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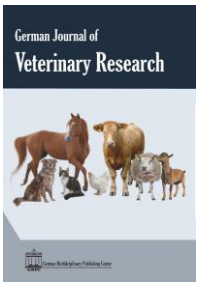
Research article

Unearthing microbial genome sequences yields genetic determinants of the probiotic potential in *Lacticaseibacillus paracasei* strains E-25 and E-49 isolated from the vagina of cows kept on an organic farm

Elena A. Yildirim[1,2], Georgi Yu. Laptev[1], Daria G. Tiurina[1], Valentina A. Filippova[1,2], Larisa A. Ilina[1,2], Natalia I. Novikova[1], Kseniya A. Sokolova[1,2], Nataliia S. Patiukova[1], Irina A. Klyuchnikova[2], Alesya A. Savicheva[1], Vasiliy A. Zaikin[1], Elena A. Korochkina[3], Darren K. Griffin[4,5], and Michael N. Romanov[4,5,6*]

[1] Molecular Genetics and Microbiomics Laboratory, BIOTROF+ Ltd, Pushkin, St. Petersburg, Russia
[2] Federal State Budgetary Educational Institution of Higher Education "St. Petersburg State Agrarian University", Pushkin, St. Petersburg, Russia
[3] Federal State Budgetary Educational Institution of Higher Education "St. Petersburg State University of Veterinary Medicine", St. Petersburg, Russia
[4] School of Natural Sciences, University of Kent, Canterbury, Kent, UK
[5] Animal Genomics and Bioresource Research Unit (AGB Research Unit), Faculty of Science, Kasetsart University, Chatuchak, Bangkok, Thailand
[6] L. K. Ernst Federal Research Center for Animal Husbandry, Dubrovitsy, Podolsk Urban Okrug, Moscow Oblast, Russia



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***Corresponding author:**
Michael N. Romanov
m.romanov@kent.ac.uk

Abstract

Study of the genomes and functional properties of autochthonous bacterial strains isolated from the bovine reproductive tract may provide a basis for assessing their probiotic potential and for developing new treatments for genital tract microbiome disorders. This study aimed to analyze the characteristics of whole genomes, primarily relevant to probiotic potential, of two *Lacticaseibacillus paracasei* strains (E-25 and E-49) isolated from the vaginas of cows kept at the Valaam Monastery organic farm (Republic of Karelia, Russia), following preliminary selection using in vitro tests. Whole-genome sequencing (WGS) of these strains was performed using the Illumina MiSeq platform. In vitro analysis revealed zones of inhibition against *Clostridium perfringens* with diameters of 32.0 ± 1.1 and 38.0 ± 1.0 mm for strains E-25 and E-49, respectively. The inhibitory effect against *Streptococcus agalactiae* was 16 ± 0.7 mm for strain E-25, and 32 ± 1.9 mm for strain E-49 against *Enterococcus faecalis*. Both strains exhibited antifungal activity against Aspergillus spp. (22 ± 0.6 and 20 ± 0.9 mm for E-25 and E-49, respectively). WGS revealed that the genome sizes of strains E-25 and E-49 were 2,913,242 and 3,050,973 bp, respectively, with GC contents of ~46%. In silico analysis revealed the presence of the EnlA and cbnCP52 bacteriocin genes in strain E-25, while strain E-49 harbored a gene cluster homologous to the sactipeptide and ribosomally synthesized and post-translationally modified peptide (RiPP) groups. The genomes of both strains contained a spectrum of genes responsible for heteroenzymatic lactic acid fermentation, the biosynthesis of vitamins B2, B6, and B7, adhesion systems, and oxidative stress resistance determinants. Comparative analysis revealed that the proportion of genes associated with carbohydrate metabolism and phosphotransferase systems in the E-49 genome is 10.0%, higher than that in strain E-25 (2.9%). The identified genetic features and phenotypic characteristics suggest that strains E-25 and E-49 are candidates for the development of targeted probiotic preparations.

Keywords: Eco-farm, Functional annotation, High-yielding cows, *Lacticaseibacillus paracasei* strains, Probiotic potential, Vaginal microbiome, Whole genome sequencing

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Introduction

The vaginal microbiota of dairy cows (*Bos taurus*) plays a key role in preventing inflammation, ensuring good fertility, and maintaining high productivity (Várhidi et al., 2025). In mammals, members of the *Lactobacillaceae* family have traditionally been assigned a dominant role in maintaining homeostasis and resistance to pathogen colonization in this ecological niche (Chee et al., 2020). These microorganisms harness their probiotic potential by synthesizing a wide range of bioactive metabolites (Amabebe and Anumba, 2018).

Lactobacilli, through the production of organic acids (lactic, acetic, etc.), maintain an acidic vaginal environment (pH 3.5–4.5), which creates unfavorable conditions for the colonization of opportunistic and pathogenic microorganisms (Liu et al., 2023). Moreover, the synthesis of hydrogen peroxide (H_2O_2) by lactobacilli, catalyzed by the lactate oxidase system, enhances the bactericidal effect, particularly against anaerobic infections (Martín and Suárez, 2010). Thirdly, the production of bacteriocins (nisin, pediocin, lactococcin, enterocin, helveticin, enterolysin, etc.) ensures highly specific suppression of targeted pathogens (Chen et al., 2025).

Lactobacilli exhibit pronounced immunomodulatory activity. They enhance the innate immune response mediated by monocytes and macrophages by activating Toll-like receptors and modulating the cytokine profile (Mitchell et al., 2021). An important aspect of their action is their ability to regulate the inflammatory response. In particular, lactobacilli and their metabolites suppress the expression of key proinflammatory cytokines, such as interleukins IL-6, IL-1β, IL-2, and tumor necrosis factor α, while simultaneously stimulating the production of anti-inflammatory IL-10 (Liu et al., 2023). This bidirectional regulation helps prevent the development of both local and systemic acute inflammation (Liu et al., 2023).

Previous studies by our team identified dysbiosis of the vaginal and endometrial microbiota in cows, partly caused by intensive industrial farming conditions (Yildirim et al., 2026a, b). A sharp decline in the proportion of lactobacilli (to the point of near-extinction) was observed, accompanied by an increase in opportunistic and pathogenic bacteria, such as *Fusobacterium* spp. and *Helococcus* spp., which was associated with a high incidence of endometritis. At a single organic farm of the Valaam Monastery (Republic of Karelia, Russia) with organic farming (without the use of antibiotics, pesticides, etc.), a relatively high proportion of lactobacilli was observed in the reproductive system of cows, which was associated with good reproductive performance (Yildirim et al., 2025). Of particular scientific interest are autochthonous lactobacillus strains from ecosystems similar to the eco-farm of the Valaam Monastery, which likely exhibit greater diversity and a broader spectrum of probiotic properties. Therefore, the aim of the study was to conduct an in vitro examination and whole-genome sequencing (WGS) of two autochthonous strains of *Lacticaseibacillus paracasei* E-25 and E-49, isolated from the vagina of cows kept at the Valaam Monastery eco-farm, in order to identify genetic determinants associated with their probiotic activity and preferable technological properties.

