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Investigating tryptophan-producing probiotic lactic acid bacteria from Lithuanian fermented foods: functional characterization, safety evaluation, and genomic analysis

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ABSTRACT

This study aimed to isolate and characterize lactic acid bacteria (LAB) from traditional Lithuanian fermented foods and evaluate their probiotic potential, safety, and ability to produce tryptophan. Around 20 LAB isolates were screened for gastrointestinal tolerance, antimicrobial activity, aggregation, and adhesion to intestinal cells. Only 5 strains were identified and further analyzed using whole-genome sequencing and *in vitro* assays. Among them, *Leuconostoc mesenteroides* KA15 showed enhanced survival under simulated gastrointestinal conditions and produced 10.48 μM tryptophan in the presence of fructooligosaccharides. Genomic analysis confirmed the absence of virulence and transferable antibiotic resistance genes in selected strains. While some isolates exhibited antimicrobial activity and adhesion capacity, phenotypic antibiotic resistance excluded certain candidates from probiotic consideration. Overall, KA15 demonstrated the most promising combination of functional and safety characteristics. These findings highlight traditional fermented foods as a source of potentially beneficial LAB supporting further studies to evaluate their physiological effects in host systems.

1. Introduction

A category of gram-positive, non-spore-forming microorganisms known as lactic acid bacteria (LAB) have been an integral part of human nutrition for millennia, known for the fermentation and preservation of various food products (Sionek et al., 2023). These bacteria can be isolated from a variety of sources, such as dairy products, meat, cereals, fermented vegetables, and traditional fermented foods (Grujović et al., 2022). By producing lactic acid and other essential metabolites, LAB plays an important role in preserving and improving food quality by enhancing flavor, texture, and aroma (Wang et al., 2021). Sourdough bread, fermented milk, and vegetables are examples of traditional fermented foods from around the globe that are rich sources of LAB isolates with unique and possibly advantageous characteristics (Wang et al., 2023) (Abdel Tawab et al., 2023).

LAB microorganisms have gained significant attention in recent years due to their probiotic properties and direct association with health benefits *via* the gut microbiota modulation (Abdel Tawab et al., 2023; Burokas et al., 2015; Khushboo et al., 2023). Probiotics are described as 'live microorganisms that, when administered in adequate amounts, confer health benefits on the host' (Hill et al., 2014). The global nutrition and pharmaceutical market have experienced substantial growth, driven by increasing consumer awareness of the link between gut health, modulation, and overall well-being (Anđela et al., 2024).

Traditional fermented foods that vary across cultures and regions serve as a rich source of diverse LAB strains with potential probiotic

Abbreviation: AMR, Antimicrobial resistance; ANI, Average nucleotide identity; CFS, Cell-free culture supernatants; CFU, Colony forming units; EFSA, European Food Safety Authority; FOS, Fructooligosaccharides; GABA, Gamma-aminobutyric acid; GOS, Galactooligosaccharides; HPLC-MS, High-performance liquid chromatography-mass spectrometry; LAB, Lactic acid bacteria; mm, Millimeter; MRS, de Man, Rogosa, and Sharpe; RAST, Rapid Annotations using Subsystems Technology; VF, Virulence factors.

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properties (Dey et al., 2023). Current findings have concentrated on the isolation and characterization of LAB from various fermented foods, aiming to identify novel probiotic strains with enhanced probiotic properties. For instance, LAB obtained from indigenous fermented cereal-based products in India has exhibited promising probiotic qualities, such as tolerance to acid and bile, antimicrobial properties, and antioxidative capabilities (Meena et al., 2022). Likewise, LAB strains derived from the traditional Korean fermented dish, Kimchi, have demonstrated significant cell surface hydrophobicity and antioxidant activity, suggesting their potential as effective probiotics (Cho et al., 2020). Finally, the safety of these strains is paramount, necessitating thorough assessment to ensure they do not pose risks such as harmful metabolite production and transferable antibiotic resistance (Holzapfel & Todorov, 2023).

The European Food Safety Authority has established stringent guidelines for the evaluation and approval of selected probiotic strains (on Biological Hazards (BIOHAZ) et al., 2017). The qualified presumption of the safety concept introduced by EFSA provides a framework for assessing the safety of microorganisms intended for food and feed applications (Binda et al., 2020). These guidelines emphasize the need for the thorough characterization of LAB strains, including their genetic makeup, metabolic activities, and potential to produce harmful substances such as biogenic amines or antibiotic resistance genes. The selection process for LAB strains involves ensuring they do not carry transferable antibiotic resistance genes and that they are free from virulence factors.

LAB are generally considered safe due to their presence in traditional fermented foods, and their safety is recognized by health authorities (Castellone et al., 2021). These bacteria are crucial in the human gut microbiota, elevating digestive health and hindering harmful pathogens by producing lactic acid and other antimicrobial compounds (Afzaal et al., 2022). As the demand for probiotics that target certain health issues is increasing, the identification of novel LAB strains is gaining attention (Pavlova et al., 2020). The novel LAB strains provide distinct advantages: reduced metabolic disorder (Yang et al., 2023), immune modulation (Bernier et al., 2020), and enhanced brain function (Missiego-Beltrán & Beltrán-Velasco, 2024; Park et al., 2020). For instance, in animal model, *Enterococcus faecium* has demonstrated significant hypolipidemic effects and immune response enhancement (Darwish et al., 2022). One particular interest is the ability of certain LAB to synthesize tryptophan, an essential amino acid with various physiological roles and a precursor for the production of serotonin (Roth et al., 2021; Xu et al., 2020). Ongoing research and development of new probiotic LAB strains are crucial for creating effective, stable products to address various mental health conditions, improve nutrition, and enhance overall well-being. Although numerous lactic acid bacteria have been isolated from fermented foods, only a limited number of studies have investigated LAB strains capable of producing tryptophan and their potential role in modulating host metabolic pathways such as serotonin biosynthesis and aryl hydrocarbon receptor (AhR) signaling (Aguirre-Garcia et al., 2025; Zhao et al., 2024). Tryptophan biosynthesis pathways in bacteria have been extensively described, the identification of probiotic LAB strains capable of producing tryptophan remains limited and is often strain-specific (Gao et al., 2020a). Recent studies have demonstrated that certain LAB strains isolated from fermented foods or dairy environments can synthesize tryptophan or related metabolites, although the production levels vary considerably between strains. A recent study reported that *Lacticaseibacillus paracasei* 11w produced approximately 9.95 μM tryptophan under glucose conditions and up to 16.63 μM when supplemented with prebiotics such GOS, while other strains produced substantially lower amounts (~2.6 μM) or none at all (Megur et al., 2023). These findings highlight the strain-dependent nature of tryptophan biosynthesis and underscore the importance of identifying new LAB isolates with enhanced metabolic capabilities. Furthermore, little information is available on probiotic LAB strains isolated from traditional Lithuanian fermented foods and their functional metabolic potential (Megur et al., 2023; Megur et al., 2024a).

The purpose of this study is to assess the potential of LAB as probiotic strains by characterizing them from traditional fermented foods from Lithuania. Their ability to synthesize tryptophan and evaluate its *in vitro* probiotic qualities, such as gastrointestinal tolerance, adhesion qualities, and antimicrobial activity, are among the specific goals. To protect consumers, EFSA safety evaluations are also carried out, such as profiling antibiotic resistance and assessing the production of toxins. The study intends to link traditional food heritage with contemporary health innovations by examining these parameters to find promising LAB strains that can aid in the creation of functional foods and nutraceuticals.

2. Materials and methods

2.1. Sample isolation and collection

Strains were isolated from different fermented foods collected at Halės Turgus market (Halle Market, Vilnius, Lithuania). The fermented foods selected were traditional Lithuanian sauerkraut, Kombucha, cheese, yogurt, and cucumber pickles. Each sample (1 g or 1 mL) was carefully placed into separate sterile test tubes containing 9 mL of sterile 0.1% (*w/v*) peptone water (Sigma-Aldrich, Poznań, Poland). Aliquots of 10 μL from appropriate 10^8 CFU/mL dilution were pentagonally streaked on the pre-solidified de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Wesel, Germany) and incubated at 35 °C for 48–72 h under aerobic conditions. Representative colonies of LAB were randomly picked and purified by repeated streak plating on MRS agar until pure colonies were obtained. Pure bacterial cultures were maintained on MRS agar plates and subcultured every 3 weeks until imperative for characterization. Cell morphology and colonial characterization were observed on MRS agar. These isolates were stored and preserved in a − 80 °C deep freezer (Froilabo, Livingston, United Kingdom) at the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Center, Vilnius University, Vilnius, Lithuania.

2.2. Survival in in vitro gastrointestinal conditions

2.2.1. Resistance to low pH

The resistance of LAB to low pH was studied according to a previously described method (Manovina et al., 2022), with minute modifications. Briefly, LAB cultures incubated at 37 °C for 24 h were centrifuged at 10,000*g* for 10 min. The pellets were suspended in sterile PBS (Sigma-Aldrich, Poznań, Poland) and adjusted to a pH of 2.0 using 1 M HCl. The mixture was then incubated at 37 °C for 4 h. A static *in vitro* gastrointestinal simulation model was used to evaluate LAB survival under acidic gastric conditions. Aliquots were taken at 0 and after 4 h. The samples were serially diluted in peptone water, and the viable cells were determined by the spread plate method using MRS agar. The plates were incubated at 37 °C for 24 h, and the percentage survival of the bacteria was calculated as follows:

$$\%\text{Survival} = \frac{\text{CFU of viable cells survied}}{\text{CFU of initial viable cells inoculated}} \times 100$$

2.2.2. Resistance to pepsin

To test the viability in the presence of pepsin, simulated gastric juice was prepared by suspending 3 mg/mL pepsin (Sigma- Aldrich, Poznań, Poland) in sterile peptone water and adjusted to pH 2.0. The fluid was inoculated with active cultures at an inoculum size of 1% (*v/v*) and incubated at 37 °C for 4 h. The spread plate method determined the viable cells before (T1) and after incubation (T2). The percentage survival of the bacteria was calculated according to resistance to low pH.

$$\%\text{Survival} = \frac{\text{CFU of viable cells survied}}{\text{CFU of initial viable cells inoculated}} \times 100$$

2.2.3. Resistance to bile salts

Resistance to intestinal juices was tested as reported (Megur et al., 2023). Briefly, 0.3% (*w/v*) bile salts (Sigma- Aldrich, Poznań, Poland) was dissolved in sterile peptone water and adjusted to pH 8.0 cell-free culture supernatants. The fluid was inoculated with 1% (*v/v*) LAB cultures and incubated at 37 °C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated according to the equation below.

$$\%\text{Survival} = \frac{\text{CFU of viable cells survied}}{\text{CFU of initial viable cells inoculated}} \times 100$$

2.2.4. Resistance to pancreatin

Resistance to intestinal juices was tested as reported (Wu et al., 2023). Briefly, 1 mg/mL of pancreatin (Sigma-Aldrich, Poznań, Poland) was dissolved in sterile peptone water and adjusted to pH 7.2 cell-free culture supernatants. The fluid was inoculated with 1% (*v/v*) LAB cultures and incubated at 37 °C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated according to the equation below.

$$\%\text{Survival} = \frac{\text{CFU of viable cells survied}}{\text{CFU of initial viable cells inoculated}} \times 100$$

2.3. DNA extraction and molecular identification

LAB strains were grown overnight in MRS broth (Oxoid, Wesel, Germany) at 35 °C. The QIAGEN DNeasy PowerSoil Pro Kit was used to isolate DNA from the bacteria following the manufacturer's protocol. Briefly, the quantity of DNA in the samples was measured using the QuantiFluor® dsDNA System with the GloMax Plate Reader System (Promega Italia, Promega France). To prepare the DNA libraries, Nextera XT DNA Library Preparation Kit (Illumina, Warszawa, Poland) was used coupled with IDT Unique Dual Indexes. An amount of 1 ng DNA was used as input. The genomic DNA was fragmented using Illumina Nextera XT fragmentation enzyme and a unique dual index was added to the sample. The library was then constructed using 12 cycles of PCR. The DNA library was purified using AMpure magnetic beads and eluted in QIAGEN EB buffer. The quantity of DNA in the library was measured with a Qubit 4 fluorometer and a Qubit dsDNA HS Assay Kit. The library was then sequenced with the Illumina NovaSeq platform with 2 × 150 bp reads.

