susceptibility*

Antibiotics are the most common tools for treating infectious diseases in the medical industry. However, the rising antibiotic resistance problem is a major challenge that is accompanied by significant complications (Frieri et al., 2017). Therefore, the assessment of antibiotic resistance is an important criterion for evaluating the safety of strains used in food and feed (Muteeb et al., 2023). Accordingly, the safety issue of LAB strains was tested by evaluating their susceptibilities to 10 clinically relevant antibiotics and the obtained results indicated the high sensitivity of isolates to most of the clinically important tested antibiotics. The data for all strains tested are presented in **Table 2**. All screened LAB strains were susceptible to chloramphenicol, penicillin, and ampicillin. In addition, many strains showed susceptibility or intermediate resistance to clindamycin, erythromycin, and tetracycline. However, the selected LAB strains were resistant to gentamicin, vancomycin (except *E. durans* W28), streptomycin, and kanamycin. It is suggested that LAB may possess natural intrinsic resistance, which helps maintain intestinal microbiota balance after antibiotic treatment (Shah et al., 2021). However, they may also carry antibiotic resistance genes, which should be tested using molecular techniques such as PCR or genome sequencing (Su et al., 2019).

Furthermore, the resistance of all LAB isolates to kanamycin and vancomycin in the current study was found to be consistent with earlier studies which found that many *Lactobacillus* and *Pediococcus* species had high resistance to streptomycin, vancomycin, and kanamycin (Mahjoory et al., 2023, 2022; Singla et al., 2018; Soleimani et al., 2023). It has been suggested that the LAB genus is thought to be intrinsically resistant to aminoglycoside antibiotics such as gentamicin, streptomycin, and kanamycin, which is due to the lack of cytochrome-mediated electron transport that facilitates the absorption of the drugs (Danielsen and Wind, 2003). In summary, the antibiotic susceptibility and resistance of LAB vary according to the targeted species (Urban-Chmiel et al., 2022). When used as probiotics, determining antibiotic resistance is essential

Table 2
Antibiotic susceptibility of isolated strains.

Antibiotics		C	CN	CD	E	VA	ST	P	K	TE	AMP
Concentration		30 μg	10 μg	2 μg	15 μg	30 μg	10 μg	10 IU	30 μg	30 μg	10 μg
E. durans	W28	S	R	S	S	S	R	S	R	S	S
E. facecium	WD3	S	R	S	S	R	R	S	R	S	S
L. casei	WG33	S	R	S	S	R	R	S	R	S	S
L. corvniformis	WD13	S	R	S	S	R	R	S	R	S	S
L. plantarum	WB1.2	S	R	S	S	R	R	S	R	S	S
	WB3.1	S	R	S	S	R	R	S	R	S	S
	WC16	S	R	S	S	R	R	S	R	S	S
	WR1	S	R	S	I	R	R	S	R	R	S
	WR2	S	R	S	S	R	R	S	R	I	S
P. acidilactici	WR6	S	R	I	I	R	R	S	R	R	S
	WR13	S	R	I	I	R	R	S	R	R	S
	WR20	S	R	S	I	R	R	S	R	R	S
	WTS17	S	R	S	S	R	R	S	R	I	S
	W9	S	R	S	S	R	R	S	R	S	S
P. pentosaceus	W17	S	R	S	I	R	R	S	R	R	S
	WB1.4	S	R	S	I	R	R	S	R	I	S
	WB1.5	S	R	S	S	R	R	S	R	I	S

Antibiotic susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100, 35th Edition (2025) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Breakpoint Table Version 15.0 (2025). S: susceptible; I: intermediate; R: resistant; C: Chloramphenicol; CN: Gentamicin; CD: Clindamycin; E: Erythromycin; VA: Vancomycin; ST: Streptomycin; P: Penicillin; K: Kanamycin; TE: Tetracycline; AMP: Ampicillin. Each strain was tested in triplicate.

for selecting suitable strains. The presence of transferable antibiotic resistance genes in LAB poses a risk of resistance transmission. Therefore, understanding their antibiotic susceptibility is critical for selecting appropriate strains, preventing resistance issues, and ensuring safety.