Material and methods

Sampling and ethical aspects

The study was conducted in 2025 using two autochthonous lactic acid bacteria (LAB) strains isolated from the endometria of two clinically healthy Ayrshire cows housed at the Valaam Monastery eco-farm (Valaam Monastery, Valaam Island, Sortavala District, Republic of Karelia) during the early dry period. Samples for microbiological analysis were collected from the upper lateral vaginal fornix at the level of the cervical canal using a sterile cytobrush. The procedure was performed under strict aseptic conditions to minimize contamination. The study complies with the ethical standards of the European Convention for the Protection of Vertebrate Animals (ETS No. 123, Strasbourg, 1986) used for research and other purposes and was approved by the Bioethics Committee of the L. K. Ernst Federal Research Center for Animal Husbandry (Protocol No. 3/2025 dated February 4, 2025).

After collection, epithelial scraping samples were immediately placed in sterile transport media and delivered to the laboratory in thermal containers at 4 °C. All animals were kept under standard conditions, and their diets complied with established zootechnical standards as

described elsewhere (Volgin et al., 2018).

Isolation and production of pure cultures

The material was seeded onto Lactobacillus Selection Agar Base (HiMedia Laboratories Private Ltd, Thane, India), a selective, dense nutrient medium modified with 0.15% bacteriological ox bile (Pushchino Laboratories, Moscow, Russia) to reduce selectivity. Pure cultures were then isolated from the grown colonies using standard microbiological methods (Netrusov et al., 2005), especially sequential streak culturing and smear microscopy using a Microscope Mikmed-1 (AO LOMO, St. Petersburg, Russia). Purity was verified by visual inspection, cell microscopy, and plating on selective media.

Evaluation of probiotic and technological properties of strains in vitro

The isolated *Lacticaseibacillus* strains were tested in vitro for their antibacterial and antifungal properties against opportunistic microorganisms. Agar block (well) and perpendicular streak methods (Netrusov et al., 2005) were used for all isolated strains (with no procedure difference depending on the strain) against 10 microbial species, including *Clostridium perfringens*, *Enterococcus faecalis*, *Enterobacter* spp., *Streptococcus uberis*, *Trueperella* spp., *Salmonella* spp., *Streptococcus agalactiae*, *Staphylococcus aureus*, *Aspergillus* spp., *Penicillium* spp., and *Escherichia coli* from the microorganism collection of the St. Petersburg State Agrarian University (Pushkin, St. Petersburg, Russia). Specifically, when using the agar diffusion method, test microorganisms were plated as a lawn on the surface of a dense agarized medium (MRS; HiMedia Laboratories Private Ltd). Wells were cut in the seeded agar using a sterile metal cylinder, into which 100 µl of the test lactobacilli strain culture was added. The dishes were incubated under optimal conditions for a test microorganism, i.e., 24 h at 37 °C for bacteria, and 48 h at 28 °C for micromycetes. After incubation, the diameter of the test culture growth inhibition zone around the well was measured. When using the perpendicular streak method, the test culture was plated as a single straight streak in the center of a Petri dish with an agarized MRS. After incubation for 24 h at 37°C, which allowed the test culture to grow, the studied lactobacilli strains were plated in perpendicular streaks, starting from the line of the grown test microorganism. The plates were then incubated under optimal conditions for the studied lactobacilli, i.e., 24–48 h at 37 °C. Antagonist activity was assessed by the presence of an inhibition (clearing) zone in the test culture at or near the intersection of the streaks. All in vitro experiments were carried out in triplicate (n = 3).

Based on the obtained values (in mm), the inhibition effect for each lactobacilli strain against the test microorganisms was classified according to the following scale: high activity, inhibition zone diameter more than 15 mm; moderate activity, inhibition zone diameter of 6 to 15 mm; weak activity, inhibition zone diameter of 1 to 6 mm; and no effect, inhibition zone diameter less than 1 mm (marked as “n/o”, i.e., not observed). The selected two strains with higher antibacterial and antifungal activity were then plated on MRS agar slants (HiMedia Laboratories Private Ltd). To obtain a working culture, one loopful of grown colonies was added to 10 ml of MRS broth (HiMedia Laboratories Private Ltd) and incubated at 37 °C for 24 h. The viable cell concentration (titer) was determined by serial dilution. For short-term storage of the strains, MRS broth was used.

The hemolytic potential of the strains was assessed by streak plating on 5% Columbia agar (HiMedia Laboratories Private Ltd) containing defibrinated sheep blood. The cultures were incubated at 37 °C for 48 h. The results were assessed visually by the presence or absence of a clearing zone (β-hemolysis) or greening (α-hemolysis) around the growth zone. The absence of visible changes in the environment was classified as γ-hemolysis (absence of hemolysis).

The pH values of the culture liquid of the strains were measured using a KL-0101 laboratory pH meter (OOO NV-Lab Company, Moscow, Russia) with a combination electrode. Titratable acidity was expressed in Terner degrees (T°). For this, 10 ml of culture liquid was titrated with 0.1 N sodium hydroxide solution in the presence of phenolphthalein indicator until a stable pink color appeared. Acidity was calculated using the following formula: $T° = V \times 10$, where V is the volume of 0.1 N NaOH (ml) used to titrate 10 ml of the sample. These measurements were also performed in triplicate.

Isolation and sequencing of genomic DNA

Whole genomic DNA was isolated from the samples using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., Waltham, MA,

USA). The extraction procedure was followed in strict accordance with the manufacturer's protocol.

Further, WGS of the strains was performed. A DNA library was prepared using the Nextera XT kit (Illumina, Inc., Sa Diego, CA, USA). Nucleotide sequences were determined using the MiSeq next-generation sequencing system (Illumina, Inc., USA) with the MiSeq Reagent Kit v3 (300-cycle; Illumina, Inc., USA).

In silico and statistical analyses

The generated WGS data were sequentially processed in silico using the appropriate bioinformatics tools that we had previously tested in similar studies (Filippova et al., 2025; Yildirim et al., 2025, 2026c; Kassymbekova et al., 2025). Trimmomatic (version 0.38; Bolger et al., 2014; Bolger, 2025) was used for read quality control and adapter trimming. De novo genome assembly was performed using the SPAdes algorithm (version 3.11.1; Nurk et al., 2013; Prjibelski et al., 2020; Prjibelski, 2026), and assembly parameters were estimated using the QUAST program (version 5.0.2; Mikheenko et al., 2018; QUAST, 2024). The taxonomy was refined by alignment with reference genomes in the National Center for Biotechnology Information (NCBI) databases (NCBI, 2025). To determine the genomic relationship between the studied strains, the average nucleotide identity (ANI) was calculated using the FastANI algorithm (version 1.34; Jain, 2024). An ANI ≥ 95% criterion was used to classify genomes as the same species. To assess genomic diversity and identify common and unique genes, a pangenome analysis was performed using the Roary pipeline (version 3.13.0; Page et al., 2015; Page, 2019). Annotated genomic sequences in GFF3 format obtained in the previous step were used as input. Gene clustering was completed with an amino acid sequence similarity threshold of 95%.