2.4. Gut colonization properties

2.4.1. Probiotic antimicrobial activity

Antibacterial activity was determined using the agar well diffusion test as previously described (Chaichana et al., 2025). *Staphylococcus aureus* ATCC 29213, *SalmonellaTyphimurium* ATCC 14028, *Streptococcus pyogenes* ATCC12384, *Streptococcus pyogenes* ATCC 19615, and *Klebsiella pneumoniae* ATCC 13883 were obtained from the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Center, Vilnius University and were used as indicator strains for the detection of antimicrobial activity. The LAB was cultured in 3 mL MRS broth medium and incubated for 24 h at 37 °C. The MRS broth tubes were centrifuged (10,000*g* for 10 min) to prepare cell-free culture supernatants (CFS). The pH values of the supernatants were adjusted to approximately 7.2 by adding NaOH. A suspension of 100 μL of 10^7 CFU/mL of each pathogenic strain was then prepared and spread onto the nutrient agar, into which 5-mm-deep wells had been dug. Approximately 100 μL of CFS was poured into each well, and nutrient agar plates were incubated for 24 h to 48 h at 37 °C. Finally, the inhibition zone diameter was measured in millimeters (mm). Inhibition zones were measured in millimeters and expressed as mean ± standard deviation.

2.4.2. Auto-aggregation

The overnight grown LAB cultures were centrifuged at 10000*g* for 10 min to harvest the cell pellets. Pellets were washed thrice with phosphate-buffered saline (PBS; pH 7.4) and re-suspended in PBS, and the initial absorbance was noted at 600 nm. The bacterial suspension was incubated at 37 °C for 24 h, and the final absorbance of the supernatant was measured at 600 nm at three different times: 4 h, 12 h, and 24 h. The formula measured the percentage of cellular auto-aggregation:

$$\text{Autoaggregation}\ (\%) = \left(\frac{\text{ODinitial} - \text{ODfinal}}{\text{ODinitial}}\right) \times 100$$

2.4.3. Co-aggregation

Equal volumes of cells (1.5 mL) of the different LAB and pathogen strains were mixed, vortexed for 10 s, and incubated at 37 °C. The absorbance was determined for the mixture and the bacterial suspensions alone. Co-aggregation was calculated as follows:

$$\textit{Coaggregation}\ (\%) = \{[(\text{Apat} + \text{Aprobio})/2 - \text{Amix}]/(\text{Apat} + \text{Aprobio})/2\} \times 100$$

where A_{pat} and A_{probio} represent A_{600}nm of the separate bacterial suspensions in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at 2 h.

2.4.4. In vitro percent adhesion on HCT116 cells

The human colonic cancer cell lines HCT116 were obtained from the Department of Biological Models, Institute of Biochemistry, Life Sciences Center, Vilnius University, Lithuania, and were prepared as mentioned in Supplementary data (methods and materials). The cell concentration in the monolayer was determined by trypsinizing the adhered cells with 3 mL of 0.25% trypsin–EDTA solution for 5–10 min at 37 °C. The final cell count in suspension was quantified with the help of a hemocytometer (Sigma-Aldrich, Poznań, Poland). For the adhesion assay, HCT116 cells were seeded individually in each well of standard 12-well tissue culture plates at a concentration of 1×10^7 cells/mL and incubated for ~48 h or more, or until a complete monolayer was formed. The medium was changed every 24–48 h. Before the adhesion assay, the spent medium was completely removed after 24 h and cells were fed with DMEM lacking antibiotics. The LAB isolates intended for adhesion assay were propagated in MRS broth and cultures obtained after 18 h of growth at 37 °C were centrifuged at 6000×*g* for 10 min. The pellet was rinsed once with PBS (pH 7.4). The cell density was adjusted to the desired levels by measuring the absorbance at 600 nm. The precise number of viable bacteria used in the assay was determined by plate counting on MRS agar.

The adhesion of LAB isolates was measured as described previously with few modifications (Megur et al., 2023). The HCT 116 cells in a monolayer were washed twice with 3 mL of PBS (pH 7.6). The 2 mL of DMEM without serum and antibiotics was added to each well and incubated at 37 °C for 40 min before inoculation of bacteria. Different LAB isolates with a concentration of approximately 1×10^7 CFU suspended in 1 mL DMEM without serum and antibiotics were used to inoculate each well of tissue culture plates. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air for 3 h. After incubation, the monolayer was washed five times with sterile PBS (pH 7.6) to remove non-adherent bacteria.

The monolayer was washed five times with sterile PBS (pH 7.4) to remove non-adherent bacteria. To enumerate the viable adhered bacteria, the cells from the monolayer were detached by trypsinization. Each well was treated with 1 mL of 0.25% trypsin–EDTA solution and incubated for 15 min at room temperature. The suspension of lysed cells and LAB was serially diluted with saline solution and plated on MRS agar. The enumeration was done after 48 h of incubation at 37 °C in an anaerobic atmosphere by plate counting method. The adhesion was expressed as the percentage of the number of bacteria adhered to the

total bacteria used for the experiment and calculated as:

$$\%\text{Adhesion} = \frac{\text{Initial count of bacteria}}{\text{Final count of bacteria}} \times 100$$

2.5. Influence of prebiotics on the growth of strains

Prebiotic influence on LAB growth was tested as described previously with slight modifications (Megur et al., 2023). Briefly, LAB cultures incubated at 37 °C for 24 h were administered in the MRS broth containing prebiotic 5% galacto-oligosaccharides (GOS) (King-Prebiotics®; New Francisco (Yunfu City) Biotechnology Corporation Limited; China) and 5% fructo-oligosaccharides (FOS) (Beneo, Mannheim, Germany), *i.e.*, the lowest concentration that elicited a significant increase in the growth of LAB. The concentration of 5% FOS and GOS was selected based on previous studies demonstrating significant stimulation of LAB growth (Megur et al., 2023). The LAB growth was noted at every 4 h interval at 37 °C by measuring absorbance at 600 nm. The optical densities were measured using a spectrometer (Eppendorf Bio spectrometer®, Hamburg, Germany). The initial optical density value of the media was deducted from the final value for each test sample.

2.6. Cell-free culture supernatant (CFCS) preparation

The following protocol was modified from previous studies (Kaewchomphunuch et al., 2022; K. M. Yang et al., 2021). The LAB strains were inoculated in MRS broth and grown at 37 °C with shaking overnight. Then, the inoculated MRS broth was transferred to 2 mL microcentrifuge tube and performed centrifugation for 3 min at 5000×*g* (Denville Micro 260D Microcentrifuge, Denville Scientific, Inc., Metuchen, NJ, USA). The supernatant was collected by sterile syringe with a needle and then filtered with a sterile polyethersulfone membrane filter with a pore size 0.22 µm (Guangzhou Jet Bio-Filtration Co., Ltd., Guangzhou, China).

2.7. Metabolite profiling of LAB

The metabolite profiling of LAB was monitored as previously described (Vaitekūnas et al., 2020). In the prebiotic-supplemented LAB CFS samples, metabolite concentrations were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS). First, the CFS of LAB were mixed with an equal volume of acetonitrile and centrifuged for 10 min at 10,000 rpm. The samples were analyzed using the Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector (Shimadzu, Kyoto, Japan) and LCMS-2020 mass spectrometer (Shimadzu, Kyoto, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro C18 column (3 × 150 mm; YMC, Kyoto, Japan) at 40 °C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) delivered in the 5–95% gradient elution mode. Mass scans were measured from *m/z* 50 up to m/z 2000 at a 350 °C interface temperature, 250 °C desolvation line (DL) temperature, ±4500 V interface voltage, and neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions software (Shimadzu, Kyoto, Japan). The detection wavelength is 254 nm and the retention time for tryptophan was 4.65 min. Production of tyrosine and gamma-aminobutyric acid (GABA) was detected by the mass spectrometry module of HPLC-MS system and quantified by the calibration curves of the standard compounds. The presence or absence of tyrosine and GABA was verified by spiking the samples with the corresponding compounds.

2.8. Gene prediction

Genome annotation was performed using Prokka v1.14.6 (Seemann, 2014) with default parameters and the UniProt bacterial database. Additional functional annotation was carried out using the RAST server (version 2.0) (https://rast.nmpdr.org/) (Meier-Kolthoff et al., 2022). Putative genes related to tryptophan metabolism and probiotic properties were further verified using BLASTp searches against the NCBI non-redundant protein database, with an identity threshold of >90% and alignment coverage was at least 60% relative to reference sequences.

2.9. Safety analysis of LAB

Safety evaluation of the LAB strains was conducted in accordance with the European Food Safety Authority (EFSA) guidelines for microorganisms intended for use in food and feed applications.

2.9.1. Determination of antibiotic susceptibility

Building upon previously reported methods (Shu et al., 2024), the susceptibility of LAB isolates to various antibiotics was evaluated. All antibiotics, rifampicin (Rif), kanamycin (Kan), streptomycin (Str), gentamicin (Gen), vancomycin (Van), erythromycin (Ery), tetracycline (Tet), ampicillin (Amp), and penicillin G (Pen) were procured from Carl Roth (Carl Roth, Karlsruhe, Germany). The disc diffusion method was employed to assess susceptibility, following established EFSA guidelines for antibiotic concentration selection (Aquilina et al., 2012). Each LAB isolate was exposed to discs containing 5 µg Rif, 30 µg Kan, 25 µg Str, 10 µg Gen, 30 µg Van, 15 µg Ery, 30 µg Tet, 10 µg Amp, and 10 µg Pen. Following incubation at 37 °C for 24 h, the agar plates were examined for the presence or absence of inhibition zones surrounding the antibiotic discs. If the zone of inhibition occurred then the strains are Susceptible, with no zone of inhibition the strains are considered resistant.

2.9.2. Mucin degradation test

The ability of the LAB strains to degrade mucin was evaluated using a previously established method with minor modifications (Megur et al., 2025). Briefly, the LAB cultures were grown in an MRS broth supplemented with mucin (0.5% *w/v*) (Thermo Fisher, Vilnius, Lithuania) as a carbon source. Following inoculation, the cultures were incubated aerobically at 37 °C for 48 h. Bacterial growth was monitored every 6 h by measuring the absorbance of the cultures at a wavelength of 600 nm using a spectrometer. *Escherichia coli* strain ATCC 25922 served as a positive control. This strain was grown in Luria broth (Sigma-Aldrich, Poznań, Poland) supplemented with mucin (0.5% w/v). Like the tested LAB isolates, *E. coli* was incubated aerobically at 37 °C for 48 h. Bacterial growth in the control cultures was also monitored by measuring optical density at 600 nm using a spectrometer. To account for the background signal, the initial optical density value of the media was subtracted from the final value for each test sample.

2.9.3. Search for antimicrobial resistance genes, virulence factors, and plasmid

In *in silico* analysis, it was conducted to identify genes associated with tryptophan, tyrosine, GABA, antimicrobial resistance (AMR) and virulence factors (VF) within the assembled genomes. Abricate software was employed to screen the genomes against reference databases, specifically Resfinder (https://genepi.food.dtu.dk/resfinder) for AMR genes and VFDB (https://www.mgc.ac.cn/VFs/) for VF genes (Florensa et al., 2022). A gene was considered present if its sequence exhibited greater than 90% nucleotide identity and aligned over 60% of the reference gene's length with the assembled genome. Additionally, MUMmer software was used to calculate the average nucleotide identity (ANI) between isolates. Finally, genome annotation of the assembled isolates was performed using the Prokka Annotation Pipeline. Multilocus sequence typing (MLST) was achieved by comparing *de novo* assemblies against the PubMLST database using BLAST (Megur et al., 2024).

2.9.4. Hemolytic activity

Hemolysis activity was determined by streaking isolates onto 5% Sheep blood Columbia agar plates (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 37 °C. Alfa(α)-hemolysis and beta(β)-hemolysis were denoted by greenish and translucent zones around the colonies, respectively. Gamma (γ)-hemolysis indicated the absence of hemolytic activity, characterized by the absence of clear zones around the colonies. *Staphylococcus aureus* ATCC 25923 was used as β-hemolytic control strain, respectively.

2.10. Statistical analysis

All experiments were performed in triplicate independent biological replicates, and each measurement was recorded as mean ± standard deviation (SD). Differences among multiple groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test to identify statistically significant pairwise differences. The significance level of $p < 0.05$ was considered statistically significant. Graphical representations include error bars indicating standard deviation. All statistical analyses were conducted using GraphPad Prism version 9.5.1 software (GraphPad Software Inc., Boston, USA).

3. Results

3.1. Isolation of LAB strains

In this study, 20 pure bacterial colonies were isolated, originating from diverse sources, including homemade cheese (1 isolate), fermented green beans (3 isolates), canned tomatoes (2 isolates), fermented cucumbers (1 isolate), sauerkraut (6 isolates), kombucha (2 isolates), yogurt (2 isolates), cucumber pickles (2 isolates), and homemade wine (1 isolate) (Supplementary table 1S). These isolates exhibited distinct morphologies (Supplementary fig. 1S). These isolates were subsequently added to the probiotic library curated by the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Center, Vilnius University, Lithuania. The LAB isolates underwent testing for probiotic properties, aiming at their resilience to acidic gastric juices they would encounter after consumption. These steps focused on assessing their suitability as probiotic candidates.