3.2.3. Mucin degradation

Mucin degradation experiments with LAB were conducted to evaluate their impact on the intestinal mucosal barrier, which is a crucial aspect in probiotic safety. Mucins are glycoproteins secreted by epithelial cells in the intestinal mucosa, which play a role in protecting the intestinal tract from pathogenic bacteria (Kang et al., 2022). Excessive mucin degradation by LAB may compromise barrier function, increasing intestinal permeability and infection risk (Qin et al., 2022).

In Fig. 4A, all strains exhibited reduced OD_{500} max values in glucose-free MRS, indicating growth limitation due to the absence of utilizable carbon sources. In both 0.5 % and 1 % mucin media, *E. coli* demonstrated significantly higher OD_{500} max values ($p < 0.05$) compared to other strains, suggesting a potential capacity for mucin utilization. In contrast, OD_{500} max values of the other strains remained low and comparable to glucose-free MRS, implying a lack of mucin degradation mechanisms. All strains showed significantly increased OD_{500} max values ($p < 0.05$) in 0.5 % and 1 % glucose media, with *P. pentosaceus* (W9), *L. corvniformis* (WD13), and *P. acidilactici* (WC16, WR1, WR13) exhibiting particularly pronounced growth enhancement, highlighting glucose as an effective growth substrate for these strains. The growth rates of all LAB strains were lower in mucin media (Fig. 4B), aligning with their OD_{500} max results, indicating limited reliance on mucin as a carbon source. In glucose media, growth rates were significantly higher ($p < 0.05$), reinforcing the role of fermentable sugars as the main energy source. Notably, while the growth rate in 0.5 % glucose MRS was higher than in 1 % glucose, the OD_{500} max was lower, suggesting that moderate glucose concentrations favor growth rate, while higher concentrations sustain prolonged growth. Among all strains, *L. casei* (WG33), *L. corvniformis* (WD13), and *E. durans* (W28) exhibited significantly higher growth rates in 0.5 % glucose MRS ($p < 0.05$), indicating their potential for fermentation under low-sugar conditions. Therefore, all tested LAB strains exhibited limited mucin degradation capacity while maintaining efficient sugar utilization, indicating an absence of mucinolytic activity that could harm the gastrointestinal mucosa. These findings are consistent with previous studies on L. *rhamnosus* and *P. acidilactici* (Daliri et al., 2022), further supporting their safety as probiotics and their potential for broader applications.

3.3. Potential probiotic characterization of selected strains

3.3.1. Resistance of isolated strains to the simulated gastro-intestinal conditions

Pepsin is a digestive enzyme secreted by the gastric glands, and pancreatin is a digestive enzyme secreted by the pancreas into the small intestine. Both enzymes play an important role in the digestive process. When supplementing or consuming probiotics orally, survival in the gastrointestinal tract is key to their beneficial effects (Zommara et al., 2023). The survival of the selected strains in simulated gastric (pepsin) and intestinal fluids (pancreatic enzymes plus bile salts) is shown in Table 3. In terms of the change in bacterial population (log CFU/mL), the initial number of bacteria ranged from 8.76 ± 0.04 log CFU/mL (*P. pentosaceus* W17) to 9.70 ± 0.05 log CFU/mL (*L. plantarum* WB3.1), with little difference. After gastric phase treatment, the colony numbers of most strains decreased slightly, but L. *plantarum* WB1.2 (9.31 ± 0.02 log CFU/mL), WB3.1 (9.55 ± 0.09 log CFU/mL), and *P. acidilactici* WR6 (9.32 ± 0.02 log CFU/mL) still maintained high numbers. However, after intestinal fluids treatment, there was a significant decrease in the number of colonies. The survival rate (SR%) of all strains remained above 89.53 % after pepsin treatment, indicating that these LAB were highly tolerant in acidic environments. Among them, *P. acidilactici* WR13 (99.87 %) and L. *plantarum* WB1.2 (99.48 %) showed the highest survival rate, whereas *E. faecium* WD3 (89.53 %) showed a relatively low survival rate. This difference may be related to the cell wall structure of the strains and the expression level of acid stress-related genes. After treatment with enteric fluid in pancreatin with bile salts, the survival of