Functional annotation was performed using the integrated Bacterial and Viral Bioinformatics Resource Center (BV-BRC) platform (Olson et al., 2023; BV-BRC, 2025), complemented by analysis in the Rapid Annotations using Subsystems Technology (RAST) system (Aziz et al., 2008; RAST, 2025). The Circos visualization tool (version 0.69-10; Krzywinski et al., 2009; Krzywinski, 2025) was used to generate circular charts of the functional annotation distribution across genomes. The visualization integrated functional annotation data obtained from the RAST server.

Metabolic potential was studied by reconstructing pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al., 1998; KEGG, 2025). Automated gene annotation was implemented using the Prokka pipeline (Seemann, 2014, 2026). The search for specific genetic determinants was executed using hidden Markov models via the HMMER software package and its hmmsearch function (Eddy et al., 2011; Finn et al., 2011; HMMER, 2025), which ensured high sensitivity in detecting evolutionarily conserved protein domains. The closest reference and representative genomes to the two strains were identified using Mash/MinHash (Ondov et al., 2016; Ondov, 2021). Bootstrapping was used to assess the reliability and robustness of the generated phylogenetic tree topology. Bootstrap support values (as percentages) were shown at the corresponding tree nodes.

The whole genome sequences of *L. paracasei* strains E-25 and E-49 have been deposited in the NCBI database. The raw sequencing data, genome assemblies, and annotated sequences are available under BioProject identifier PRJNA1426175. This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions JBVLSY000000000 (BioSample SAMN55848473)/JBVLSX000000000 (BioSample SAMN55848474).

Mathematical processing of the results was performed using RStudio software (version 1.1.453; RStudio Team, 2018) and Microsoft Excel. Multifactorial analysis of variance (multi-factor ANOVA) was used to determine the statistical significance of differences. Data in the text and tables are presented as M ± m (mean ± standard error of the mean). To compare the antagonist activity of two selected strains (E-25 and E-49) based on the inhibition zone diameter for each test microorganism, a parametric Student's t-test for independent samples was used. For statistical comparison of microorganism titers, cell concentration data (CFU/ml) were transformed into decimal logarithms ($\log_{10}$). The statistical significance of differences was also assessed using a Student's t-test for independent samples.

Results and discussion

Probiotic and technological characteristics of

strains at the phenotype level

Fifteen isolates with characteristic features of lactobacilli were isolated from the vagina of cows. The results of an in vitro evaluation of the antibacterial and antifungal properties of these isolates against pathogenic and opportunistic microorganisms were produced. The results revealed a wide range of activity for a number of the studied isolates (Supplementary Table S1, Table 1). Some demonstrated a high degree of antimicrobial activity, causing significant zones of growth inhibition, while others had less pronounced effectiveness or showed no effect on pathogens at all. According to the established activity scale, only four of 15 isolates tested exhibited a high degree of antimicrobial activity (inhibition zone>15 mm) against at least one test microorganism (Figure 1). These were strains E-25, E-49, E-42, and E-40. The remaining 11 isolates mostly demonstrated moderate or weak activity or showed no effect at all. Among the tested lactobacilli isolates, further work was continued with two *L. paracasei* strains, E-25 and E-49, which demonstrated the highest activity (Table 1). In particular, strain E-49 was a more potent antagonist against two gram-positive test microorganisms, i.e., C. perfringens and *E. faecalis*, with mean inhibition zones of 38.0 ± 1.0 and 32.0 ± 1.9 mm, respectively ($p < 0.05$; Table 1). On the other hand, no antagonism was observed in relation to *Enterobacter* spp. and *S. uberis*, while there was a minimum antagonism observed against *S. aureus* (2.0 ± 0.1 mm, $p < 0.05$).

In contrast, strain E-25 demonstrated a slightly different pattern of activity. It maintained high activity against *C. perfringens* (32.0 ± 1.1 mm), although it was slightly inferior to strain E-49, and showed moderate activity (10.0 ± 0.9 and 3.0 ± 0.2 mm) against *Enterobacter* spp. and *S. uberis*, i.e., microorganisms the growth of which was not affected by strain E-49. However, the efficacy of E-25 against *E. faecalis* was lower (5.0 ± 0.5 mm, $p < 0.05$), in contrast to the results for the other *Lacticaseibacillus* strain. Antimicrobial activity against *Salmonella* spp., *E. coli*, and *Trueperella* spp. was comparable (Figure 1A, B, C). Both lactobacilli isolates showed significant antifungal activity, inhibiting the growth of filamentous fungi *Aspergillus* spp. (22.0 ± 0.6 and 20.0 ± 0.9 mm for E-25 and E-49, respectively) and *Penicillium* spp.

The data obtained may indicate a possible qualitative difference in the spectra of antimicrobial metabolites produced by strains E-25 and E-49, despite their isolation from a similar ecological niche. This observation of phenotypic differences drew our attention to the need for further study of the extent of their genetic relationship (Caulier et al., 2019). The pronounced antimicrobial activity of the isolated strains likely suggests that they produce antimicrobial factors. Such a broad spectrum of activity is often associated with the manifestation of both nonspecific factors (organic acids, hydrogen peroxide, etc.) and specific peptide bacteriocins, to which many microorganisms, including fungi, are sensitive (Teneva and Denev, 2023).

Microscopic examination revealed that the isolated strains E-25 and E-49 have straight or slightly curved rods with rounded ends (Figure 2A, B). Cells are arranged singly, in pairs, or in short chains, and exhibit uniform staining.

When assessing hemolytic activity in vitro, a complete absence of clearing or greening of the medium around the colonies was noted for both strains. This type of growth is classified as γ-hemolysis, pointing out the absence of hemolysin synthesis (Pivard et al., 2023) and, therefore, the biological safety of strains E-25 and E-49.

A study of the dynamics of biomass accumulation and metabolic activity after 24 h of cultivation revealed differences in viable cell concentrations between the strains, with strain E-25 demonstrating an order-of-magnitude higher growth rate ($p < 0.01$, Table 2). Despite the significant difference in population density, both strains exhibited a relatively high and virtually identical acid-forming capacity.