3.2. Survival in in vitro gastrointestinal conditions

3.2.1. Resistance to low pH

The LAB strains were subjected to pH 2.0 to observe their survival ability in *in vitro* conditions. Out of 20 isolates, only 15 strains showed survival abilities of 50% and above. Among the strains that were tested, KA13, KA18, KA19, and UV11 showed the highest survival abilities of 93.76 ± 3.71%, 83.16 ± 8.20%, 94.67 ± 3.39%, and 85.95 ± 7.65% respectively. The strains showing <50% survival abilities were KA3.2.2, KA8.2, KA9.2, KA14, KA15.1 with survival abilities of 30.78 ± 8.81%, 24.22 ± 1.69%, 23.81 ± 8.24%, 37.74 ± 8.24%, 27.62 ± 12.88% (Fig. 1). Acid tolerance suggests that the LAB isolates can endure in the stomach and the intestine without being degraded. The selected pH levels resemble the stomach environment, which helps to select probiotics that show resistance to significant acidic conditions. The strains exhibiting survival abilities <50% were excluded from the further steps.

3.2.2. Resistance to pepsin

The study examined the impact of pepsin activated at a low pH 2.0, and we tested the viability of 15 LAB isolates, testing their endurance in 0.3% bile salts for 4 h. Results exhibited that all LAB strains-maintained viability across these conditions. Lowest resistance to pepsin was observed in 8 strains KA2.1 (19.01 ± 5.22%), KA3.1 (17.23 ± 2.26%), KA9.2 (13.10 ± 1.93%), KA11.1 (9.25 ± 1.23%), KA14 (19.52 ± 3.35%), KA16 (12.91 ± 0.55%), KA17 (19.69 ± 1.31%), and UV11 (17.46 ± 2.13%) indicating their reduced survival abilities (< 20%). Furthermore, 3 LAB isolates showed pepsin resistance above 80% in the order UV16 (96.32 ± 0.30%), KA9 (94.45 ± 2.06%), and KA18 (82.80 ± 1.57%), while KA11.1 (9.253 ± 1.23%) showed the least survival ability (Fig. 2). This indicates that UV16, KA9, and KA18 might possess

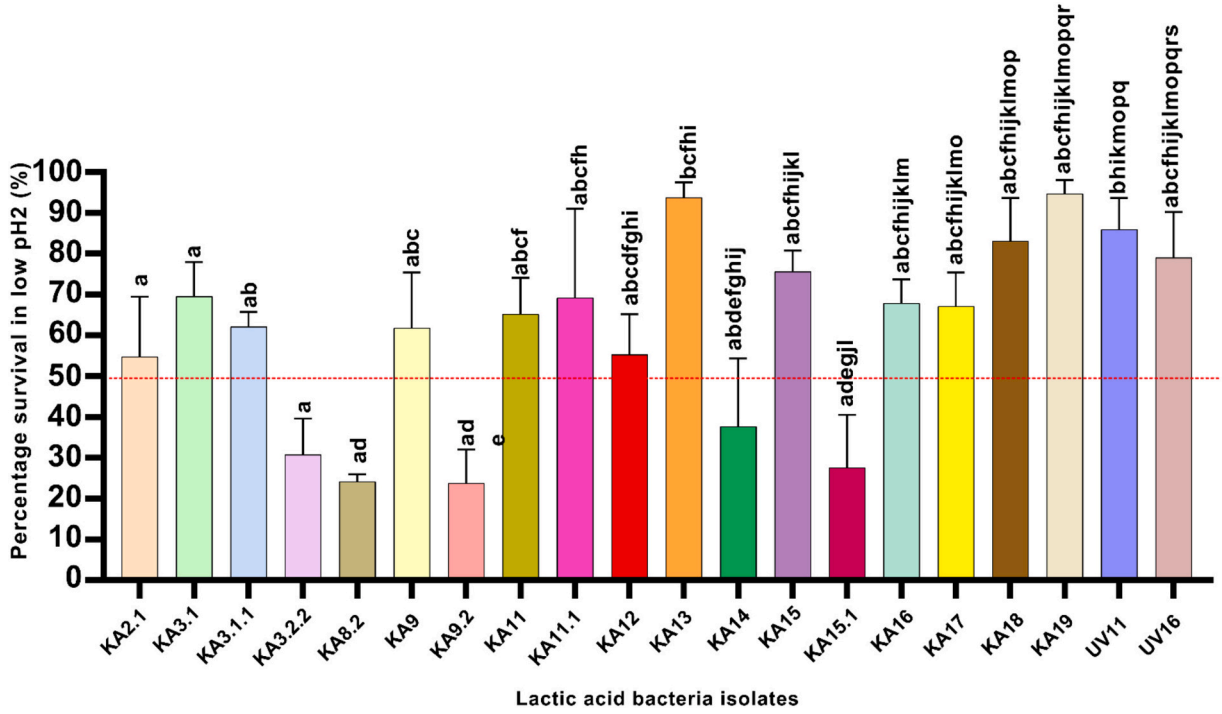

Fig. 1. Acid resistance of LAB in phosphate-saline buffer (pH 2.0). Values are expressed as mean ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significantly different whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimum percentage survival requirement of the individual isolates.

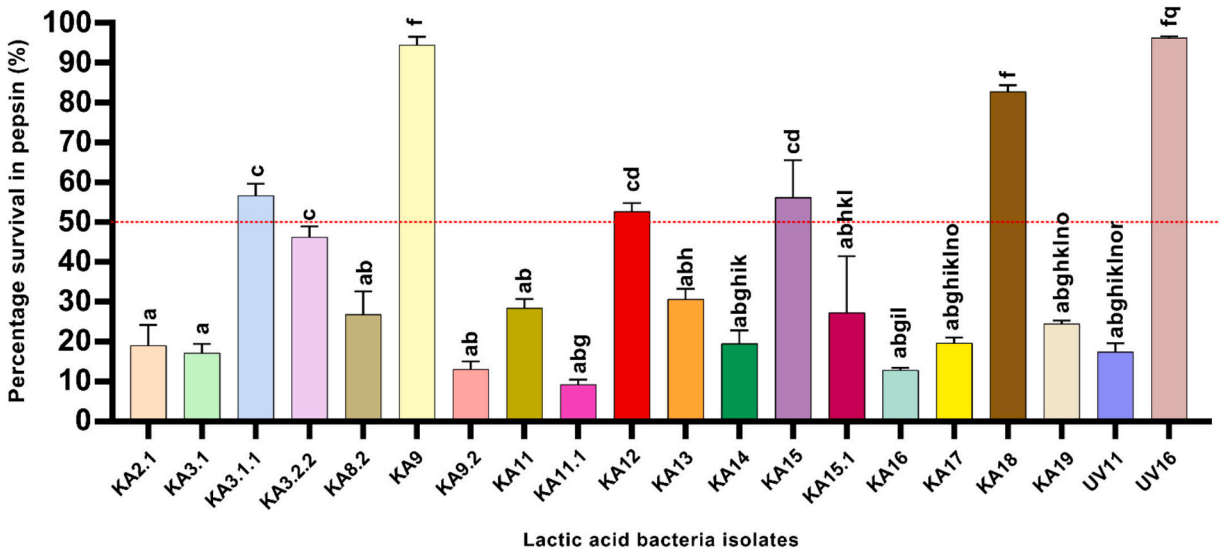

Fig. 2. Resistance of LAB to pepsin (pH = 2.0). Values are expressed as mean ± standard deviation (n = 3). Bars with the same lower-case letters are not significant, whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimal requirement for survival of the individual isolates.

adaptive mechanisms that confer an enhanced ability to withstand the harsh conditions posed by pepsin.

3.2.3. Resistance to bile salts

The study examined the impact of bile salts on the viability of 6 LAB strains which passed pepsin resistance, testing their endurance in 0.3% bile salts concentrations over 6 h. Results displayed all strains-maintained viability across these conditions. Specifically, strain KA12 exhibited remarkable resilience, with survival rates of 92.37 ± 2.04%. Following that, strains KA3.1.1 and KA15 showed resistance with 77.77 ± 0.69% and 73.94 ± 3.78%, respectively (Fig. 3). This indicates that LAB strains, KA12, KA3.1.1, and KA15, may have developed adaptive mechanisms that confer an enhanced ability to withstand the harsh conditions presented by bile salts.

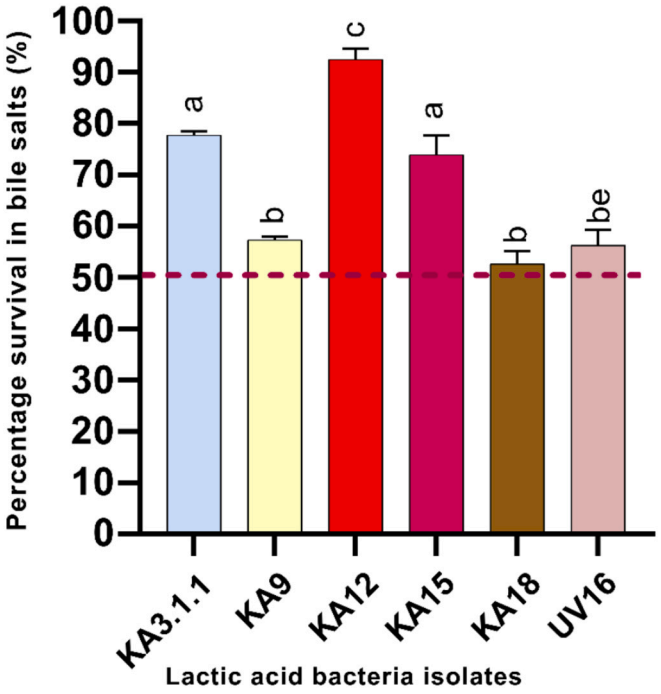

Fig. 3. Viability of LAB in the presence of simulated intestinal fluid (0.3% (*w/v*) bile salts in peptone water, pH 7.2). Values are expressed in mean ± standard deviation ($n = 3$). The dotted line represents the minimal requirement for survival of the individual isolates. Bars with the same lower-case letters are not significantly different, whereas those with different lower-case letters are significantly different ($p < 0.05$).

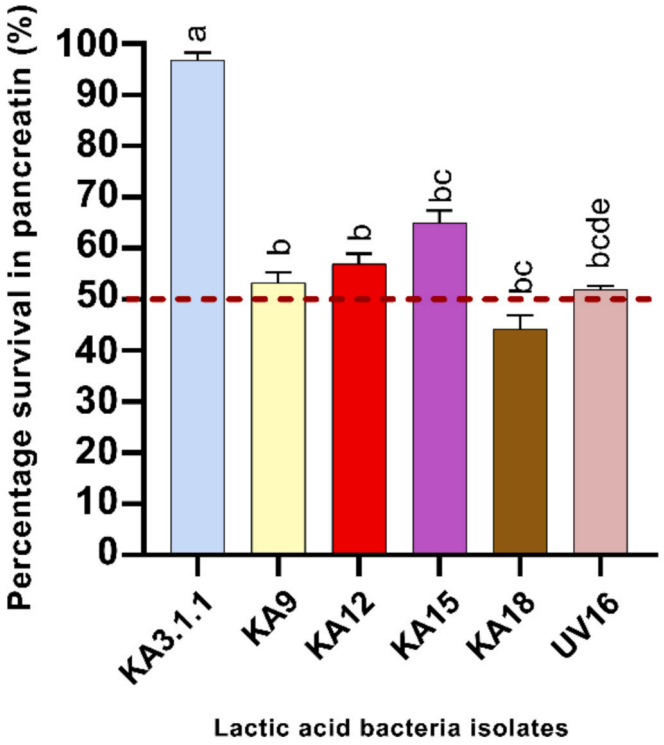

Fig. 4. Viability of LAB strains in the presence of 1 mg/mL pancreatin in 0.85% NaCl, w/v, pH 8.0). Values are expressed as the mean ± standard deviation (n = 3). Bars with the same lower-case letters are not significantly different, whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimal requirement of survival of the individual isolates.

3.2.4. Resistance to pancreatin

The study elevated the gastric tolerance of the LAB strains, particularly emphasizing their survival in highly acidic conditions mimicking the stomach. The LAB strain KA3.1.1 exhibited survival in an alkaline pancreatin solution with a viability of 96.82 ± 1.49%, and KA15 exhibited a viability of 67.04% (Fig. 4). The strain KA18 exhibited the least survival ability of 45.89%, hence excluded from further steps of probiotic identification and characterization. The strains that showed a survival ability of more than 50% highlight the acid-resistant characteristics of specific bacteria, underscoring their ability to endure the passage through the stomach and deliver health benefits in the gut.

