










Original Research

Lactobacillus paragasseri Ig94 With High L-Lactic Acid Production and Probiotic Potential

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Academic Editor: Tünde Pusztahelyi

Submitted: 8 October 2025 Revised: 21 December 2025 Accepted: 8 January 2026 Published: 30 May 2026

Abstract

Background: *Lactobacillus paragasseri* strains are widely used as probiotics. According to modern requirements, it is necessary to characterize their morphological, cultural, physiological-biochemical, and antagonistic properties, as well as to determine their virulence, toxigenicity, toxicity, and secondary metabolite production, to study their potential and safety. The main aim of the study was to determine the probiotic potential of the *L. paragasseri* Ig94 strain based on its biochemical features and genomic landscape. **Methods:** *L. paragasseri* Ig94 was isolated from human feces. The genus and species were determined by mass spectrometry using the MALDI Biotyper. The main cultural, biochemical, and genomic characteristics of *L. paragasseri* Ig94 were established. The biochemical profile and L-lactic acid content of the strain were determined. Additionally, the strain’s resistance to bile and low pH stress, as well as its antibiotic susceptibility, were assessed. The genomic sequence of strain Ig94 was obtained using Illumina NextSeq550 sequencing. **Results:** In this study, the strain Ig94 isolated from the feces of a healthy child was identified as *L. paragasseri*. After 24 h of cultivation in API 50 CH/CHL medium, strain Ig94 exhibited positive results in the fermentation of 25 carbohydrates. Strain Ig94 exhibited intense L-lactic acid production, reaching a concentration of 6.41 g/L in 24 h of cultivation, indicating high acid-forming capacity. The results showed that *L. paragasseri* Ig94 demonstrated low resistance to acid stress (pH 2.0) but high resistance to 0.3% bile, with no changes in colony morphology observed. The disk diffusion assay revealed that *L. paragasseri* Ig94 was phenotypically susceptible to several antibiotics, including ampicillin, amoxicillin, penicillin G, tetracycline, erythromycin, and chloramphenicol, with intermediate resistance to oxacillin and resistance to fosfomicin, gentamicin, and clindamycin. The whole-genome of *L. paragasseri* Ig94 was sequenced. The absence of antibiotic resistance, pathogenicity, and virulence determinants in the genome was confirmed. Strain Ig94 has genes for the production of gassericin T, enterolysin A, and helveticin J. **Conclusions:** The results of the studies showed that *L. paragasseri* Ig94 has probiotic potential, and it is recommended to continue studying its antagonistic activity and secondary metabolite production.

Keywords: *Lactobacillus paragasseri*; probiotic; genome; lactic acid; bioinformatics

1. Introduction

Recent and ongoing advances in research on the gut microbiome have shown that gut microbiota compounds are a robust, yet understudied, source of probiotic research [1,2]. There is growing awareness and broader acceptance of probiotic supplements, especially in light of these recent findings. Therefore, academic and industrial efforts are focused on identifying and characterizing new microbial strains to develop effective probiotics [3].

We have accumulated extensive experience in studying the human gut microbiota and its relationship with var-

ious health disorders [4,5]. Hence, our focus is on treatments to address dysbiotic disorders, primarily through research on probiotics [6]. The isolation of probiotic strains from healthy people facilitates the creation of innovative multicomponent probiotics via improved methods. Previous research has shown that the biocompatibility of candidate strains and a potential synergistic effect, marked by increased antagonistic activity from the consortium compared to single strains, are crucial for the effective formation of a probiotic consortium [7,8].

Lactobacillus paragasseri strains are widely used in probiotic selection and formulation. The positive effects of



L. paragasseri are strain-specific and can be used to correct various diseases. For example, the probiotic strain *L. paragasseri* K7, isolated from the gut of an infant, maintains intestinal homeostasis, participates in the regulation of the immune system, reduces allergic symptoms, prevents bacterial and viral infections, and helps alleviate infectious diseases [9]. It has been experimentally proven that taking *L. paragasseri* OLL2809 helps increase the content of commensal *Akkermansia muciniphila*, *Bifidobacterium*, and *Lactobacillus* in the intestinal microbiota and reduces depressive behavior in mice [10]. A placebo-controlled study found that long-term use of a drug based on the *L. paragasseri* OLL2809 strain had a positive effect on menstrual-related symptoms [11]. The *L. paragasseri* LPG-9 strain has gastroprotective properties and the ability to inhibit the growth of *Helicobacter pylori* in the stomach of mice [12]. Another probiotic effect of *L. paragasseri* was demonstrated by Kim *et al.* [13], who noted a decrease in white adipose tissue and normalization of the blood lipid profile after adding *L. paragasseri* LG2055 lyophilisate to the diet of obese mice. In addition, the mechanisms for increasing lipid excretion by the *L. paragasseri* LG2055 strain were determined due to the regulation of mRNA of proteins involved in lipid metabolism [13].