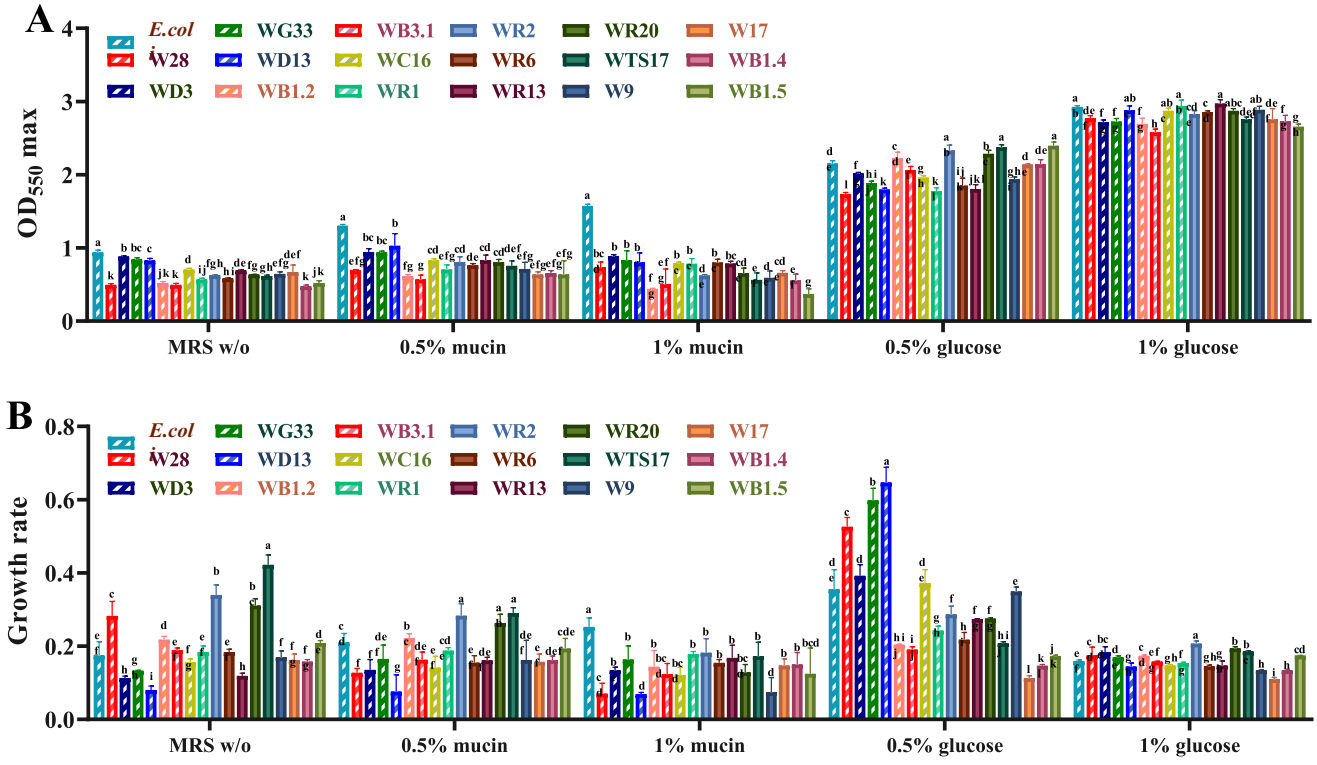

Fig. 4. Growth of isolates in modified MRS with various carbon sources: basal medium (glucose-free MRS), basal medium supplemented with 0.5 % mucin, 1.0 % mucin, 0.5 % glucose, or 1.0 % glucose. (A) Maximum OD_{550}; (B) Growth rate constant.

Table 3
Survival under in vitro gastro-intestinal conditions (gastric and intestinal phases), cell surface hydrophobicity, and auto-aggregation activity of selected strains of LAB.