3.3. Molecular identification of LAB candidates

The identification of LAB strains was executed by whole-genome sequencing. Table 1 details the identification of each isolate and provides the GenBank accession number for its closest neighbor in the National Center for Biotechnology Information (NCBI) database. Based on sequence similarity, the isolates were classified as: *Lactiplantibacillus plantarum* KA3.1.1, *Lactiplantibacillus plantarum* KA9, *Pediococcus pentosaceus* KA12, *Leuconostoc mesenteroides* KA15, and *L.fermentum* UV16. Supplementary Figs. 2S–6S depict the phylogenetic trees constructed using core genome SNP data, visually representing the evolutionary relationships among these LAB isolates. The genetic analysis revealed that these isolated strains share a high degree of similarity, implying a close relationship among them (Table 1). This insight is vital for understanding the characteristics of the strain and its potential applications in the development of the probiotic product.

3.4. In vitro indicators of gut colonization potential

LAB strains isolated from Lithuanian fermented foods were evaluated for *in vitro* properties associated with intestinal colonization, including antimicrobial activity, aggregation capacity, and adhesion to epithelial cells. These tests aimed to assess their viability as potential probiotic candidates. Moreover, their capability to inhibit microbial growth, maintain their properties, and produce metabolites essential for epithelial functions was examined, paving the way for the development of beneficial probiotic strains.

3.4.1. Probiotic antimicrobial activity

The antimicrobial activity of LAB strains was scrutinized using a disc diffusion method, focusing on their cell-free supernatant (CFS) that contains bioactive compounds. The assay involved testing these strains against 5 different enteropathogenic bacteria. Post 24 h and 48 h of incubation, the CFS of isolated LAB displayed a notable antagonistic activity, forming clearance zones indicative of bacterial inhibition (Table 2). Specifically, *L. plantarum* KA9 and *L. fermentum* UV16 CFS exhibited substantial antimicrobial activity against all tested pathogens, thus the results highlight beneficial impact against a range of enteric pathogens. Isolate *L. plantarum* KA3.1.1 CFS exhibited no activity to the pathogenic strains *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC27853 suggesting no broad-spectrum antimicrobial properties. However, all the strains showed low efficacy against *S. enteritidis* ATCC25928.

3.4.2. Auto-aggregation of LAB strains

The auto-aggregation rate of *L. fermentum* UV16 was notably high, reaching 71.09 ± 0.81% after 0.25 h and 93.50 ± 1.01% after 24 h of incubation, as outlined in Fig. 5. The strain *P. pentosaceus* KA12 showed the second highest auto-aggregation ability by reaching 59.73 ± 0.67% after 0.25 h and 74.60 ± 0.63% after 24 h. The least auto-aggregation ability was observed by *L. plantarum* KA3.1.1 exhibiting 41.68 ± 0.45% after 0.25% and 63.12 ± 0.83% after 24 h. They confirmed the clustering of cells, indicating strong self-adhesion properties. This trait is crucial for probiotics, as it enhances their stability and persistence in the gastrointestinal tract.

3.4.3. Co-aggregation of LAB strains

The co-aggregation between probiotic strains and pathogens is shown in Fig. 6. Among the probiotic strains tested, *L. plantarum* KA9 showed the highest co-aggregation abilities with *P. aeruginosa* ATCC27853 (49.74 ± 0.65%), *K. pneumoniae* ATCC 13883 (49.76 ± 0.55%) and *S. enteritidis* ATCC 25928 (52.48 ± 0.45%). The *L. fermentum* UV16 strain showed the most co-aggregation ability with *K. pneumoniae* ATCC 13883 (54.81 ± 0.42%). All probiotic strains tested were highly co-aggregated with *S. enteritidis* ATCC 25928 (35.79 ± 1.79 to 52.48 ± 0.45%). Among the probiotic strains, KA12 showed the least coaggregation abilities with *K. pneumoniae* ATCC 13883 (32.43 ± 0.96%), *S. enteritidis* ATCC 25928 (35.79 ± 1.79%), and *S. typhimurium* ATCC 14028 (34.51 ± 0.76%). *L. plantarum* KA3.1.1 demonstrated the least co-aggregation ability with *K. pneumoniae* ATCC 13883 (29.13 ± 1.22%). In general, this ability is particularly important for preventing intestinal colonization by harmful bacteria. Refer to Table 5 for strain overview.

3.4.4. Percentage adhesion on HCT116 cells

The study examined the colonization ability of 5 LAB strains using the HCT116 intestinal epithelial cell model after 48 h (Fig. 7). Adhesion levels of these LAB isolates on HCT116 cells varied from 16.63% to 53.33%. LAB strain *L. fermentum* UV16 showed the best adhesion ability of 53.33 ± 0.45% compared to isolate *L. plantarum* KA3.1.1, which had the least adhesion ability of 16.63 ± 0.95% (Fig. 7). Among the 5 isolates, *L. plantarum* KA9, *L. mesenteroides* KA15, and *L. fermentum* UV16 showed adhesion percentages >40%. Replication of results showed minimal variation, demonstrating consistency in the attachment capabilities of LAB strains (Table 5).

3.5. Influence of prebiotics on the growth of LAB strains

The study highlighted the prebiotic role enhancing the growth of LAB strains, particularly emphasizing their synbiotic potential to

Table 1
Species identification of LAB isolates.

Arbitrary name	Strain	Source	Closest Homolog	Core genome coverage	Gene Bank Accession (NCBI)
KA3.1.1	*Lactiplantibacillus plantarum* KA3.1.1	Fermented green beans	*Lactiplantibacillus plantarum* WLP04	74.3%	https://www.ncbi.nlm.nih.gov/nuccore/JAUKUH000000000
KA9	*Lactiplantibacillus plantarum* KA9	Sauerkraut	*Lactiplantibacillus plantarum* strain ZFM9	72.7%	https://www.ncbi.nlm.nih.gov/nuccore/JBCITF000000000.1
KA15	*Leuconostoc mesenteroides* KA15	Kombucha	*Leunostoc mesenteroides* strain SRCM102733	68.4%	https://www.ncbi.nlm.nih.gov/nuccore/JBCHJT000000000
KA12	*Pediococcus pentosaceus* KA12	Homemade Wine	*Pediococcus pentosaceus SL4GCF*	78.9%	https://www.ncbi.nlm.nih.gov/nuccore/JAUEMC000000000
UV16	*Limosilactobacillus fermentum* UV16	Cucumber pickle	*Limosilactobacillus fermentum* strain VHProbi O48	60.9%	https://www.ncbi.nlm.nih.gov/nuccore/JBCNWP000000000

Table 2
Antimicrobial activity of LAB strains on pathogenic enteropathogenic bacteria strains. (–) stands for not detected.

Pathogens causing dysentery	Isolated LAB strains				
	L. plantarum KA3.1.1	*L. plantarum* KA9	*P. pentosaceus* KA12	*L. mesenteroides* KA15	*L. fermentum* UV16
Salmonella enteritidis ATCC25928	0	19.62 ± 0.75	8.25 ± 0.28	12.05 ± 0.17	11.22 ± 0.22
E. coli P42	15.20 ± 0.33	16.32 ± 0.08	15.23 ± 0.24	–	11.13 ± 0.19
Salmonella Typhimurium ATCC 14028	17.14 ± 0.19	20.99 ± 0.12	–	21.55 ± 0.48	19.22 ± 0.27
Klebsiella pneumoniae ATCC 13883	–	17.30 ± 0.37	8.22 ± 0.33	20.11 ± 0.17	22.05 ± 0.07
Pseudomonas aeruginosa ATCC27853	–	21.01 ± 0.46	19.38 ± 0.14	22.57 ± 0.27	19.49 ± 0.56

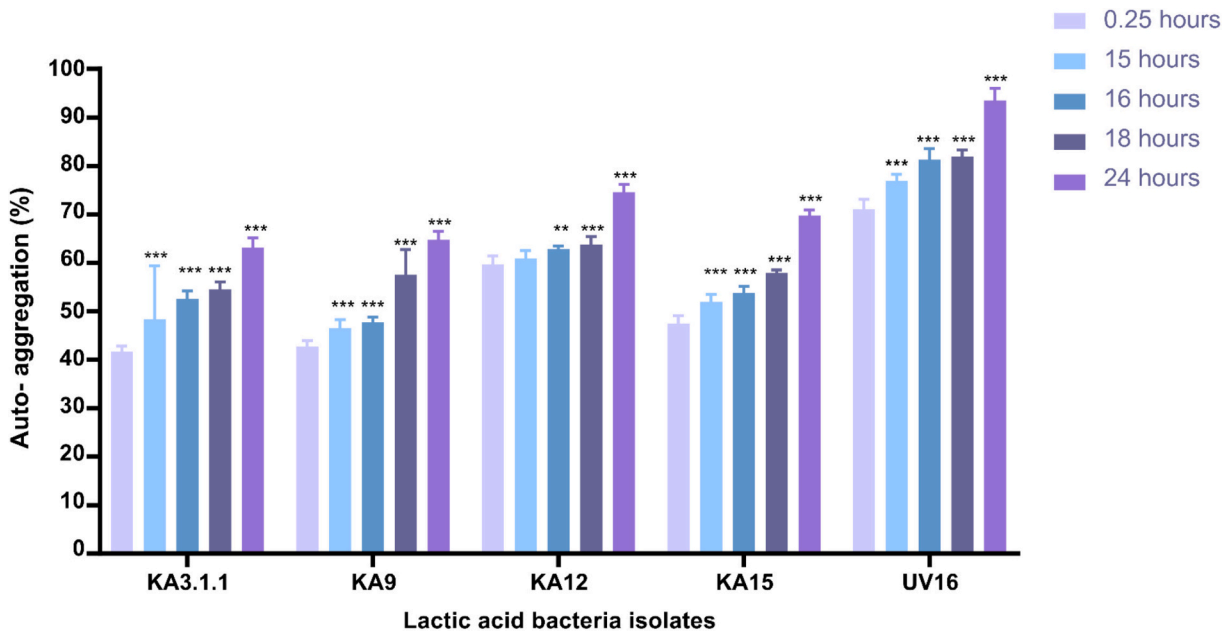

Fig. 5. Auto-aggregation abilities of potential probiotic LAB strains after 0.25 h, 15 h, 16 h, 18 h, and 24 h incubation at 37 °C. Each strain of auto-aggregation ability was compared at the initial time of 0.25 h. This prolonged incubation period allows for the observation of aggregation behavior over time, which is essential for assessing the robustness and viability of the probiotic strain under conditions like those in the human gut. The table likely includes quantitative data on the extent of auto-aggregation, possibly presented as percentages or through a specific metric that quantifies the degree of cell-to-cell adhesion. Such data is invaluable in determining the suitability of LAB as a probiotic, as effective auto-aggregation can enhance the bacteria's ability to form stable colonies in the gut, resist flushing out by intestinal movements, and exert their beneficial effects.

increase the number of beneficial bacteria in the gut. The LAB strains *L. plantarum* KA3.1.1, *L. plantarum* KA9, *L. mesenteroides* KA15 and *L. fermentum* UV16 exhibited enhanced growth in presence of 5% FOS and 5% GOS when compared to MRS supplementation. The strain *P. pentosaceus* KA12 showed enhanced growth in presence of 5% GOS as compared to 5% FOS and MRS supplementation (Fig. 8). Overall, FOS and GOS are utilized by the probiotic LAB as carbohydrate source, facilitating their proliferation while suppressing pathogenic bacteria in the gut, which leads to gut modulation.

3.6. Amino acid production by LAB

Tryptophan, an essential amino acid not synthesized by humans, is produced by beneficial microorganisms in the gut. The test designed to assess the tryptophan levels in the supernatant of the LAB bacteria emphasizes the potential of strains to produce the essential metabolite in the presence of glucose, with a notable increase in production of tryptophan in presence of 5% FOS and 5% GOS. Among all five strains tested, *L. mesenteroides* KA15 and *P. pentosaceus* KA12 produced tryptophan, with strain *L. mesenteroides* KA15 demonstrating a particularly significant increase in tryptophan production, reaching 10.48 μM when grown in MRS media supplemented with 5% FOS (Table 3). This concentration is comparable to levels reported for other tryptophan-producing LAB strains, which typically range from approximately 2–16 μM depending on strain and culture conditions. For strain *P. pentosaceus* KA12, MRS and 5% GOS supplementation led to tryptophan production of 2.69 μM and 1.69 μM, respectively, while no tryptophan production in 5% FOS supplementation (Table 3). This finding suggests that different prebiotics supplementation may enhance tryptophan production by certain LAB strains. Refer to supplementary 10S and 11S for calibration and detection curve in HPLC.