Nowadays, no safety group has been established for *L. paragasseri*. However, according to the classification of the Canadian Food Inspection Agency, *L. gasseri* is a sister taxon of *L. paragasseri* [14] and belongs to risk group 1, meaning that this species is not a pathogen for terrestrial animals, including humans. This suggests that the safety information for *L. gasseri* may be applicable to *L. paragasseri*. However, in 2021, the first case of cavernous abscess caused by *L. paragasseri* was described [15]. Therefore, the safety of strains of this species still needs to be confirmed.

According to modern requirements, pre-registration preclinical safety studies of potential probiotic strains of microorganisms require characterization of physiological, biochemical, and antagonistic properties, determination of virulence, toxigenicity, and toxicity [16]. The taxonomic affiliation of strains must be established using molecular methods, and the presence of antibiotic resistance genes, plasmid material, and prophages had to be studied [17].

The complete 16S ribosomal RNA gene sequence of *L. gasseri* and *L. paragasseri* differs only in two single-nucleotide polymorphisms [18]. Whole-genome sequence analysis can reliably distinguish between two species [18–20]. Moreover, distinguishing between *L. paragasseri* and *L. gasseri* species using matrix-assisted laser desorption ionization (MALDI) is difficult, whereas biochemical identification allows for the accurate differentiation of these two related species [14,15].

Currently whole-genome sequencing allows to distinguish taxonomy between closed species and establish of the absence of the required gene determinants (plasmid mate-

rial and prophages). The metabolic potential of strains can also be predicted, possible mechanisms of interaction with the host organism can be determined, and probiotic effects can be substantiated using this approach.

Studying the biochemical properties of microbial strains is important for identification and evaluation of their probiotic and biotechnological potential. Despite the published data on the phenotypic properties of *L. paragasseri* strains [14], the ability to use different substrates may vary. Therefore, it is necessary to establish metabolic features for candidate probiotics.

Therefore, this study aimed to determine the probiotic potential of the *L. paragasseri* lg94 strain based on its biochemical features and genomic landscape.

2. Materials and Methods

2.1 Cultivation and Identification of the Strain

L. paragasseri lg94 strain was isolated from the human feces. Cultivation was performed in MRS lactobacillus broth (HiMedia, Mumbai, Maharashtra, India) at 37 °C for 48 h. Identification was performed using a bacteriological method that assessed the morphological, cultural, tinctorial, and biochemical properties of the isolates, with confirmation provided via MALDI-TOF direct protein profiling [21]. The strain genus and species were determined by mass spectrometry using the MALDI Biotyper system. In brief, isolates were grown at 37 °C for 24 h after isolation of pure cultures. Protein extraction was performed according to the manufacturer's recommended standard operating protocol. A Microflex instrument (Bruker, Billerica, MA, USA) was used for mass spectrometric analysis. Spectra were collected manually or automatically. The server interface (version 4.3.1) and server module (version 4.3.18) were used for identification.

2.2 Studying the Biochemical Profile

The biochemical profile of the *L. paragasseri* lg94 strain was studied using API 50 CH/CHL kits (Biomérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions, and the plates were incubated at 37 °C. The results were visually assessed by the change in well color after 24, 48 and 72 h of incubation.

2.3 Quantitative Determination of L-Lactic Acid Content

A 24-h culture was used for the inoculum, and the inoculum cell titer was 1.8×10^9 CFU/mL. In this study, 0.1 mL of the inoculum was added to 9.9 mL of liquid MRS medium (Table 1). The strain lg94 was grown at 37 °C for 24 h. After that, we pelleted the cells by centrifugation at 9000 rpm for 5 min and transferred the supernatant to a new tube.

We determined the concentration of L-lactic acid in the culture fluid by photometric method using the commercial Lactate-Vital kit (Vital Development Corporation, Saint Petersburg, Russia), according to the manufacturer's

Table 1. The composition of the MRS medium used in the experiment.