LAB strains	Identification	Population (log CFU/mL)			Survival rate (SR%)		Cell surface hydrophobicity (%)	Auto-aggregation (%)
		Control (Initial)	Gastric phase	Intestinal phase	Gastric phase	Intestinal phase		
E. durans	W28	9.03 ± 0.04^{defg}	8.43 ± 0.15^{h}	4.52 ± 0.05^{i}	93.33	49.99	48.23 ± 3.0^{5d}	59.83 ± 1.95^{b}
E. facecium	WD3	8.99 ± 0.06^{efg}	8.05 ± 0.06^{j}	3.60 ± 0.06^{k}	89.53	39.98	31.02 ± 2.51^{e}	73.29 ± 0.58^{a}
L. casei	WG33	9.12 ± 0.07^{cdef}	8.70 ± 0.03^{f}	5.47 ± 0.10^{g}	95.40	59.97	45.15 ± 2.98^{d}	51.84 ± 1.36^{c}
L. coryniformis	WD13	9.13 ± 0.14^{cde}	8.89 ± 0.10^{de}	3.64 ± 0.16^{k}	97.42	39.89	82.10 ± 1.61^{b}	43.81 ± 3.70^{d}
L. plantarum	WB1.2	9.36 ± 0.14^{b}	9.31 ± 0.02^{b}	6.80 ± 0.11^{bc}	99.48	72.69	87.06 ± 1.44^{a}	31.60 ± 2.91^{f}
	WB3.1	9.70 ± 0.05^{a}	9.55 ± 0.09^{a}	6.71 ± 0.03^{cd}	98.39	69.15	24.97 ± 1.59^{f}	24.43 ± 2.61^{gh}
P. acidilactici	WC16	8.92 ± 0.09^{gh}	8.60 ± 0.05^{fg}	5.04 ± 0.04^{i}	96.43	45.30	62.05 ± 1.07^{c}	54.73 ± 0.92^{c}
	WTS17	8.96 ± 0.01^{fg}	8.81 ± 0.10^{e}	6.60 ± 0.09^{d}	98.35	73.60	26.66 ± 1.62^{f}	27.08 ± 2.67^{g}
	WR1	9.05 ± 0.02^{cdefg}	8.86 ± 0.05^{de}	6.37 ± 0.06^{e}	97.85	70.34	32.44 ± 2.81^{e}	31.76 ± 1.98^{f}
	WR2	9.21 ± 0.08^{c}	9.12 ± 0.01^{c}	7.07 ± 0.01^{a}	99.08	76.82	28.43 ± 2.55^{ef}	23.21 ± 1.52^{h}
	WR6	9.59 ± 0.11^{a}	9.32 ± 0.02^{b}	6.92 ± 0.05^{b}	97.18	72.14	44.54 ± 2.70^{d}	27.34 ± 1.72^{g}
	WR13	9.16 ± 0.09^{cd}	9.15 ± 0.01^{c}	7.07 ± 0.06^{a}	99.87	77.11	26.46 ± 2.81^{f}	12.55 ± 2.91^{ij}
	WR20	9.13 ± 0.03^{cde}	8.96 ± 0.04^{d}	6.43 ± 0.06^{e}	98.16	70.44	18.86 ± 3.13^{g}	10.77 ± 1.14^{j}
P. pentosaceus	W9	8.81 ± 0.18^{hi}	8.51 ± 0.25^{gh}	5.48 ± 0.14^{g}	93.59	48.17	59.14 ± 0.38^{c}	61.74 ± 0.86^{b}
	W17	8.76 ± 0.04^{i}	8.31 ± 0.05^{i}	5.20 ± 0.12^{h}	94.88	59.37	59.19 ± 1.32^{c}	35.88 ± 2.77^{e}
	WB1.4	9.04 ± 0.02^{defg}	8.71 ± 0.03^{f}	6.75 ± 0.05^{c}	96.34	74.71	31.09 ± 3.97^{e}	20.91 ± 1.85^{h}
	WB1.5	9.57 ± 0.07^{a}	8.94 ± 0.03^{d}	6.12 ± 0.07^{f}	93.42	63.97	26.85 ± 1.02^{f}	14.53 ± 2.08^{i}