Finally, for the metabolite production of tyrosine and GABA, none of the strains showed the ability to produce these metabolites. The prebiotic administration of 5% GOS and 5% FOS did not have an influence on metabolite production (Supplementary table 3S – 4S).

3.7. Probiotic gene manual prediction

An in-depth study of the whole-genome sequence of the strains identified unique probiotic genes. Manual analysis and prediction were performed on the genome of *L. plantarum* KA3.1.1, *L. plantarum* KA9, *P. pentosaceus* KA12, *L. mesenteroides* KA15, and *L. fermentum* UV16 using the existing information available in the literature. This study aimed to identify genetic constituents linked to diverse probiotic features, including stress tolerance, bile salts hydrolase functionality, adhesive capacity, and immunomodulatory properties, thereby elucidating genomic probiotic efficacy. In our genomic analysis, a repertoire of genes encoding proteins associated with stress response was ascertained,

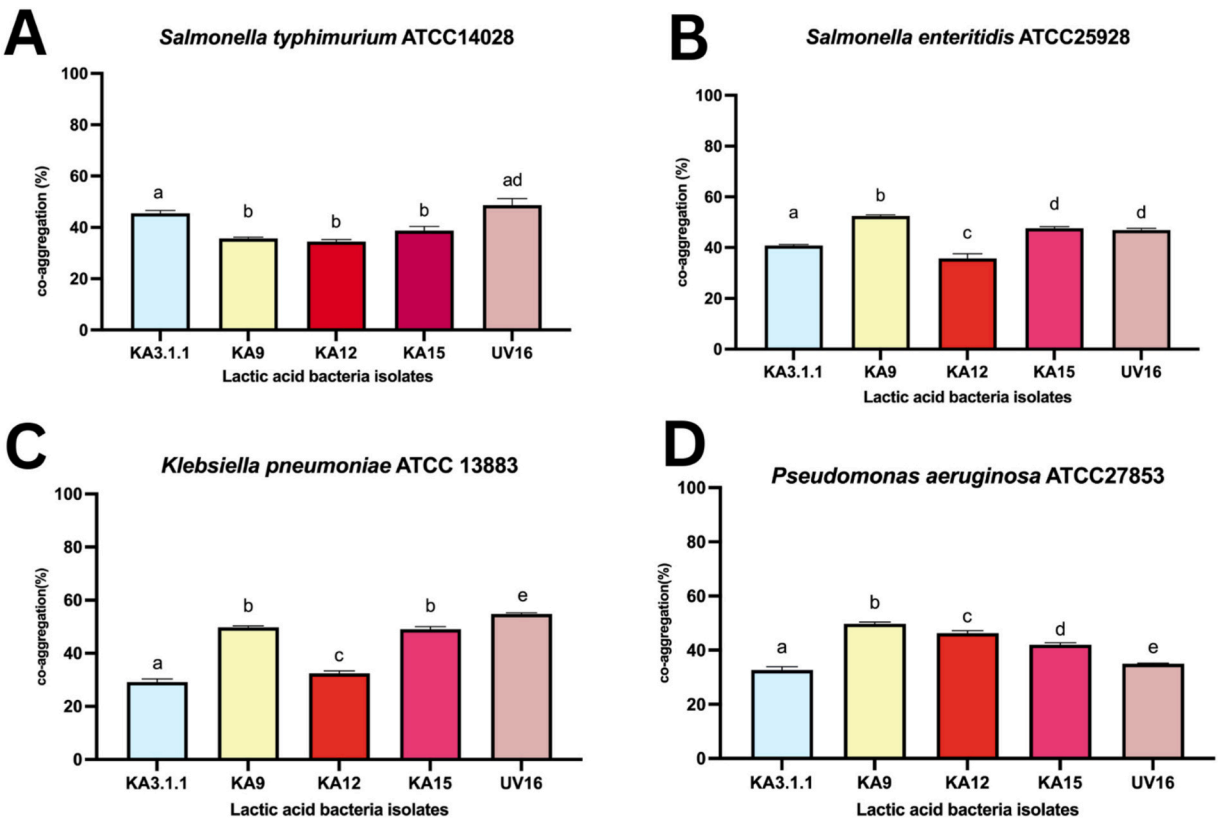

Fig. 6. Co-aggregation abilities of probiotic strains and food-borne pathogens, *Salmonella typhimurium* ATCC 14028 (A), *Salmonella enteritidis* ATCC 25928 (B), *Klebsiella pneumoniae* ATCC 13883 (C), and *Pseudomonas aeruginosa* ATCC 27853 (D) after 2 h incubation at 37 °C. Bars with the same lower-case letters are not significant, whereas those with different lower-case letters are significantly different ($p < 0.05$).

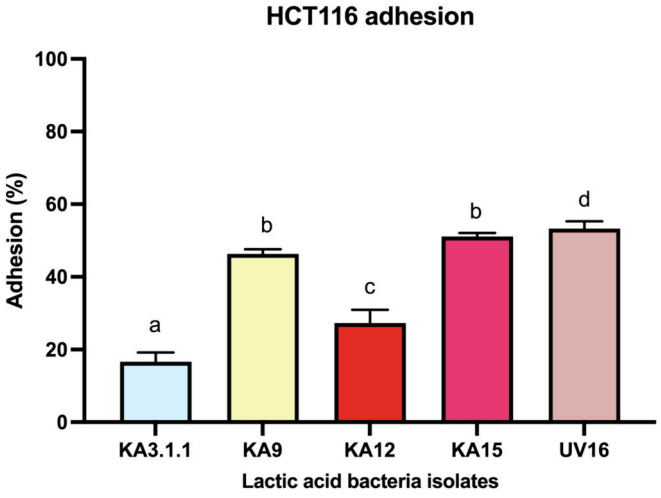

Fig. 7. Adhesion of LAB strains to HCT116 cells after 48 h incubation at 37 °C. Results are expressed as mean of triplicate values ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significant, whereas those with different lower-case letters are significantly different ($p < 0.05$).

as shown in Table 4.

Two genes related to L-tryptophan production were revealed. KA9 and KA15 possessed *trp*A gene, whereas KA9, KA12, and KA15 possessed *trp*B gene. For GABA metabolite production, *gad*B gene was found in KA9 and UV16. At last, for tyrosine metabolite production three genes *aro*C, *tyr*C, and *tyr*S were explored. All the strains possessed *aro*C gene, whereas KA15 possessed *tyr*C. Moreover, KA3.1.1, KA9, and KA15 possessed *tyr*S genes. KA15 harbored tryptophan and tyrosine production genes suggesting it as an ideal candidate for tyrosine and tryptophan production, compared to all the other strains.

A comparative evaluation of probiotic characteristics across the five selected LAB strains revealed clear strain-specific functional profiles. All strains demonstrated survival under simulated gastrointestinal conditions; however, their performance varied across antimicrobial activity, aggregation, adhesion, and metabolic activity parameters. *L.plantarum* KA9 and *L. fermentum* UV16 exhibited strong antimicrobial activity against multiple pathogens and high adhesion capacity, indicating good colonization potential. *P. pentosaceus* KA12 showed high bile tolerance and aggregation ability but was excluded from probiotic consideration due to antibiotic resistance and plasmid presence. *L. plantarum* KA3.1.1 showed good acid and enzymatic tolerance but limited metabolic activity and adhesion compared to other strains. Among all strains, *L. mesenteroides* KA15 demonstrated the most balanced probiotic profile, combining gastrointestinal survival, antimicrobial activity, aggregation ability, adhesion capacity, absence of virulence and resistance genes, and significant tryptophan production under prebiotic supplementation (Table 5). Overall, based on the combined functional, and metabolic characteristics, KA15 was identified as the most promising candidate for further safety food applications.

3.8. Safety analysis of LAB strains

3.8.1. Antibiotic susceptibility

All five LAB strains were resistant to kanamycin, gentamycin, and streptomycin. The *P. pentosaceus* KA12 strain showed important results because this culture was resistant to penicillin, kanamycin, vancomycin, and tetracycline (Table 6). Even though *L.fermentum* UV16 isolate showed resistance to only three of all tested antibiotics, it was still included in the subsequent safety steps.

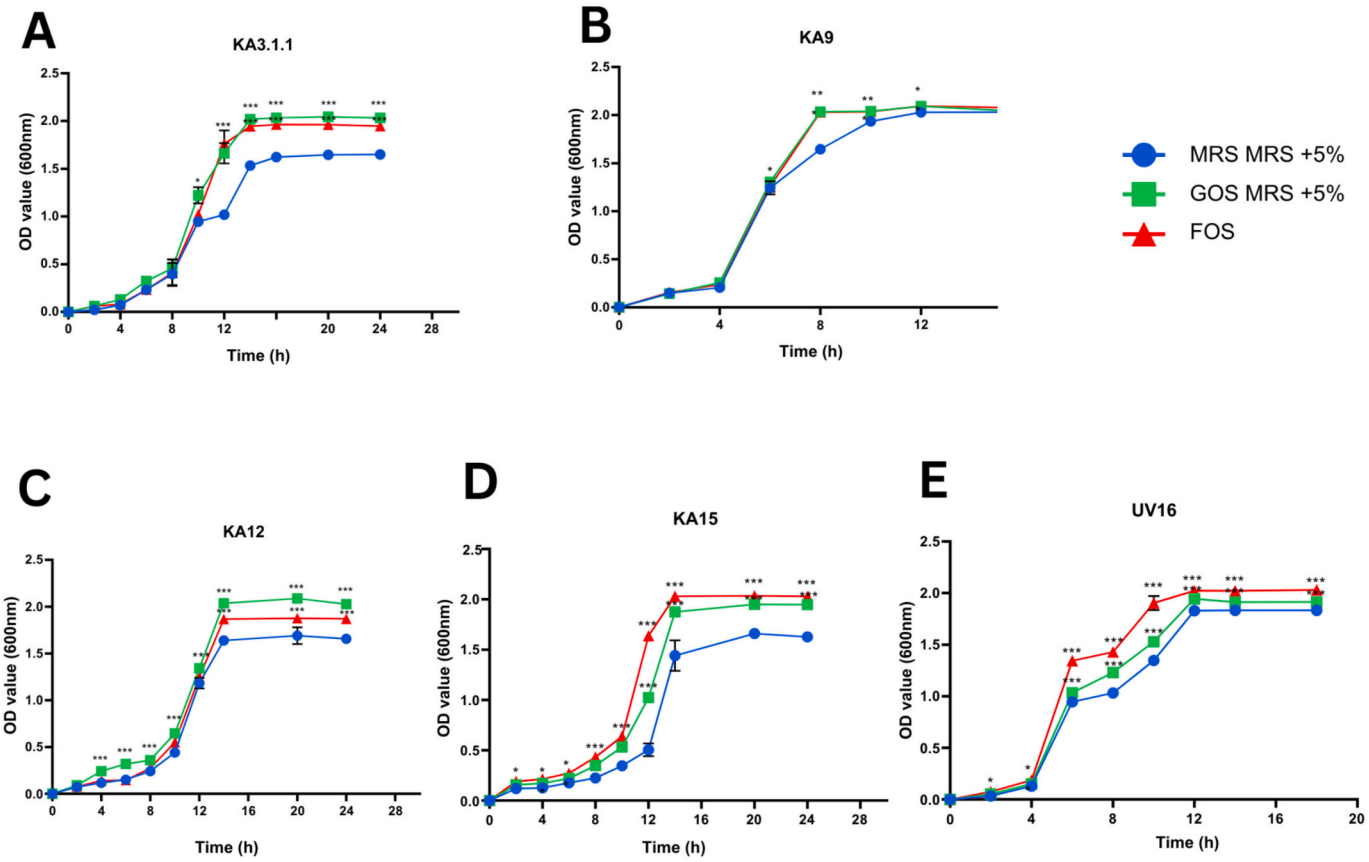

Fig. 8. The growth curves of LAB isolates were measured at 600 nm with 5% GOS and 5% FOS. Growth curve of (A) *L. plantarum* KA3.1.1 supplemented with 5% GOS and 5% FOS compared with *L. plantarum* KA3.1.1 with MRS, (B) growth curve of *L. plantarum* KA9 supplemented with 5% GOS, 5% FOS compared with *L. plantarum* KA9 with MRS, (C) growth curve of *P. pentosaceus* KA12 supplemented with 5% GOS and 5% FOS compared with *P. pentosaceus* KA12 with MRS, growth curve of (D) *L. mesenteroides* KA15 supplemented with 5% GOS and 5% FOS compared with *L. mesenteroides* KA15 with MRS, (E) growth curve of *L. fermentum* UV16 supplemented with 5% GOS and 5% FOS compared with *L. fermentum* UV16 with MRS. *Significant differences at $p < 0.05$, and ***significant difference at $p < 0.001$.