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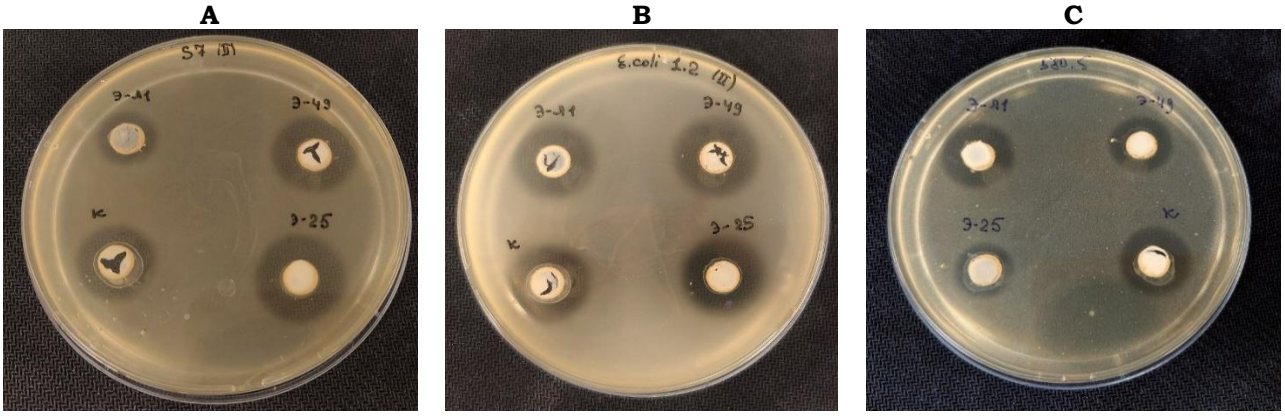
filamentous fungi *Aspergillus* spp. (22.0 ± 0.6 and 20.0 ± 0.9 mm for E-25 and E-49, respectively) and *Penicillium* spp.

Table 1: Antimicrobial activity of *Lacticaseibacillus* strains E-25 and E-49 against opportunistic microorganisms. The length of the growth inhibition zone (in mm) is presented as mean ± standard error of the mean.

Opportunistic test microorganisms	Strain E-25	Strain E-49
Clostridium perfringens	32.0 ± 1.1	38.0 ± 1.0*
Enterococcus faecalis	5.0 ± 0.5	32.0 ± 1.9*
Enterobacter spp.	10.0 ± 0.9	n/o**
Streptococcus uberis	3.0 ± 0.2	n/o
Trueperella spp.	5.0 ± 0.2	8.0 ± 0.2*
Streptococcus agalactiae	16.0 ± 0.7	12.0 ± 0.7*
Staphylococcus aureus	4.0 ± 0.6	2.0 ± 0.1*
Aspergillus spp.	22.0 ± 0.6	20.0 ± 0.9
Penicillium spp.	18.0 ± 0.5	18.0 ± 0.4
Escherichia coli	7.0 ± 0.6	9.0 ± 0.3
Salmonella spp.	11.0 ± 0.8	9.0 ± 0.4

* $p < 0.05$ when comparing the diameters of the inhibition zones between strains E-25 and E-49 according to Student's *t*-test; ** n/o, no observed antagonistic activity.



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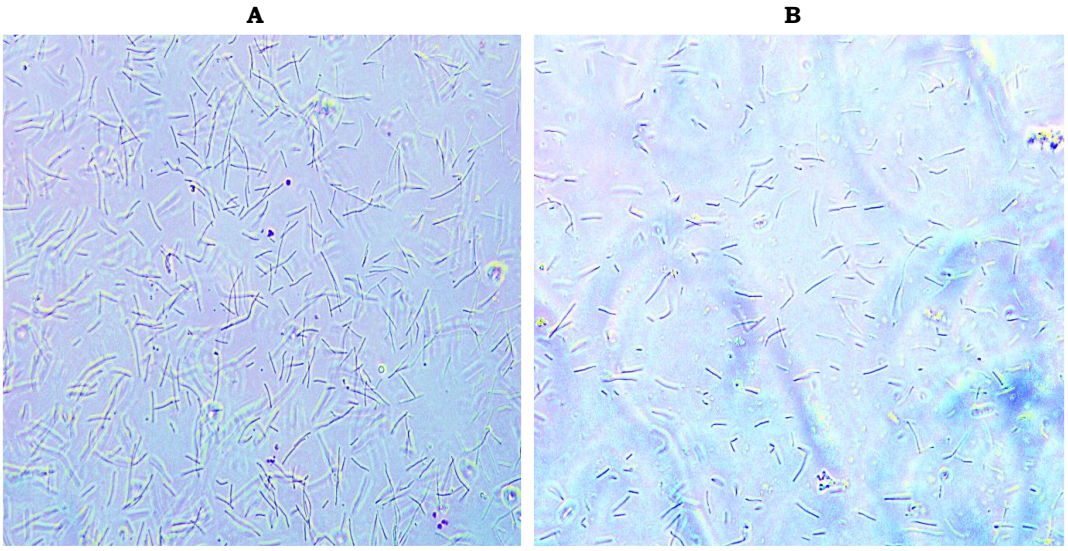

Figure 2: Micrographs of *Lacticaseibacillus paracasei* strains (A) E-25 and (B) E-49. Cells are straight or slightly curved rods with rounded ends. Magnification 400×.

Table 2: Results of the assessment of growth and acid-forming capacity of *Lacticaseibacillus* strains E-25 and E-49 (mean ± standard error of the mean, $n = 3$).

Characteristics	Strain E-25	Strain E-49
Titer, CFU/mL*	$6.9 \times 10^9 \pm 3.5 \times 10^8$	$1.5 \times 10^8 \pm 8.0 \times 10^{6}$**
Medium pH (after 24 h of cultivation)	3.90 ± 0.34	4.10 ± 0.30
Titratable acidity (T°) (after 24 h of cultivation)	88.0 ± 5.9	87.0 ± 7.1

* CFU/mL, Colony Forming Units per milliliter; ** $p < 0.01$ when comparing the titer values between strains E-25 and E-49 according to Student's *t*-test.

Genome assemblies and primary functional annotation

Preliminary phenotypic analysis revealed pronounced antimicrobial activity of the strains and some positive technological characteristics, implying a genetic basis for their probiotic properties. Therefore, whole-genome sequencing of strains E-25 and E-49 was conducted, thereby verifying their classification as *L. paracasei*. The taxonomic affiliation of *L. paracasei* strains E-25 and E-49 was also confirmed by comparative genomic analysis. The Mash/MinHash procedure was used to compute genomic distances between the studied strains and representative reference genomes. Phylogenetic dendrograms were constructed based on these distances (Figure 3A, B). Both strains formed clusters with reference genomes of the genus *Lactobacillus*. Identification of the strains as representatives of the species *L. paracasei*, which is one of the currently recognized lineages within a single complex of the respective species, along with *L. rhamnosus* and *L. zeae* (Huang et al., 2018), confirms their species identification.