Survival rates (%) under simulated gastric (pH 3.0, pepsin) and intestinal (pH 8.0, pancreatin and bile salts) conditions were determined after 3 h and 4 h incubation, respectively. SR (%) = [log CFU N/ log CFU N_0] × 100, where N_0 and N are the population values before and after the assay, respectively. Cell surface hydrophobicity (%) was measured by microbial adhesion to hydrocarbons using chloroform, and auto-aggregation (%) was evaluated based on optical density reduction over 5 h. Data are presented as mean ± SD of three independent experiments. Statistical differences were determined using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

the strains decreased significantly ($p < 0.05$), ranging from 39.89 % (*L. coryniformis* WD13) to 77.11 % (*P. acidilactici* WR13). On the contrary, *E. faecium* WD3 (39.98 %) and L. *coryniformis* WD13 (39.89 %) showed lower survival rates in this condition, which may be related to their higher sensitivity to bile salts or the lack of associated tolerance mechanisms. The overall performance of L. *plantarum* and *P. acidilactici* strains, especially WB3.1, WR2, and WR13, were excellent, maintaining high survival rates in the gastrointestinal environment, suggesting their high potential as probiotics. In contrast, *E. facecium* (WD3) and *L. coryniformis* (WD13) showed lower survival in the presence of bile salts, which may limit their probiotic applications.

3.3.2. Cell surface hydrophobicity assay

For orally administered probiotics, the high hydrophobicity of LAB usually facilitates their adhesion to host intestinal epithelial cells, thereby enhancing colonization. In addition, hydrophobicity correlates with the ability of LAB to cell auto aggregation and co-aggregation, which is important for the formation of microbial biofilms, competition for space with pathogens, and inhibition of pathogen adhesion (Han et al., 2022). Chloroform was used to assess the cell surface hydrophobicity of LAB isolates to determine their capacity for adhering to hydrophobic surfaces (**Table 3**). *L. plantarum* WB1.2 (87.06 ± 1.44 %) and *L. coryniformis* WD13 (82.10 ± 1.61 %) had the highest surface hydrophobicity, indicating that its surface has a stronger hydrophobic interaction capacity, which may contribute to its adhesive properties in the host gut, easier attachment to the surface of intestinal epithelial cells, and thus enhanced colonization. According to (Del Re et al., 2000), a minimum hydrophobicity of 40 % is required when selecting probiotics, as this affects adhesion and interaction with host cells. *L. plantarum* WB3.1 (24.97 ± 1.59 %), *P. acidilactici* WTS17 (26.66 ± 1.62 %), WR13 (26.46 ± 2.81 %), WR20 (18.86 ± 3.13 %) and *P. pentosaceus* WB1.5 (26.85 ± 1.02 %) had the lower hydrophobicity, which may be related to its surface chemical properties (e.g. protein or polysaccharide composition). The hydrophobicity of WB1.2 (87.06 ± 1.44 %) was much higher than that of WB3.1 (24.97 ± 1.59 %) in L. *plantarum*, highlighting considerable intraspecies variability in cell surface properties. This finding underscores the importance of strain-specific evaluation when selecting probiotic candidates, as functional traits can differ significantly even among strains of the same species.