Table 3
Tryptophan production by LAB CFS by HPLC-MS in MRS, MRS + 5% GOS, and MRS + 5% FOS. Not detected (ND) indicates no tryptophan production.

LAB isolates	MRS (μM)	MRS + 5% GOS (μM)	MRS + 5% FOS (μM)
L. plantarum KA3.1.1	ND	ND	ND
L. plantarum KA9	ND	ND	ND
P. pentosaceus KA12	2.69	1.69	ND
L. mesenteroides KA15	ND	0.62	10.48
L. fermentum UV16	ND	ND	ND

3.8.2. Mucin degradation

The mucin-degrading ability of LAB strains was assessed by incorporating mucin into MRS media. This modification notably prolonged the exponential growth phase of *E. coli* ATCC 35150 compared to conditions without mucin. However, the growth of *L. plantarum* KA3.1.1, *L. plantarum* KA9, *L. mesenteroides* KA15, *P. pentosaceus* KA12, and *L. fermentum* UV16 strains were not enhanced by mucin supplementation (Fig. 9), indicating differing response to mucin between *E. coli* and LAB the two bacterial strains. This finding highlights the unique metabolic capabilities of LAB in the presence of mucin, contrasting with the growth patterns observed in *E. coli*.

3.8.3. Investigation for plasmids, virulence factors, antimicrobial-resistance genes, and multilocus sequence typing in LAB

To evaluate the genetic safety of the LAB strains, we screened their genomes for the presence of genes associated with antibiotic resistance and VF and checked for harbored plasmids. This screening utilizes

Table 4
Probiotic metabolite genes in isolated bacteria. (−) indicates no tryptophan production genes. (+) indicates presence of tryptophan production genes.

Genes	Function	Strains				
		KA3.1.1	KA9	KA12	KA15	UV16
Tryptophan production						
*trp*A	catalyzes the conversion of indole-3-glycerol phosphate into indole and glyceraldehyde-3-phosphate	−	+	−	+	−
*trp*B	responsible for the synthesis of L-tryptophan from indole and L-serine	−	+	+	+	−
GABA production						
*gad*B	encodes glutamate decarboxylase isoforms that catalyze the decarboxylation of glutamate to GABA	−	+	−	−	+
Tyrosine						
*aro*C	encodes chorismate synthase	+	+	+	+	+
*tyr*C	responsible for encoding prephenate dehydrogenase	−	−	−	+	−
*tyr*S	encodes tyrosyl-tRNA synthetase	+	+	−	+	−

Table 5
Summarized table presenting key probiotic and metabolic characteristics to facilitate direct comparison between strains. (−) indicates no production. (+) indicates growth observation.

Tests	KA3.1.1	KA9	KA12	KA15	UV16
Survival in gastro-intestinal tract					
Survival in pH 2.0 (%)	62.11	61.8	55.30	74.64	79.05
Survival in pepsin (%)	54.55	97.78	52.81	56.17	96.32
Survival in 0.3% bile salt (%)	77.77	55.13	92.37	73.94	54.98
Survival in pancreatin (%)	96.82	52.14	55.32	67.04	51.17
Antimicrobial activity					
Salmonella enteritidis ATCC25928 (mm)	0	19.62	8.25	12.05	11.22
E. coli P42 (mm)	15.20	16.32	15.23	–	11.13
Salmonella Typhimurium ATCC 14028 (mm)	17.14	20.99	–	21.55	19.22
Klebsiella pneumoniae ATCC 13883 (mm)	–	17.30	8.22	20.11	22.05
Pseudomonas aeruginosa ATCC27853 (mm)	–	21.01	19.38	22.57	19.49
Auto-aggregation after 24 h (%)					
24 h	63.12	64.76	74.60	69.73	93.50
Co-aggregation (%)					
Salmonella Typhimurium ATCC14028	42.52	35.70	34.51	38.80	48.73
Salmonellaenteritidis ATCC25928	40.81	52.48	35.79	47.63	46.94
Klebsiella pneumoniae ATCC 13883	29.13	49.76	32.43	49.04	54.81
Pseudomonas aeruginosa ATCC27853	32.67	49.74	46.33	41.99	34.99
Adhesion to HCT116 colon cell lines					
Adhesion (%)	16.63	46.30	27.32	51.14	53.33
L-Tryptophan Detection in HPLC-MS analysis					
MRS, Concentration (μM)	–	–	2.69	–	–
MRS + 5% GOS, Concentration (μM)	–	–	1.69	0.62	–
MRS + 5% FOS, Concentration (μM)	–	–	–	10.48	–
Growth of LAB under prebiotic supplementation					
MRS	+	+	+	+	+
MRS + 5% GOS	++	++	++	++	++
MRS + 5% FOS	++	++	+++	+++	+++

Abricate, a software tool, and databases like Resfinder AMR and VFDB VF (Supplementary fig. 7S). Encouragingly, no genes associated with antibiotic resistance or VF were detected in LAB isolates *L. mesenteroides* KA15, *L. plantarum* KA9, and *L. fermentum* UV16. In contrast, the isolate *L. plantarum* KA3.1.1 and *P. pentosaceus* KA12 harbored plasmid repA (pR18) (Table 7). To provide a clearer overview of genomic characteristics and safety-related features of the LAB isolates, a comparative genome feature table summarizing species identification, genome similarity, virulence factors, antimicrobial resistance genes, plasmid presence, and tryptophan biosynthesis genes was constructed (Tables 5 and 7). The isolates *L. mesenteroides* KA15, *L. plantarum* KA9, and *L. fermentum* UV16 were selected as they fit the criteria of safety and passed the EFSA requirements, hence continuing with testing for hemolysis.

3.8.4. Hemolytic activity

According to the EFSA criteria, the LAB strains should not exhibit hemolytic activity, harming red blood cells and leading to anemia. The test was critical to evaluate the safety of the *L. mesenteroides* KA15, *L. plantarum* KA9, and *L. fermentum* UV16 before selection as the potential probiotic. No hemolysis was observed on the blood agar plates of all three isolates, but the control strain *Staphylococcus aureus* ATCC 25923 exhibited hemolysis (Supplementary fig. 8S). This test suggests that *L. mesenteroides* KA15, *L. plantarum* KA9, and *L. fermentum* UV16 strains do not possess hemolytic enzyme activity, aligned with the safety criteria expected by EFSA probiotics selection.

4. Discussion

LAB are commonly found in fermented foods and play a crucial role in preserving and enhancing nutritional content through fermentation and the production of numerous metabolites essential for human well-being (Banan-Mwine Daliri et al., 2023; Daliri et al., 2025). To qualify as probiotics, LAB strains must survive in the gut, remain viable in large quantities, be beneficial, safe, and stable during storage. They function by generating beneficial compounds, boosting the immune system, and improving the host immune response and gastrointestinal functions. In this study, 20 morphologically distinct probiotic LAB strains were isolated from fermented foods obtained from Lithuania, with a focus on discovering new probiotic strains with specific benefits. LAB strains, recognized for their high acid tolerance and stability, are being extensively investigated for their probiotic properties. However, safety concerns associated with these strains necessitate comprehensive evaluations. Probiotics must comply with guidelines set by the EFSA, confirming their safety, non-toxicity, and inability to transfer antimicrobial resistance.

Furthermore, once consumed by humans, these LAB should be able to survive in the gastrointestinal tract and adhere to the colon. The human gastrointestinal tract contains various enzymes and low pH conditions that may inhibit microbial survival (Stasiak-Różańska et al., 2021). Therefore, these probiotics need to survive these harsh conditions to elicit their health benefits. The initial phase of digestion begins in the mouth, where the strains interact with lysozymes present in saliva, then proceed to the gastrointestinal juices and finally reach the intestine (Sionek et al., 2024). As the pH of gastric juice ranges from 1.5 to 3.5, it is necessary to test the survival ability of probiotic candidates in low pH environments and in the presence of pepsin (Bisson et al., 2023; Corcoran et al., 2005). A low pH in gastric juices significantly influences the membrane pathway and cellular mechanisms of LAB by affecting their survival and functionality. LAB relies on the F1Fo-ATPase enzyme to regulate their intracellular pH and generate proton motive force (Corcoran et al., 2005). LAB can neutralize acidic environments through various mechanisms, including biofilm formation and maintaining high

Table 6
Susceptibility of LAB isolates to nine antibiotics (R, Resistant; S, Susceptible). List of used antibiotics and explanation of their abbreviations. RIF, Rifampicin; ERY, Erythromycin; PEN, Penicillin G; AMP, Ampicillin; KAN, Kanamycin; GEN, Gentamycin; STR, Streptomycin; VAN, Vancomycin; TET, Tetracycline.

LAB isolates	RIF	ERY	PEN	AMP	KAN	GEN	STR	VAN	TET
L. plantarum KA3.1.1	S	R	S	S	R	S	R	R	S
L. plantarum KA9	S	S	R	S	R	S	R	R	S
P. pentosaceus KA12	S	S	R	S	R	S	S	R	R
L. mesenteroides KA15	R	R	S	S	R	R	R	R	S
L. fermentum UV16	S	S	S	S	R	S	R	R	S

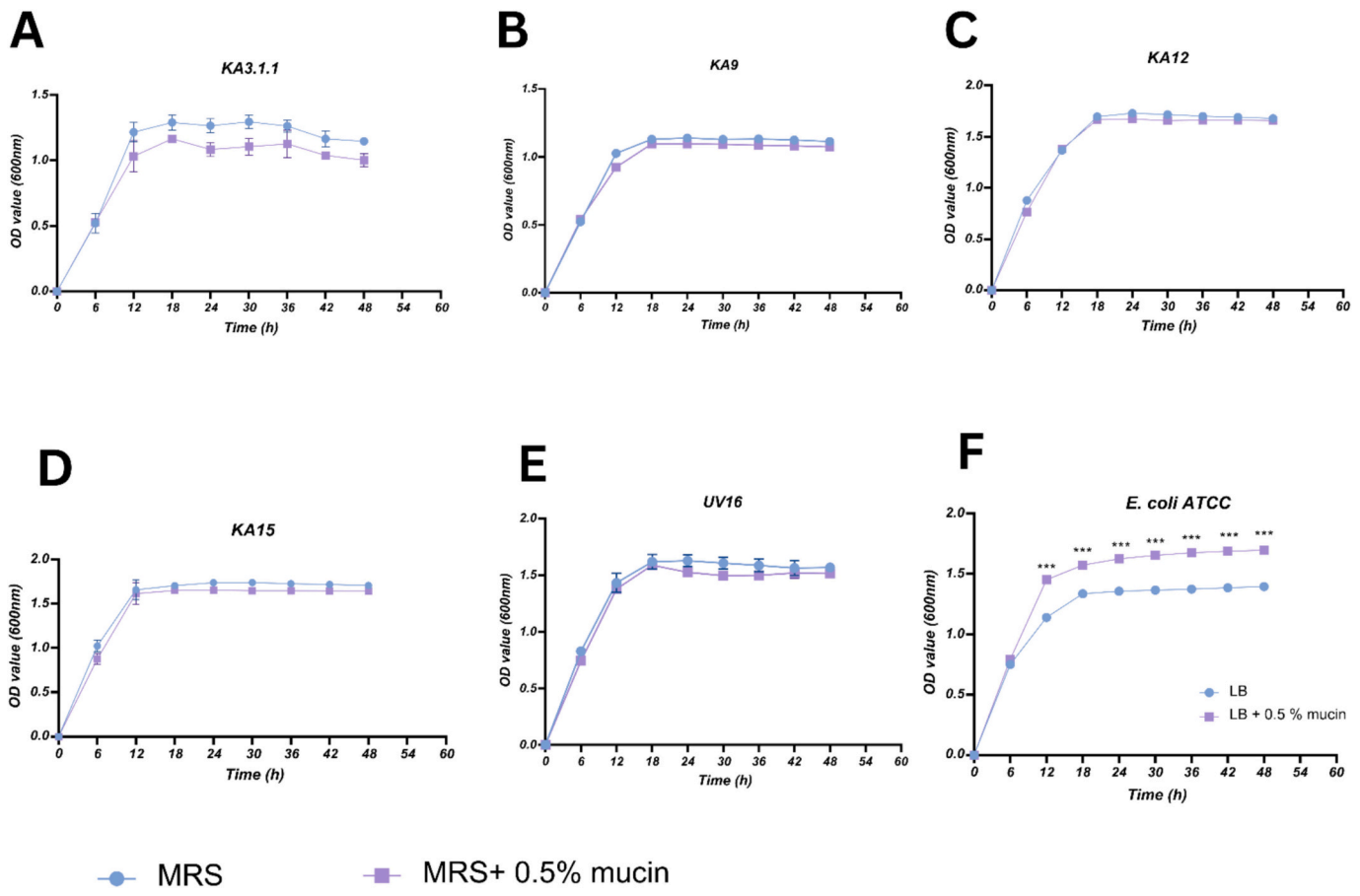

Fig. 9. Mucin degradation ability of probiotic candidates. (A) growth curve of *L. plantarum* KA3.1.1 supplemented with 0.5% mucin compared with *L. plantarum* KA3.1.1 without mucin, (B) growth curve of *L. plantarum* KA9 supplemented with 0.5% mucin compared with *L. plantarum* KA9 without mucin, (C) growth curve of *P. pentosaceus* KA12 supplemented with 0.5% mucin compared with *P. pentosaceus* KA12 without mucin, (D) growth curve of *L. mesenteroides* KA15 supplemented with 0.5% mucin compared with *L. mesenteroides* KA15 without mucin, (E) growth curve of *L. fermentum* UV16 supplemented with 0.5% mucin compared with *L. fermentum* UV16 without mucin, (F) growth curve of *E. coli* ATCC 3515 supplemented with 0.5% mucin compared with *E. coli* ATCC 3515 without mucin. **Significant difference at $p < 0.01$, and ***significant difference at $p < 0.001$.