Strains E-25 and E-49, although belonging to the same species, occupy somewhat different positions within a larger species cluster (Figure 3A, B). In particular, despite being part of the same species, strains E-25 and E-49 differed in

their genomic proximity to some reference isolates within their respective larger species clusters. This difference was clearly reflected in their phylogenetic positions in individual trees. For example, strain E-25 demonstrated a certain genomic proximity to *L. saniviri*, while this species was absent from the tree for strain E-49 among its closest taxa. Instead, *L. paracollinoides* was present among the closest taxa in the tree of strain E-49, which was absent from the tree of strain E-25. This indicates the presence of intraspecific genetic diversity and possible differences in physiological and biochemical profiles (Konstantinidis et al., 2006) that we observed based on phenotypic data analysis in this study. These differences are likely due to the fact that the strains were isolated from different biotopes, the vaginas of two separate cows, which could have led to adaptation to specific microenvironments and the formation of subpopulations within the species. Similar intraspecific genetic variability in LAB, associated with the specific characteristics of the isolation biotope, has been described in other studies (Mejía-Caballero et al., 2025; Ito and Ito, 2025). It is well known that the adaptation of strains to specific niches (intestines, fermented foods, plants, etc.) can lead to the formation of subpopulations with unique genomic and phenotypic traits (Sun et al., 2025).

For further analysis, the genomes of *L. paracasei* strains E-25 and E-49 were assembled and annotated. The genome assembly details for the strains are presented in Table 3. The assembly parameters suggested their high quality for both strains. The E-49 genome was approximately 138 kb longer than that of E-25, likely due to the presence of additional genetic elements. The GC content of both strains was within the range characteristic of L. paracasei (Chen et al., 2025) and was virtually identical, i.e., 46.43% and 46.25%, respectively, which further confirms their taxonomic affinity (Li et al., 2014). The next step in the study was to functionally annotate the genomes using the RAST server (Aziz et al., 2008; RAST, 2025). The summarized quantitative characteristics of the annotated genomes of the two studied *L. paracasei* strains are presented in Table 4. The genome of strain E-25 includes 3,139 predicted coding sequences (CDS), 56 transfer RNA genes, and 20 ribosomal RNA genes. For strain E-49, similar indicators were 3,205 CDS, 47 tRNA genes, and 22 rRNA genes. In both genomes, a small number of partial CDS were identified, while various non-coding RNAs and repetitive regions were not detected.

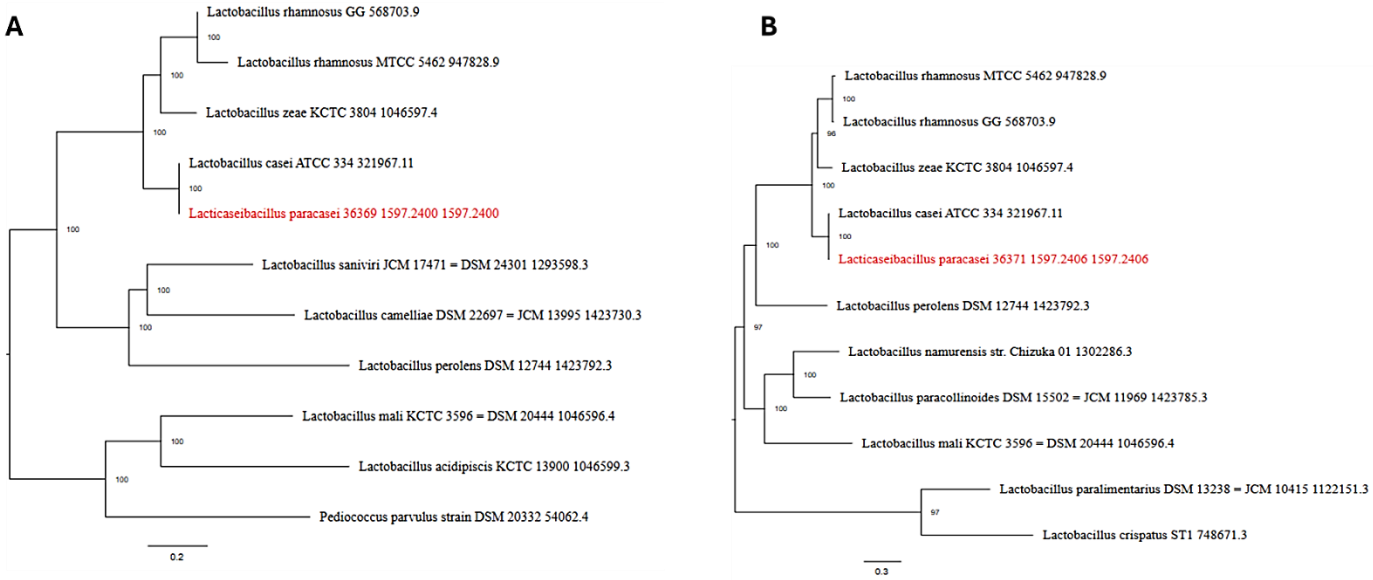

Figure 3: Phylogenetic trees based on the Mash/MinHash genomic distances for *Lacticaseibacillus paracasei* strains (A) E-25 and (B) E-49. Representative genomes of closely related taxa were used as reference sequences. Branch sizes reflect the measure of genomic difference (1, Mash distance). The positions of the two strains are designated in red. The numbers in the tree nodes indicate bootstrap support values (in percentages).

Table 3: Genome assembly characteristics of *Lacticaseibacillus paracasei* strains E-25 and E-49.

Parameter	*L. paracasei* E-25	*L. paracasei* E-49
Contigs	305	121
Genome length, bp	2,913,242	3,050,973
Average G+C content, %	46.43	46.25
Contig N50, bp	59,137	124,879
Contig L50	14	8
Plasmids	0	0

Table 4: Annotated characteristics of the genomes of *Lactobacillus paracasei* E-25 and E-49 strains.

Characteristics	*L. paracasei* E-25	*L. paracasei* E-49
Coding sequences (CDS)	3,139	3,205
tRNA	56	47
rRNA	20	22
Partial CDS	4	2
Various non-coding RNAs	0	0
Repetitive regions	0	0

For visual analysis of the functional content of the two genomes, a circular graphical representation of the annotation distribution was used (Figure 4). Comparative pan-genome analysis of *L. paracasei* strains E-25 and E-49

An assessment of genomic relatedness between the studied strains using the FastANI algorithm yielded a value of 98.3%, which is consistent with generally accepted interpretation criteria, i.e., ANI > 95% for high-coverage (Richter and Rosselló-Móra, 2009). This confirms that strains E-25 and E-49 belong to the same species. Furthermore, this value suggests that they belong to different strains within a single species, which is consistent with their positions on the phylogenetic trees.