Component of the bacterial medium	Concentration, per 1 L
Glucose/lactose	10 g
Yeast extract	5 g
Peptone	10 g
Potassium phosphate trihydrate	4 g
Magnesium sulfate heptahydrate	0.2 g
Ammonium citrate	5 g
Manganese sulfate	0.05 g
Meat-peptone broth	100 mL
Distilled water	Up to 1000 mL

instructions. To ensure the linearity and accuracy of measurements, serial dilutions of the culture fluid were made with sterile distilled water in the following ratios: 1:50, 1:100, and 1:200. A solution of L-lactic acid with a concentration of 2 mmol/L, included in the commercial Lactate-Vital kit, was used as a positive standard. We determined the concentration of L-lactic acid by measuring the optical density at a wavelength of 540 nm using an F300TP photometer (JSC Vityaz, Vitebsk, Republic of Belarus).

The concentration of L-lactic acid (C , mmol/L) was calculated using the following formula:

$$C \left(\frac{\text{mmol}}{\text{L}} \right) = \frac{2 * E_L}{E_P}$$

- E_L —the value of the optical density of the culture liquid.
- E_P —the value of the optical density of the positive standard.

To ensure the accuracy of the calculations, the optical density of the working solution of Lactate-Vital was measured, and its value was subtracted from the optical density values of all other samples resulting in the E_L value. The obtained concentration of L-lactic acid in mmol/L was converted to g/L according to the formula:

$$C \left(\frac{\text{g}}{\text{L}} \right) = \frac{C \left(\frac{\text{mmol}}{\text{L}} \right)}{1000} \times D \times M_{L\text{-lactic acid}}$$

- D —dilution factor of culture fluid (50, 100, 200 or 1).
- $M_{L\text{-lactic acid}} = 90 \text{ g/mol}$.

All calculations were performed for each dilution, and the average value between all dilutions was calculated.

2.4 Determination of the Titratable Acidity of Different Bacterial Media

The *L. paragasseri* lg94 strain was grown in liquid MRS medium with lactose/glucose and sterile milk at 37 °C for 24 h. Table 1 shows the medium composition. Sterile milk was obtained from sterilization of ultra-pasteurized commercial milk with 3.2% fat content. Titratable acidity

was determined by titration of the culture liquid with 0.1 N NaOH solution in the presence of 1% phenolphthalein solution as an indicator.

2.5 Definition of Bile and Low pH Stressful Effects

A second-generation *L. paragasseri* lg94 culture was used for inoculum preparation. 5 mL of the *L. paragasseri* lg94 culture were inoculated into 100 mL of liquid MRS medium, resulting in a 5% (v/v) inoculum. Cultivation was performed at 37 °C for 24 h under thermostatically controlled conditions. Following incubation, the culture was centrifuged at 8000 rpm for 10 min at 10 °C to collect the bacterial cells. The supernatant was discarded, and the cell pellet was washed twice with 100 mL of sterile 0.9% (w/v) NaCl solution. Finally, the pellet was resuspended in 10 mL of sterile 0.9% (w/v) NaCl solution. Stress exposure was tested under the following conditions:

- Control: MRS liquid medium.
- Low pH: Britton-Robinson buffer, pH 2.0.
- Bile: Britton-Robinson buffer containing 0.3% bile, pH 7.0.

In the experiment, 1.5 mL of concentrated *L. paragasseri* lg94 biomass was added to 8.5 mL of the corresponding solution, thoroughly mixed, and incubated at 37 °C for 2 h. The viable cell count (CFU/mL) was determined by serial dilution in tubes containing semi-solid MRS medium, followed by incubation at 37 °C for 48 h. CFU/mL values were recorded before and after exposure to the respective stress factors (low pH and bile).