Table 7
Comparative genomic features of LAB isolates identified by whole-genome sequencing.

Strain	Species identification	AMR genes	Virulence genes	Plasmids	Tryptophan genes (*trp*A/*trp*B)
KA3.1.1	*Lactiplantibacillus plantarum*	Not detected	Not detected	repA detected	–/–
KA9	*Lactiplantibacillus plantarum*	Not detected	Not detected	Not detected	+/+
KA12	*Pediococcus pentosaceus*	Not detected	Not detected	repA detected	–/+
KA15	*Leuconostoc mesenteroides*	Not detected	Not detected	Not detected	+/+
UV16	*Limosilactobacillus fermentum*	Not detected	Not detected	Not detected	–/–

cell density, which collectively help in buffer the surrounding pH (Matsui & Cvitkovitch, 2010). Additionally, LAB can undergo pre-adaptation to acidic conditions, which enhances their survival (Bisson et al., 2023; Sionek et al., 2024). Cross-protection mechanisms also play a role, where exposure to one stressor increases resistance to another (Sionek et al., 2024). In our study, out of 20 isolates subjected to pH 2.0, only 15 LAB isolates showed survival rates above 50%, indicating generally high acid tolerance. These findings are consistent with several studies reporting similar acid tolerance ranges for probiotic LAB strains. The *L. plantarum* isolates-maintained viability above 85% after exposure to pH 2 for 2 h, comparable to our highest surviving isolates (X. Huang et al., 2023). Similarly, in another study, *Lactobacillus* isolates from fermented food survived at low pH with survival rates of 70–90% (Bhatt et al., 2024), supporting the adaptability of LAB isolates to harsh gastric conditions.

Moreover, the isolates tested in 0.3% bile salts exhibited survival and viability, with strain KA12 showing the highest survival (>92%) after 6 h. Bile tolerance is associated with adaptive mechanisms such as the presence of bile salts hydrolase enzymes, which deconjugate bile salts and reduce their toxicity. Bile salts hydrolase enzymatic activity is documented mostly in *Lactobacillus* and *Bifidobacterium*, where it helps bacteria to remove bile salts and maintain membrane integrity (Ruiz et al., 2013; Ruiz-Ramírez et al., 2023). Some bile-resistant LAB isolates can regulate their membrane composition by activating efflux pumps to extrude bile salts from cells, enhancing their survivability (Ruiz et al., 2013). The observed bile tolerance of the current isolates is consistent with previous research, where LAB survived and thrived at bile concentrations from 0.3% to 2%, emphasizing their potential to colonize the small intestine (Ashraf & Smith, 2016; Hassanzadazar et al., 2012; Nawaz et al., 2017; Prete et al., 2020).

Exposure of LAB isolates to pancreatin stimulates the small intestinal environment, including lipase and protease activity at alkaline pH

(Kieliszek et al., 2021). Protection of LAB isolates against protease and lipase suggests a strong cell wall structure, production of protease inhibitors, or rapid repair mechanisms (Sénéchal et al., 2014). In the current study KA3.1.1 showed high survival ability, while KA18 had lower viability. Survival in pancreatin indicates the strains' ability to withstand proteolytic degradation and alkaline stress, which is crucial for reaching the lower intestinal tract (Masco et al., 2007). This enzyme and low acid resistance are often related to probiotic strains' cell wall function and synthesis of protective stress proteins (Ashraf & Smith, 2016; Tokatl et al., 2015).

Furthermore, LAB exhibits antimicrobial activity against various enteropathogens, including *S. aureus*, *S.Typhimurium*, *S. pyogenes*, and *K. pneumoniae* (Kiousi et al., 2023). The agar well diffusion assay revealed that *L. plantarum* KA9 and *L. fermentum* UV16 exhibited the strongest antimicrobial activity, as indicated by the formation of clear inhibition zones. These findings are consistent with previous research suggesting that LAB possess significant inhibitory effects against both Gram-positive and Gram-negative pathogens (Cirat et al., 2024). The primary mechanism involves the production of lactic acid and other inhibitory compounds. LAB, such as *L. acidophilus* and *L. rhamnosus*, produce lactic acid, which lowers the pH and creates an inhospitable environment for pathogens like *S.Typhimurium* and *S. aureus* (Anumudu et al., 2024; Ibrahim et al., 2021). Some LAB strains produce additional antimicrobial substances. For instance, *L. johnsonii* and *L. plantarum* produce unknown inhibitory compounds that work synergistically with lactic acid to inhibit pathogens (Scillato et al., 2021; F. Huang et al., 2024). Similarly, *Weissella*spp. produces metabolic extracts that interfere with biofilm formation and quorum sensing in *Salmonella*, enhancing antimicrobial efficacy (Kostoglou & Giaouris, 2025). LAB can also produce bacteriocins, which are proteinaceous toxins that inhibit closely related bacterial strains (Darbandi et al., 2022). In the present study, the CFS was neutralized to a pH of 7.2, eliminating the inhibitory effect due to acidification. Thus, antimicrobial activity can be attributed to other non-acidic antimicrobial compounds, likely peptide-based inhibitors and bacteriocins.

The high auto-aggregation percentage of probiotics, which enables them to adhere to each other, is a crucial property for successful colonization of the gastrointestinal tract (Isenring et al., 2021). In our study, *L. fermentum* UV16 (93.5%) displayed robust self-adhesion, making it more likely to adhere and resist intestinal transit, thereby maintaining its persistence in the gut environment (Chantanawilas et al., 2024; Zawistowska-Rojek et al., 2022). Strong auto-aggregation can also be related to the formation of biofilms, creating stable communities that enhance probiotic survival and activity within the host's gastrointestinal tract (Chantanawilas et al., 2024). Auto-aggregating probiotics occupy adhesion sites which reduces the chances of pathogen colonization in the gut (Chantanawilas et al., 2024). In a recent study, *L. fermentum* SD7 was found to exhibit high auto-aggregation (63%), which correlated with stronger colonization ability, while *L. casei* Shirota showed lower auto-aggregation (22%) (Chantanawilas et al., 2024). Moreover, studies on lactobacilli have observed similar ranges (15–62%), indicating that the values observed in the current study are high for strains like *L. fermentum* UV16 and *P. pentosaceus* KA12 (Izhar et al., 2024). In addition to auto-aggregation, probiotic bacteria utilize exopolysaccharides, surface proteins, and various other adhesion molecules to bind to both host epithelial cells and pathogenic bacteria (Kaur & Dey, 2023; Singh et al., 2021). This co-aggregation ability allows probiotics to outcompete the pathogens for mucosal binding and physically block their colonization (Vinayamohan et al., 2024). Studies have shown that the co-aggregation ability of *L. plantarum* against pathogens *E. coli*, *S. typhimurium*, and *K. pneumoniae*, ranging from 20% to over 50%, aligns closely with our findings of 30% to 54% (Izhar et al., 2024; Sohn et al., 2020; Zawistowska-Rojek et al., 2022). Many LAB strains can produce bacteriocins and organic acids that lower intestinal pH and directly harm pathogens, further hindering pathogen growth and colonization (Golletz et al., 2025; Pessione, 2012). The high co-aggregation potential observed in *L. plantarum* KA9 and *L. fermentum* UV16 is consistent with previous research highlighting strain-specific functional characteristics among probiotic bacteria.

Moreover, the ability of probiotics to adhere to the intestinal mucosa is a critical characteristic, as it ensures their persistence in the gut and enables them to suppress pathogenic bacteria and interact with the host's immune system. In exploring this aspect, the study investigated the adherence capabilities to HCT116 cells. HCT116 cells are derived from human colorectal carcinoma and may exhibit differences in surface glycoprotein composition, receptor expression, and mucus production compared to normal intestinal epithelial cells (Yeung et al., 2010). Consequently, adhesion results obtained using this model should be interpreted as an initial screening of colonization potential rather than a direct representation of *in vivo* intestinal adhesion (Rosidi et al., 2023). The results showed strong adhesive properties of LAB isolates (16.63%–53.33%). A similar adhesion range was observed in the study of numerous LAB strains on HCT116 cells (Emmawati et al., 2016). For instance, some LAB strains were able to adhere up to 60%, while others fell in the range of 10–20% (Megur et al., 2023). The values observed in this study for five LAB isolates are within the expected spectrum and underscore the strain-specific nature of adhesion. The highest adhesion of *L. fermentum* UV16 to HCT116 cells is also observed in *L. fermentum* strains, which are increasingly recognized for their robust mucosal binding and probiotic properties (Shirvanian et al., 2023). The adhesion ability observed for *L. fermentum* UV16 (53.3%) falls within or above the range reported for established probiotic strains. For example, *Lactobacillus rhamnosus* GG exhibits adhesion rates of approximately 20–24% to HCT116 cells under comparable experimental conditions (Yan et al., 2026). Moreover, the role of bacterial surface proteins in mediating adhesion is well-studied. In one study, removal of surface proteins from *L. plantarum* HC-2 reduced adhesion to HCT116 cells from 98.1% to 20.9% (Du et al., 2022). This highlights the importance of surface protein molecules in host interaction, suggesting that high-adhesion strains may possess specific exopolysaccharides or surface adhesins that enhance their attachment. While the *in vitro* assays performed in this study provide valuable information regarding probiotic characteristics such as adhesion, aggregation, and pathogen inhibition, these results should be interpreted cautiously when considering intestinal colonization *in vivo*. The human gastrointestinal tract is a highly complex and dynamic environment influenced by host factors, immune responses, resident microbiota, and mucus layer interactions that cannot be fully replicated in simplified *in vitro* models (Skok et al., 2025). Therefore, the aggregation and adhesion properties observed in this study should be considered preliminary indicators of colonization potential rather than definitive evidence of stable gut colonization (Kim et al., 2026). Future investigations using advanced intestinal models, such as mucus-producing epithelial cell lines, intestinal organoids, or animal models, would be valuable to further validate the colonization capacity and functional activity of these LAB strains *in vivo*.