Pangenome analysis performed using the Roary tool (Page et al., 2015; Page, 2019) revealed a total gene pool of 3,482, of which 2,293 genes were assigned to the core genome and 1,189 genes to the shell genome (Table 5). The distributions of unique genes for each strain were as follows: 522 for E-25 and 667 for E-49. Furthermore, functional annotation of unique genes in the studied strains revealed differences in their potential specialization (Table 6). Strain E-49 was characterized by a significantly higher proportion of genes (10.0%) associated with carbohydrate metabolism and phosphotransferase systems (PTS) compared to strain E-25 (2.9%). This indicates higher adaptive plasticity in strain E-49 and its ability to efficiently utilize a wide range of carbohydrates in a competitive environment, both of which are important probiotic traits (Latif et al., 2024). At the same time, the unique genome of E-25 has a higher relative content of mobile element genes (3.3%).

This includes, from outer to inner circle: contigs, coding sequences (CDS) on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content, and GC skew. The subsystems to which these genes belong are indicated by the colors of the CDS on the forward and reverse strands.

Genome annotation of *L. paracasei* strains E-25 and E-49 revealed several genes associated with antimicrobial resistance (Figure 4, Table 6). Most of them belonged to the category of constitutive housekeeping genes (e.g., *rpoB*, *gyrA/B, ddl, murA*), which are targets for various classes of antibiotics in susceptible microorganisms (Gustaw et al., 2021). Their presence alone does not generally indicate acquired resistance and is characteristic of many probiotic lactobacilli (Campedelli et al., 2018; Anisimova et al., 2022). However, the genomes also contained genes that provide adaptive resistance mechanisms, such as the *liaFSR* system, which regulates the stress response to cell wall damage (Papadimitriou et al., 2016). The difference between the two strains was that E-25 was found to contain genes for the putative *mdtABC-tolC* efflux system and the *fabK* gene,

which is associated with triclosan resistance (De Gaetano et al., 2023; Radovanovic et al., 2023), whereas these genes were absent from the E-49 genome. In the context of antibiotic resistance gene spread during probiotic use, strain E-49 may be considered safer. At the same time, the presence of additional efflux and detoxification mechanisms in strain E-25 could, in principle, contribute to its survival under conditions of combined use with certain antimicrobials (Jin et al., 2021), a finding that requires further experimental confirmation.

It is important to note that determinants of acquired resistance to clinically significant antibiotics (e.g., the *erm* or *tetM* genes) were not detected in the genomes of both strains, which meets the safety criteria for probiotic microorganisms (Ammor et al., 2007; Campedelli et al., 2018).

A detailed analysis of the most abundant annotated genes (Table 7) corroborated the identified trends. The unique E-49 genome was dominated by multiple copies of genes encoding components of the PTS systems for mannose, sorbose, and fructose, as well as enzymes involved in sugar alcohol metabolism. In E-25, in addition to the hypothetical proteins, genes associated with vitamin B12 metabolism (*BtuD*), bacitracin transport (*BcrA*), recombination (*XerC*), and SOS response repression (*LexA*) were prominent.

Figure 5 shows the distribution of functionally significant genes in the *L. paracasei* E-25 and E-49 genomes across subsystems, as generated using the RAST database (Aziz et al., 2008; RAST, 2025). Analyzing the distribution of functions, it can be noted that, in both genomes, subsystems associated with the metabolism of carbohydrates (165 for E-25 and 177 for E-49, as noted above), amino acids and derivatives (119 genes for E-25 and 122 for E-49), and proteins (119 for E-25 and 117 for E-49) predominated. A significant number of genes were also associated with regulation and cellular signaling (22 genes in both strains), which may play a role in interactions with the host microbiota and the immune system (Wiertsema et al., 2021).

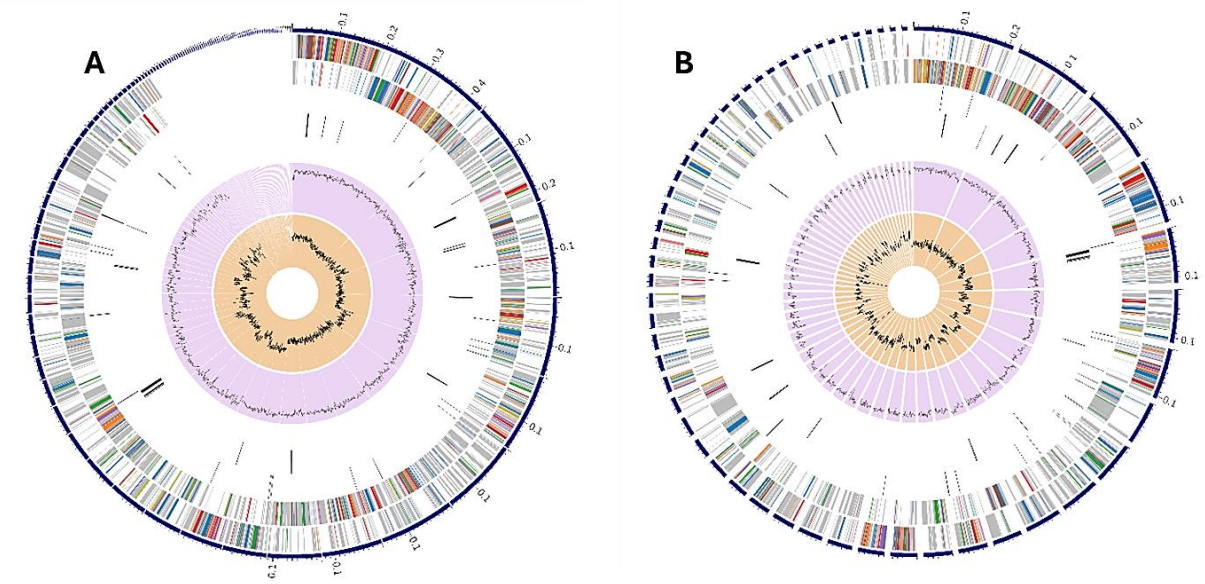

Figure 4: Graphical representation of the distribution of genome annotations for *Lacticaseibacillus paracasei* strains (A) E-25 and (B) E-49.

Table 5: Pangenome statistics of strains E-25 and E-49.

Gene category	Number of genes
Core genes	2,293
Soft core genes	0
Shell genes	1,189
Cloud genes	0
Total genes	3,482

Table 6: Functional profile of unique genes (n) of strains E-25 and E-49.

Functional category	Strain E-25 (*n* = 522)	Strain E-49 (*n* = 667)
Hypothetical proteins	393 (75.3%)	432 (64.8%)
Other/uncategorized	458 (87.7%)	529 (79.3%)
Carbohydrates/phosphotransferase systems	15 (2.9%)	67 (10.0%)
Transporters	13 (2.5%)	25 (3.7%)
Regulatory genes	14 (2.7%)	24 (3.6%)
Transposable elements	17 (3.3%)	14 (2.1%)
Protection/stress	4 (0.8%)	6 (0.9%)
Antimicrobial resistance	4 (0.8%)	2 (0.3%)

Table 7: Most represented annotated unique genes.