2.6 Antimicrobial Susceptibility Testing Using the Disk Diffusion Method

The *in vitro* susceptibility of the strain to antibiotics was determined using the disk diffusion method [22]. A 24-h culture was grown on MRS agar and suspended in 0.9% NaCl until a turbidity of 0.5 according to McFarland (1.5×10^8 CFU/mL) was achieved and 100 μL of the bacterial suspension was spread evenly on MRS agar plates. Ten antibiotics (Scientific Research Center of Pharmacy, St. Petersburg, Russia) from different groups were used: β -lactams (ampicillin, 2 $\mu\text{g/mL}$), penicillins (penicillin G, 1 U; oxacillin, 1 $\mu\text{g/mL}$; amoxicillin, 30 $\mu\text{g/mL}$), tetracyclines (tetracycline, 30 $\mu\text{g/mL}$), lincosamides (clindamycin, 2 $\mu\text{g/mL}$), macrolides (erythromycin, 15 $\mu\text{g/mL}$), and amphenicols (chloramphenicol, 30 $\mu\text{g/mL}$), aminoglycosides (gentamicin, 10 $\mu\text{g/mL}$), other antibacterial drugs (fosfomicin, 200 $\mu\text{g/mL}$). The results of the study were interpreted according to the criteria specified in the methodological recommendations: if the inhibition zone diameter was ≤ 8 –10 mm, the strain was considered resistant to antibiotics (R), if it was 11–20 mm, it was considered susceptible with increased exposure (I), and if it was ≥ 21 mm, it was considered susceptible (S) [22].

2.7 Antagonistic Activity Assay

Antagonist activity was assayed using two methods: the perpendicular streak method and the well method, each in triplicate.

Test cultures included the reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, as well as clinical isolates with confirmed multidrug resistance (MDR) obtained from the collection of Laboratory of Microbiome and Microecology (Scientific Center for Family Health and Human Reproduction Problems, Irkutsk, Russia). They were *E. coli* 235-LMM, *P. aeruginosa* ODKB-58, *Klebsiella pneumoniae* ODKB-81 and *K. pneumoniae* ODKB-82.

For the well method, the strain was grown in MRS broth (37 °C, 24 h) under anaerobically maintained conditions. Anaerobiosis was achieved using an Anoxomat atmospheric generation system (Advanced Instruments, Inc., Norwood, MA, USA): sealed anaerostats containing culture tubes were filled with a gas mixture (80% N₂, 10% H₂, 10% CO₂). The optical density of the culture at 600 nm (OD₆₀₀) was measured in triplicate using a Nano-500 spectrophotometer (Allsheng Instruments Co., Ltd., Hangzhou, China) with sterile MRS broth as the reference medium. The average OD₆₀₀ value was 2.42 ± 0.01, corresponding to 2 × 10⁹ CFU/mL for this strain in the stationary growth phase. Petri dishes with Mueller-Hinton agar (HiMedia, Mumbai, Maharashtra, India) were seeded with a continuous lawn of tested strains, adjusted to 0.5 on the McFarland scale (1 × 10⁸ CFU/mL). Wells with a diameter of 10 mm were aseptically formed in the agar, and 100 µL of *L. paragasseri* Ig94 culture fluid was added. The strain was incubated aerobically at 37 °C for 24 h.

For the perpendicular streak method, the strain Ig94 was streaked onto Bifidum agar medium (State Research Center of Applied Microbiology and Biotechnology, Obolensk, Russia) and cultured in an anaerobic jar at 37 °C for 48 h. After incubation, an exponential culture of the test strains was streaked perpendicular to the *L. paragasseri* Ig94 growth line. Further incubation was carried out under aerobic conditions (37 °C, 24 h) to ensure the growth of the test strains.

The sizes of the inhibition zones were measured in millimeters (mean ± standard deviation) and interpreted according to established criteria: for the perpendicular streak method, 4–9 mm means weak activity, 9–14 mm means moderate activity, and ≥14 mm means high activity; for the well method, the inhibition zone is 10–15 mm—weak activity, 15–20 mm—moderate activity, and >20 mm—high activity (diameters include the well, ±1 mm) [17].

2.8 DNA Extraction

The 24 h cell culture of *L. paragasseri* Ig94 was used for DNA extraction. Genomic DNA was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA). DNA quantity was estimated

using a Qubit 4 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit ds-DNA HS Assay Kit (Invitrogen, Waltham, MA, USA). DNA concentration >4 ng/µL was considered acceptable for whole genome sequencing of the sample.

2.9 Whole-Genome Sequencing

The nucleotide sequence of the *L. paragasseri* Ig94 genome was obtained by sequencing on the Illumina NextSeq550 platform (Illumina, San Diego, CA, USA) using the Illumina® DNA Prep Tagmentation, IDT® for Illumina® DNA/RNA UD Indexes Set Tagmentation, and NextSeq 500/550 High Output Kit v2.5 (300 Cycles) library preparation reagents according to the manufacturer's recommendations (Illumina, San Diego, CA, USA).