3.3.3. Cell auto-aggregation activity assay

Potential probiotics should have the ability to colonize the intestine and prevent pathogens colonization (Méndez-Galarraga et al., 2023). The auto-aggregation capacity of LAB made an important contribution to the adhesion of intestinal cells. As shown in **Table 3**, *E. faecium* WD3 (73.29 ± 0.58 %) exhibited the highest auto-aggregation capacity and was favorable for the formation of biofilms in the intestine, thereby increasing antipathogenic competitiveness. However, *P. acidilactici* WR13 (12.55 ± 2.91 %), WR20 (10.77 ± 1.14 %), and *P. pentosaceus* WB1.5 (14.53 ± 2.08 %) had the lowest auto-aggregation capacity and were weak in competitive colonization, which may affect their stability. Similar to *P. acidilactici*, the auto-aggregation capacity of WR13 (12.55 ± 2.91 %) and WR20 (10.77 ± 1.14 %) were much lower than that of WC16 (54.73 ± 0.92 %), suggesting that strains differed greatly in this characteristic. In addition, *L. plantarum* WB1.2 (87.06 ± 1.44 %) had the highest surface hydrophobicity and high gastrointestinal conditions survival rate (72.69 %), but the auto-aggregation ability was only 31.60 ± 2.91 %, suggesting that it may easily attach to intestinal epithelial cells but is less likely to form biofilms. These results indicate that surface hydrophobicity alone is insufficient to fully account for bacterial aggregation behavior. Auto-aggregation is a multifactorial process influenced by various cell surface characteristics, including electrostatic interactions, surface charge, proteinaceous surface structures, and extracellular polysaccharides, all of which can affect intercellular aggregation (Tokatlı et al., 2015; Zhang et al., 2022). Strains with high auto-aggregation capacity are generally regarded as advantageous, as it facilitates biofilm formation in the gut, thus improving competitiveness against pathogens.

3.4. Selection of LAB strains with favorable potential probiotic properties

To comprehensively assess and compare the properties of the isolated strains, the probiotic characterization data (Gastric phase and intestinal phase survival rate, cell surface hydrophobicity, and auto-aggregation activity) using PCA and heat maps were analyzed. As shown in **Fig. 5A**, principal component 1 (PC1) and principal component 2 (PC2) accounted for 63.69 % and 26.33 % of the variance, respectively. The strains were distributed in four quadrants: *L. plantarum* (WB1.2) and *P. acidilactici* WR6 were positioned in Quadrant I, showing a strong correlation with both PC1 and PC2, indicating that they are