Strain	Gene/gene product	Number of copies
E-25	Hypothetical protein	393
	Glycerol kinase	3
	Vitamin B_{12} import ATP-binding protein (*BtuD*)	3
	IS5 family transposase ISCaa8	3
E-49	Hypothetical protein	432
	PTS system mannose-specific EIID component	9
	PTS system sorbose-specific EIIC component	5
	PTS system mannose-specific EIIAB component	5

Analysis of the genetic apparatus associated with probiotic functions and safety

A complex of the above-described phenotypic properties of the studied strains, revealed in vitro, correlated with the profiles of identified genes for organic acids and bacteriocins. In particular, genomic analysis of *L. paracasei* strains E-25 and E-49 suggested the presence of a complete genetic apparatus for heterofermentative lactic acid fermentation, resulting in the formation of a spectrum of end metabolites characteristic of heterofermentative lactobacilli (Wang et al., 2021). The dominant pathway in both strains was the production of L-lactic acid, as confirmed by the presence of multiple copies of the L-lactate dehydrogenase (ldh) gene (Zhang et al., 2015). The potential for the synthesis of the D-isomer of lactic acid is provided by the dld genes and the full-fledged larABCDEF operon, which encodes racemase enzymes, indicating the ability to interconvert L- and D-isomers (Desguin et al., 2014, 2015). The presence of a complete set of genes for acetic acid production via both anaerobic (pta-ackA) and aerobic (pox) pathways was established, indicating the strains' metabolic flexibility under varying oxygen availability (Bueno et al., 2012). Additionally, genes associated with the ability to synthesize succinic (*fumC, ifcA,* and *mdh*) and formic (*pflB*) acids were identified. These data point out a high and diverse enzymatic potential of the studied strains, aimed at the efficient fermentation of carbohydrates with the formation of lactic and acetic acids as the main products, as well as other organic acids that contribute to the phenotypically observed acidification of the environment and antimicrobial activity (Leyva et al., 2018). In addition, strain E-25 possesses the EnlA and cbnCP52 genes, encoding the bacteriocins enterolysin A and carnocin CP52, respectively. Enterolysin A is a class III bacteriolysin (lysins) exhibiting endopeptidase activity (Nilsen et al., 2003). Its N-terminal catalytic domain is homologous to that of enzymes that degrade cell wall glycan in some Gram-positive bacteria (Nilsen et al., 2003). This may explain the high activity of E-25 against *S. agalactiae*; however, this bacteriocin has not previously shown activity against *Clostridium* spp. (Nilsen et al., 2003). Carnocin CP52 is a class IIb (two-peptide) bacteriocin (Cui et al., 2012). Carnocins are known for their activity against clostridia, which is consistent with our data on E-25's antimicrobial activity (Blom et al., 2001). Listeria monocytogenes also belongs to the phylum Firmicutes (like *C. perfringens* and *S. agalactiae*), which may suggest evolutionary "specialization" of bacteriocins. Identification of associated genes (sensor histidine kinase, two-component signaling system response regulators

and ABC transporters) in the E-25 genome indicates that the respective regions represent functional operons likely capable of regulating, biosynthesizing and secreting active bacteriocins (Mascher et al., 2006). The genome of strain E-49 also demonstrated a complex and diverse genetic potential for synthesizing antimicrobial peptides. Similar to strain E-25, the presence of the enterolysin A gene may have been a key factor in its pronounced activity against *S. agalactiae*. In addition, the genome of *L. paracasei* strain E-49 contained genes encoding bacteriocin-like peptides LSEI_2386 and LSEI_2163. Homologs of these genes were previously established in the genomes of other representatives of the *L. paracasei* species (Surachat et al., 2017). Genes predicting the synthesis of a relatively rare class of bacteriocins, sactipeptides (Mathur et al., 2015), were also identified. These are class I bacteriocins characterized by a unique post-translational modification, the formation of thioether bonds between the sulfur atom of cysteine and the α-carbon of another amino acid in the peptide, with known activity against clostridia (Mathur et al., 2015). This potentially explains the pronounced antimicrobial activity of strain E-49 against *C. perfringens* observed in our study. In addition to sactipeptides, comparative genomic analysis identified a large gene cluster (24,000 bp) in the E-49 strain genome responsible for producing ribosomally synthesized and post-translationally modified peptides (RiPPs). These peptides represent a chemically diverse class of natural compounds with often unique mechanisms of antimicrobial action (Wang et al., 2024).