The combination of prebiotics and probiotics, known as synbiotics, aims to enhance probiotic properties and improve the host functions by increasing metabolite production, modulating the gut environment, and suppressing pathogenic bacteria in the gut. This study demonstrated that the supplementation with FOS and GOS significantly enhanced the growth of LAB isolates compared to the MRS medium. The increased proliferation with prebiotic supplementation highlights their synbiotic potential. The isolated *P. pentosaceus* KA12 responded best to GOS supplementation, displaying strain-dependent utilization of specific prebiotics. Many LAB strains differ in their ability to utilize prebiotics. The probiotic strain *L. plantarum* has consistently shown improved growth with both GOS and FOS (Cao et al., 2019a), a finding similar to our study. Moreover, *P. pentosaceus* strains, as observed with KA12 in our study, preferentially use GOS for proliferation (Megur et al., 2025; Plaza-Díaz et al., 2018). Recent research on FOS and GOS supplementation indicates an increase in LAB populations while reducing *E. coli* and *Clostridium perfringens* in animal and human models (Chang et al.,

2022; Davani-Davari et al., 2019; Ding et al., 2019). These LAB isolates possess specific enzymes that pathogenic bacteria lack, such as β-galactosidase and β-fructosidase, enabling them to metabolize FOS and GOS (Maske et al., 2021). The metabolism of these prebiotics leads to increased production of SCFAs and tryptophan, a precursor of serotonin (Gao et al., 2020b; Megur et al., 2022). Tryptophan biosynthesis in bacteria occurs through the shikimate pathway, which generates aromatic amino acids from central metabolic intermediates (Shende et al., 2024a). In this pathway, phosphoenolpyruvate and erythrose-4-phosphate serve as key precursors leading to the formation of chorismate, the common precursor of aromatic amino acids including tryptophan (Tzin & Galili, 2010). Moreover, the concentration of tryptophan produced by *L. mesenteroides* KA15 reached 10.48 μM in the presence of FOS. Previous studies have shown that micromolar concentrations of tryptophan and its derivatives can influence host metabolic and immune pathways through activation of the aryl hydrocarbon receptor (AhR) and modulation of serotonin synthesis in intestinal cells (Li, 2023). In the intestinal environment, microbial metabolism of tryptophan contributes to the pool of bioactive metabolites involved in gut–brain signaling (Rosell-Cardona et al., 2025). Therefore, the production of tryptophan at micromolar concentrations by KA15 suggests potential biological relevance, although further *in vitro* or *in vivo* studies are required to confirm host physiological effects.

In this study, the genome LAB isolates *L. plantarum* KA9, *L. fermentum* UV16, and *L. mesenteroides* KA15 revealed the presence of *gad*B gene which is associated with GABA production. Despite the genetic potential, no metabolite production was observed in the *in vitro* assays. Similarly, the isolates also possessed the tyrosine producing genes *aro*C, *tyr*C and *tyr*S, but HPLC analysis did not identify any corresponding metabolite production. Tryptophan biosynthesis in LAB occurs *via* the shikimate pathway, where chorismate serves as a precursor for aromatic amino acids through enzymes encoded by the *trp* operon (Shende et al., 2024b). These observations suggest that the presence of genes does not guarantee the active metabolite production in these strains. It can even be possible that these genes are pseudogenes, which as present in the genome but are not functionally expressed. This phenomenon has been previously reported in studies investigating GABA production by LAB isolates (Phonghanpot & Jarintanan, 2022; Weber et al., 2015). Although the strains might harbor the *gad*B gene, the GABA production levels vary widely and depend on strain-specific factors and substrate availability (Otaru et al., 2021; Pizzi et al., 2025). Moreover, the presence of *trp*A and *trp*B genes in the isolated LAB strains indicates the genetic potential for tryptophan biosynthesis. Enzymes encoded by the *trp* operon, particularly *trp*A and *trp*B, catalyze the final steps of converting indole-3-glycerol phosphate to L-tryptophan. The presence of these genes in the genomes of several isolates indicates their genetic capacity to synthesize tryptophan (Ferrer et al., 2022; Michalska et al., 2021). Interestingly, *P. pentosaceus* KA12 produced detectable tryptophan despite the absence of the annotated *trp*A gene, while *L. plantarum* KA9 harbored both *trp*A and *trp*B genes but did not produce detectable tryptophan. This discrepancy may be explained by the presence of alternative metabolic routes contributing to tryptophan synthesis (Li et al., 2025). Additionally, the expression of tryptophan biosynthesis genes is tightly regulated and depends on environmental conditions and substrate availability (Ramos-Valdovinos & Martínez-Antonio, 2024). Previous studies have shown that the presence of biosynthetic genes does not necessarily guarantee metabolite production due to transcriptional regulation or metabolic flux limitations (Baral et al., 2018; Reshi et al., 2023). On the other hand, tryptophan production is vigorously regulated and may only be activated under specific nutritional conditions, such as specific carbon/nitrogen sources (Raethong et al., 2025). GOS and FOS serve as carbohydrate sources for LAB (Cao et al., 2019b). The observed increase in tryptophan production in the presence of prebiotics such as FOS and GOS may be explained by changes in metabolic flux through central carbon metabolism (Shende et al., 2024b). Both FOS and GOS are fermentable carbohydrates that can be metabolized by LAB *via* glycolysis and the pentose phosphate pathway, generating key intermediates such as phosphoenolpyruvate (PEP) and erythrose-4-phosphate (Liu et al., 2023). These metabolites serve as essential precursors for the shikimate pathway, which ultimately leads to the synthesis of aromatic amino acids including tryptophan (Shende et al., 2024b). Increased availability of these precursors may therefore enhance flux toward chorismate formation, the central branching metabolite for aromatic amino acid biosynthesis (Shende et al., 2024b). From chorismate, the tryptophan pathway proceeds through several enzymatic steps culminating in the final reactions catalyzed by the tryptophan synthase complex encoded by the *trp*A and *trp*B genes (Liu et al., 2023). Consequently, supplementation with fermentable prebiotics may indirectly stimulate tryptophan biosynthesis by increasing carbon flux toward the shikimate pathway and aromatic amino acid metabolism (Monteiro et al., 2026). Such metabolic interactions highlight the potential role of synbiotic combinations in modulating microbial metabolite production in the gut environment (Gänzle & Follador, 2012; Parthasarathy et al., 2018).

Despite the favorable and productive traits of LAB strains, it is necessary to confirm the safety of LAB isolates by conducting safety tests, even though they have wide potential in food and food. Certain strains may have acquired genes that confer antibiotic resistance or may produce harmful metabolites, making them a health hazard to people and animals. The EFSA safety requirements include the QPS (Qualified Presumption of Safety) approach, which is carried out rigorously and focuses on strain identity, ensuring that strains do not possess transferable antimicrobial resistance traits and lack toxigenic potential. These requirements not only guarantee that only safe LAB isolates are approved for consumption but also ensure that consumers and the environment are protected from contamination and that the risk of spreading antibiotic resistance is reduced.

While testing antibiotic resistance, the LAB isolates in this study showed resistance to kanamycin, gentamycin, and streptomycin, with *P. pentosaceus* KA12 isolate showing resistance to all the antibiotics. Numerous studies have demonstrated that LAB is innately resistant to aminoglycoside antibiotics, such as kanamycin, gentamycin, and streptomycin. This resistance is mainly due to their unique cell wall structure and the lack of oxygen-dependent transport systems necessary for antibiotic uptake (Nunziata et al., 2022a). In a comprehensive assessment, resistance to these antibiotics was prevalent among LAB, with about 37% and 26% of strains showing resistance to aminoglycosides and tetracyclines, respectively (Nunziata et al., 2022b; Stefańska et al., 2021). Another study highlighted that several *Lactobacillus* and *Leuconostoc* species were highly resistant to gentamycin and kanamycin, which is consistent with the present results (Yilmaz et al., 2025). Additionally, *L. fermentum* UV16 was found to be resistant to only three antibiotics. Some LAB isolates can remove aminoglycosides by producing modified enzymes such as aminoglycoside phosphotransferase (Serio et al., 2018). These enzymes convert the drug structure and lead to decreased drug efficacy (Poole, 2005; Stefańska et al., 2021). *P. pentosaceus* KA12 showed resistance to all tested antibiotics, exhibiting broad-spectrum resistance linked to the presence of mobile genetic elements and attributed to intrinsic mechanisms (Stefańska et al., 2021).

After further testing for mucin degradation, the isolates displayed differing responses to mucin, highlighting the diverse metabolic capabilities of microbes. The strains L. *plantarum* KA9, *L. fermentum* UV16, *P. pentosaceus* KA12, and L. *mesenteroides* KA15 did not show mucin degradation when supplemented with MRS. The mucin degradation assay was conducted in MRS medium supplemented with mucin to evaluate whether these LAB strains possess strong mucinolytic activity. While MRS contains readily available carbon sources that may suppress mucin utilization through catabolite repression, the absence of enhanced growth in mucin-supplemented conditions suggests that the these LAB strains do not exhibit significant mucinolytic activity. This observation aligns with studies on *Lactobacillus* species, including L. *gasseri*, *L. johnsonii*, *L. brevis*, and L. *acidophilus*, which did not show

enhanced growth with mucin supplementation (Glover et al., 2022). The mucin-degrading potential of strains is linked to the presence and induction of genes encoding extracellular glycosidases such as *afc*A and *eng*BF (Ruas-Madiedo et al., 2008). *E. coli* can degrade mucin through enzymatic cleavage of glycan chains, releasing sugars for metabolism (Tailford et al., 2015). The LAB strains rely on other substrates other than mucin (Ayivi et al., 2020). This indicates that mucin degradation is strain- and species-specific, highlighting their unique ecological niches and metabolic capacities within the gut environment.

The genomic safety assessments of the LAB isolates provide important insights aligned with EFSA criteria for probiotic dosage. Rigorous screening for AMR genes and VF using the ResFinder AMR and VFDB databases revealed that all the isolates in the current study lacked any detectable resistance or virulence determinants, indicating a favorable safety profile. This absence of potentially harmful genes in probiotics is crucial, as EFSA guidelines strictly exclude strains harboring transferable antibiotic resistance and VF from probiotics supplementation to prevent horizontal gene transfer and ensure safety (Rychen et al., 2018). Moreover, the detection of the plasmid repA (pR18) in *P. pentosaceus* KA12 and *L. plantatrum* KA3.1.1 raises safety concerns as it can mediate horizontal gene transfer. Hence these strains were excluded, and the findings were aligned with EFSA guidelines emphasizing genomic evaluation for probiotic safety (Rychen et al., 2018). Comparative genomic analysis confirmed the absence of virulence and transferable antibiotic resistance genes in the selected strains KA9, KA15, and UV16, supporting their safety for probiotic applications (Table 7).

The strains later went through the hemolytic testing, an assessment which is key safety parameter for probiotic testing. The hemolysis of the isolates indicates the ability to lyse red blood cells, which could lead to adverse effect such as anemia in the host. In this study, *L. plantarum* KA9, *L. mesenteroides* KA15, and *L. fermentum* UV16 isolates exhibited no hemolytic activity on blood agar, aligning with the EFSA safety standards. This aligns with multiple research projects where various *L. plantarum* and *L. fermentum* strains isolated from fermented foods have lacked hemolysis, expressing their non-pathogenic profile (Echegaray et al., 2023; Sun et al., 2025). The absence of hemolytic activity in the isolates from the current study adds further evidence to the safety profile of these strains and supports their continued evaluation and use in probiotic formulations.

Despite the promising probiotic characteristics identified in this study, several limitations should be acknowledged. First, genome annotation was performed using automated bioinformatics pipelines, which may not fully capture all functional genes involved in metabolic pathways such as tryptophan biosynthesis. Therefore, some metabolic capabilities may remain undetected or incompletely annotated. Second, although the *in vitro* assays used in this study provide valuable preliminary insights into probiotic potential, they cannot fully replicate the complex conditions of the gastrointestinal tract. Factors such as host immune interactions, microbiota competition, and metabolic cross-feeding may influence probiotic functionality *in vivo*. Additionally, the biological relevance of tryptophan production observed *in vitro* was not evaluated in host models. Future studies involving transcriptomic analysis and *in vivo* experiments are therefore necessary to confirm the physiological effects and probiotic efficacy of the identified strains.

5. Conclusion

Considering all the *in vitro* tests conducted, along with the survival ability under acidic and environmental stress, gut colonization potential, improved growth in the presence of prebiotic FOS and GOS, enhanced tryptophan production in the presence of prebiotics and passing all the safety assessments, *L. mesenteroides* KA15 demonstrated impressive probiotic characteristics. Although *P. pentosaceus* KA12, *L. fermentum* UV16, *L. plantarum* KA9, and *L. plantatrum* KA3.1.1 demonstrated certain probiotic characteristics, its resistance to multiple antibiotics, including tetracycline, raises safety concerns. According to EFSA safety criteria, strains exhibiting potential transferable antibiotic resistance should be excluded from probiotic development. Therefore, KA12, KA9, KA16 were not considered among the final probiotic candidates. Therefore, *L. mesenteroides* KA15 yielded the most promising results. Further *in vivo* testing is needed for understanding the probiotic potential of the *L. mesenteroides* KA15 strain.

CRediT authorship contribution statement

Ashwinipriyadarshini Megur: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Conceptualization. **Kamilė Ambrutaitytė:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Mikas Sadauskas:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation. **Jonita Stankevičiūtė:** Writing – review & editing, Software, Resources, Methodology, Investigation, Data curation. **Rolandas Meškys:** Writing – review & editing, Software, Resources, Methodology. **Eglė Lastauskienė:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition. **Aurelijus Burokas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Data curation.

Ethical statement

This study involves the collection and analysis of traditional fermented food samples. Ethics approval was not required for this work.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2026.107281.

Data availability

Data will be made available on request.

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