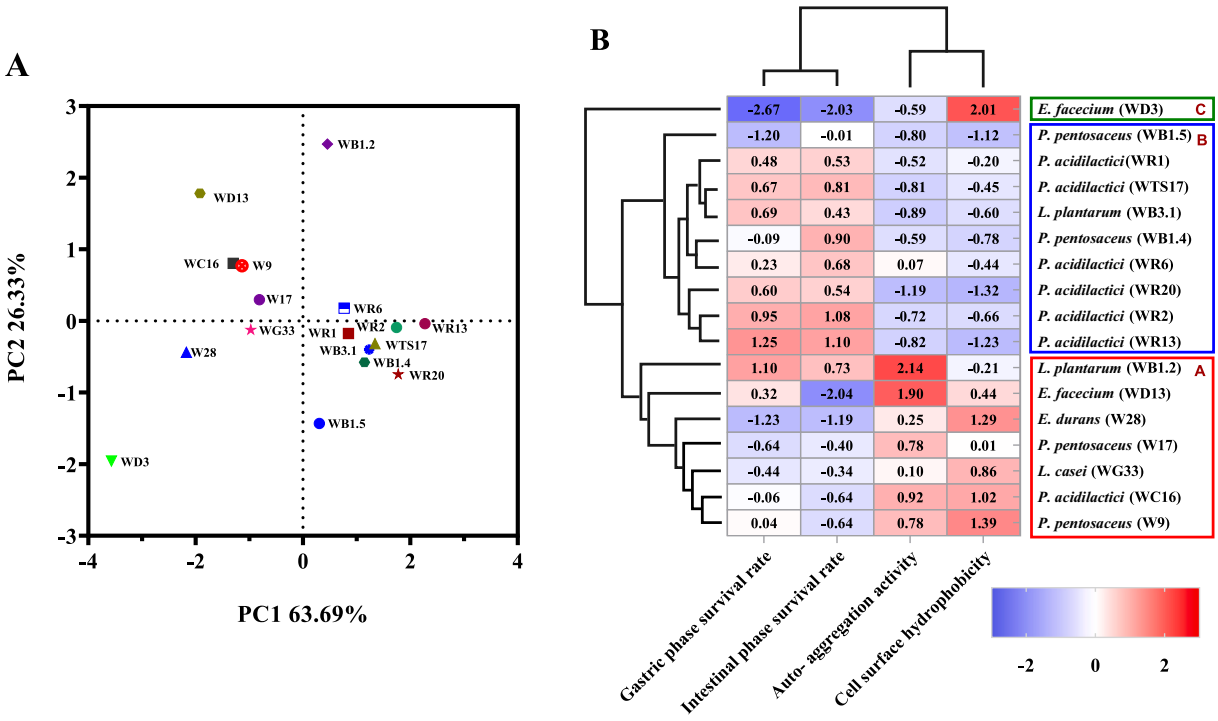

Fig. 5. Selection of LAB strains with potential probiotic properties (Gastric phase survival rate, intestinal phase survival rate, cell surface hydrophobicity, and auto-aggregation activity). (A) Principal component analysis (PCA), the percentages: the values contributed by the principal components to the sample differences; (B) Heat map, the redder the color is, the better the probiotic property is.

likely the most promising strains. *P. acidilactici* (WR1, WR2, WR13, WR20, WTS17), and *P. pentosaceus* (WB1.4, WB1.5) were located in Quadrant IV, exhibiting a stronger correlation with PC1. *L. coryniformis* (WD13), *P. acidilactici* (WC16), and *P. pentosaceus* (W9, W17) were positioned in Quadrant II, where they correlated more strongly with PC2 and were considered promising probiotic candidates.

Heat maps (Fig. 5B) were used to classify the LAB strains into three distinct groups based on their properties. Group A exhibited superior cell surface hydrophobicity and auto-aggregation, suggesting enhanced intestinal adhesion, which supports biofilm formation and colonization. Group B, on the other hand, displayed significant tolerance to gastrointestinal fluids, with higher survival rates in both gastric and intestinal conditions, indicating its potential for probiotic applications. Group C consisted of a single strain, *E. faecium* (WD3), which showed poor survival under gastrointestinal conditions and relatively weak adhesion-related traits, suggesting limited probiotic potential in its current form. These results highlight the diverse functional attributes of LAB strains, with each group offering unique advantages for probiotic development.

4. Conclusions

In conclusion, this study provides a detailed and integrative analysis of lactic acid bacteria (LAB) associated with wheat grains and spikes, uncovering significant genetic diversity and identifying eight distinct LAB species through molecular and phylogenetic approaches. The predominance of *Enterococcus faecium* species suggests a strong ecological adaptation to the wheat environment, although its safety profile limited its probiotic potential. Seventeen non-hemolytic, most of them belong to *Lactobacillus* and *Pediococcus* genera, were sensitive to most antibiotics commonly used in clinical practice and were not able to degrade mucin. They were further analyzed, with *Lactiplantibacillus plantarum* (WB1.2) and *Pediococcus acidilactici* (WR6) strains emerging as the most promising probiotic candidates due to their high surface hydrophobicity, auto-aggregation, and tolerance to simulated gastrointestinal conditions. This work not only fills a knowledge gap regarding LAB diversity in cereal-based ecosystems, but also establishes a robust and reproducible workflow for selecting safe and functionally relevant LAB strains aligning with the global demand for sustainable, plant-based microbial resources. Although the selected strains in this study exhibit promising probiotic and safety-related characteristics, positioning them as potential candidates for use as starter cultures in novel probiotic fermented products, several limitations must be acknowledged. Notably, these properties have not yet been validated through in vivo studies, and important aspects such as antibiotic-resistant genes remain untested. Therefore, these further comprehensive studies are essential before these strains can be considered for practical application in food systems.

CRediT authorship contribution statement

Weilan Gao: Writing – original draft, Software, Methodology, Formal analysis. **Laura Hernandez-Garcia:** Writing – original draft, Software, Methodology, Formal analysis. **Juan Manuel Castagnini:** Writing – review & editing, Validation, Software, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Noelia Pallares:** Writing – review & editing, Supervision, Resources, Project administration. **Francisco J. Barba:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Pedro Vicente Martínez Culebras:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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