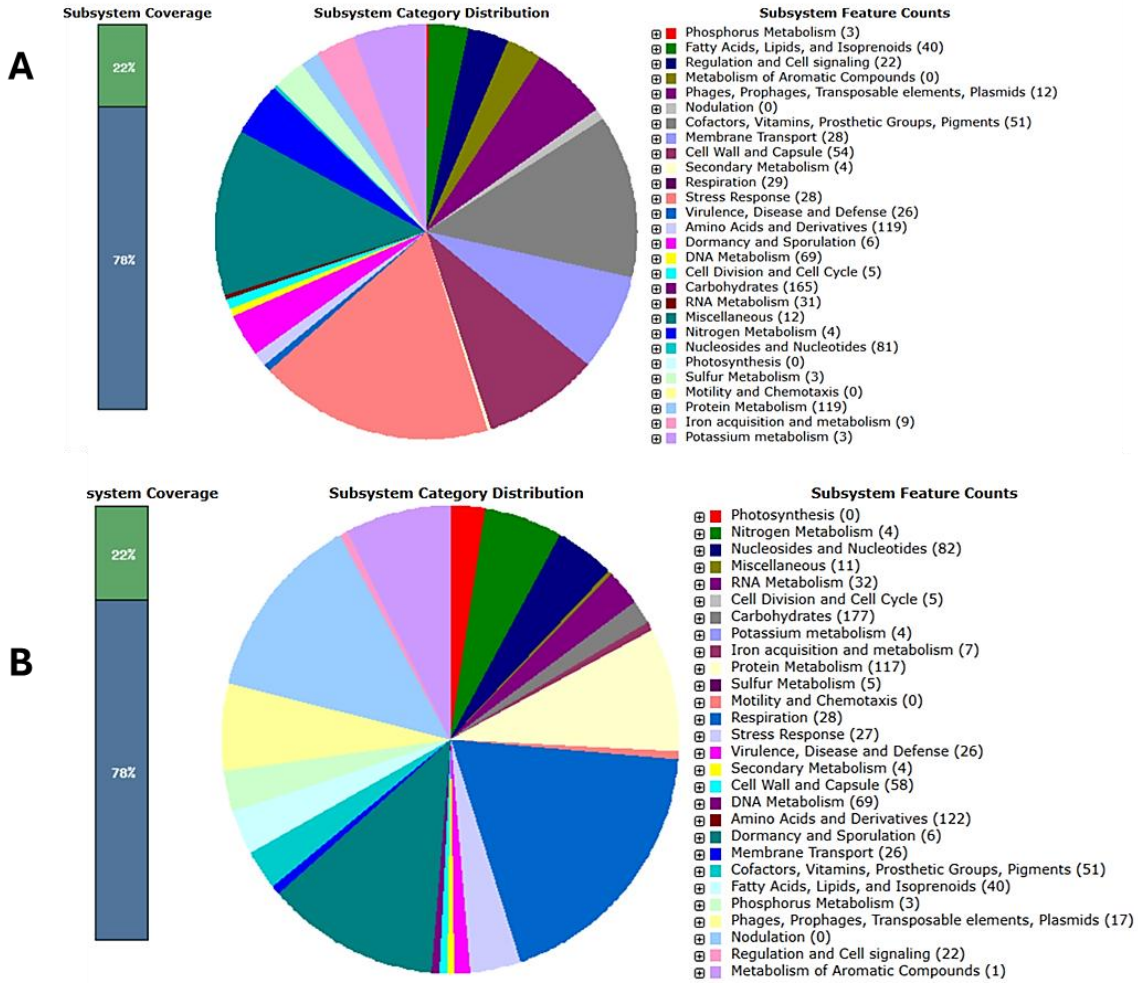

Figure 5: Predicted metabolic networks of *Lacticaseibacillus paracasei* strains (A) E-25 and (B) E-49 using the RAST databases (Aziz et al., 2008; RAST, 2025). These diagrams are based on the analysis of subsystems unique to each genome. 22% is the proportion of the genome that remained unannotated or was classified as "unknown functions," and 78% is the proportion of the genome that was successfully annotated by RAST and assigned to specific subsystems. Subsystems are indicated by colors; these are sets of predicted proteins that together implement a specific biological process or structural complex.

The ability of probiotic strains to survive physiological stress is essential for their in vivo efficacy (Kim et al., 2026). Genome annotation of *L. paracasei* strains E-25 and E-49 revealed several systems that mediate resistance to key stressors. Among them, 11 to 12 protein factors, including key components of the glutathione cycle and the glutaredoxin family, were predicted in both strains' genomes. These systems detoxify reactive oxygen species, the formation of which can be induced by both endogenous metabolic processes and exogenous factors, including competitive interactions with other microorganisms and the host inflammatory response (Zhang et al., 2010). The presence of these systems may, for example, enhance the strains' potential for persistence under local inflammatory conditions (Dagah et al., 2024).

Synthetic pathways for several vitamins, including riboflavin (B2), pyridoxine (B6), and biotin (B7), have been identified in the genomes of *L. paracasei* strains E-25 and E-49. Vitamin production (particularly riboflavin) may exert immunomodulatory effects and influence the composition of the microbiota, thereby representing an additional probiotic mechanism (Dricot et al., 2024).

The presence of diverse systems in the genome of the studied strains, such as sortase, *WxL, LysM,* and *SLH* domains, and LPXTG-motif proteins, suggests a high potential for colonization of the mucous membrane (Chapot-Chartier et al., 2014). Adhesion is the first and necessary step for long-term persistence of the probiotic, the formation of a protective biobarrier, and the effective local production of bacteriocins and acids directly on the epithelial surface (Monteagudo-Mera et al., 2019). Strong adhesion of lactobacilli to the vaginal epithelium can displace and suppress pathogens (Kawahara et al., 2022).

Moreover, analysis of the *L. paracasei* E-49 strain genome enabled us to identify conserved loci homologous to the so-called mycobacterial virulence operons. It is important to emphasize that the term "virulence" as applied to these genetic elements historically reflects their initial description in the context of mycobacterial pathogenesis but does not define their direct toxigenic function (Chen et al., 2025). The established homologs encode universal components of fundamental cellular processes. Among these, two loci are homologous to genes involved in the synthesis of RNA polymerase and associated transcription factors, seven loci are homologous to genes responsible for the synthesis of ribosomal proteins (four operons for the small ribosomal subunit, and three for the large ribosomal subunit). Their primary biological role is not to damage host tissues directly, but to ensure the viability, efficient replication, and metabolic adaptation of the bacterial cell, especially under stressful conditions in vivo (Torres et al., 2001). Thus, the identified genetic systems belong to the category of indirect, or "host," virulence factors. The presence of similar highly conserved systems, evolutionarily associated with intracellular survival in pathogens (e.g., mycobacteria), in lactobacilli apparently contributes to their stability and persistence in the host niche (Altavas et al., 2024). Previously, the presence of genes homologous to microbial surface components recognizing adhesive matrix molecules (MSCRAMM) in the genomes of some lactobacilli strains (e.g., *Lactiplantibacillus* plantarum), which have been studied in detail in pathogenic staphylococci (Wang et al., 2013), was also reported. In *S. aureus*, such proteins (e.g., *ClfA, ClfB, SdrC*, and *SdrD*) mediate binding to host extracellular matrix proteins (fibrinogen and fibronectin), cell aggregation, biofilm formation, and even immune evasion by capturing complement factors (O'Brien et al., 2002). Meanwhile, in *Lactococcus lactis*, expression of these genes enhances adhesion to abiotic surfaces and can improve in vitro survival without resulting in pathogenicity (Barbu et al., 2014).

While probiotic lactobacilli are generally recognized as safe (GRAS status; Burdock and Carabin, 2004), rare but significant cases of members of this genus acting as opportunistic pathogens in humans have been reported in clinical practice (Goldstein et al., 2015). In recent years, there has been an increasing number of reports of lactobacilli-associated infections, including peritonitis, bacteremia and infective endocarditis (Ambesh et al., 2017; Teng et al., 2017). Such infections typically develop against the background of impaired immune defense mechanisms and severe underlying diseases. Therefore, the absence of true toxigenic and invasive factors in the genomes of the studied strains indicates a favorable safety profile for further consideration as probiotic candidates. Further phenotypic studies in animals are necessary, however, to confirm the therapeutic

safety and efficacy of these strains for the prophylaxis of the bovine reproductive system microbiome definitively.

Conclusions

Based on the above preliminary analysis, *L. paracasei* strains E-25 and E-49, isolated from the vaginal biotope of healthy cows kept on an organic farm, are promising probiotic candidates for their use in animal nutrition. Their high antimicrobial activity, confirmed in vitro, particularly against significant reproductive tract pathogens, and pronounced acid-forming capacity are beneficially combined with the presence of a complex of genes in their genomes that are responsible for bacteriocin synthesis, stress resistance, vitamin synthesis, and adhesion. An important feature of the E-49 genome, such as an expanded set of carbohydrate metabolism genes, implies its potentially higher adaptive plasticity than that of strain E-25. The practical significance of these strains is determined by their autochthonous origin, which suggests a probable natural adaptation to the mucous membrane of the reproductive system of ruminants. To harness this probiotic potential, further research on the two L. paracasei strains is needed to clarify their adhesive properties in cell culture, optimize the technological parameters for biomass production, and conduct controlled clinical trials to evaluate therapeutic efficacy in vivo.

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