2.10 Data Processing

The genome sequence was obtained by aligning the obtained reads to the reference genome of *Lactobacillus paragasseri* JCM 5343 (NCBI RefSeq assembly GCF_003584685.1) using Bowtie2 v.2.5.4 [23] (Langmead Lab, Baltimore, MD, USA). MOB-suite v3.0.3 [24] (Public Health Agency of Canada, Guelph, ON, Canada) was used to search and identify plasmid sequences. Annotation was performed using Prokka v1.14.6 [25] (Victorian Bioinformatics Consortium, Melbourne, Victoria, Australia) and Prodigal v2.6.3 [26] (Oak Ridge National Laboratory, Oak Ridge, TN, USA). The search for prophages and CRISPR-Cas loci was performed using PHASTEST v1.0.1 [27] (University of Alberta, Edmonton, Alberta, Canada) and CRISPRCasFinder v4.2.20 [28] (Institut Pasteur, Paris, France). The search for gene functions was performed using BlastKOALA v3.0 [28] (Kanehisa Laboratories, Tokyo, Japan). KEGG Mapper v5.0 [29,30] (Kanehisa Laboratories, Tokyo, Japan) was used to analyze potentially implemented metabolic pathways. The search for antibiotic resistance and disinfectant resistance genes was performed using the ResFinder v2.4.0 and DisinFinder v2.0.1 databases [31,32] (Technical University of Denmark, Copenhagen, Denmark). The online tool MiGA Web v2.0 (Georgia Institute of Technology, Atlanta, GA, USA) was used to confirm the identification of the strain under study [33]. antiSMASH v8.0 (Technical University of Denmark, Copenhagen, Denmark) was used to search for secondary metabolism genes [34]. The sequencing results are presented in the NCBI database in PRJNA1248600 as sample SAMN47864912.

3. Results

Strain Ig94 was isolated from the feces of a healthy child in the Laboratory of Biomedical Microecology of the Scientific Center for Family Health and Human Reproduction Problems (Irkutsk, Russian Federation) on MRS medium. The strain was a facultative anaerobe; it grew well under conditions with low oxygen content both in test tubes

on liquid nutrient media and in conditions of complete absence of oxygen: on agar media in an anaerobic jar with gas-generating bags.

Optimum cultivation conditions were determined in the temperature range of 25–41 °C with a step of 4 °C and pH range of 5.5–7.0 with a step of 0.5. The optimal temperature for the growth of strain lg94 was determined to be 37 °C and pH 6.5, at which the strain reached a concentration of live cells of 2×10^9 CFU/mL after 24 h of incubation on MRS broth.

L. paragasseri lg94 is gram-positive, non-motile, and does not form spores. The cells are rod-shaped, short with rounded ends, located singly, in pairs, or in short chains. When grown on MRS medium, growth was observed in the form of broth turbidity and precipitation of a white-gray sediment at the bottom of the test tube. *L. paragasseri* lg94 cells formed small, mucous, and rounded white colonies on MRS agar.

For strain lg94, biochemical profile tests were conducted, as well as the quantitative determination of L-lactic acid content and full-genome sequencing, followed by genome assembly and gene landscape analysis.

3.1 Biochemical Characteristics of *L. paragasseri* lg94

3.1.1 Biochemical Profile Analysis

The study of carbohydrate metabolism of the *L. paragasseri* lg94 strain using the API 50 CH/CHL test revealed pronounced saccharolytic activity, which was characterized by different carbohydrate fermentation rates depending on the incubation duration. Data on the biochemical profile of the strain are presented in the **Supplementary Table 1**.

After 24 h of cultivation in API 50 CH/CHL medium, *L. paragasseri* lg94 showed positive results regarding the fermentation of 25 carbohydrates (**Supplementary Table 1**). In particular, the ability to utilize monosaccharides (D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, and L-sorbose), sugar alcohols (D-mannitol, D-sorbitol), α -glycosides (methyl- α -D-glucopyranoside), N-acetylglucosamine, salicin, D-cellobiose, D-maltose, D-lactose (bovine), D-sucrose, D-trehalose, D-melezitose, and D-turanose were noted. Interestingly, di- and trisaccharides (amygdalin, arbutin, and aesculin) were utilized by strain lg94 in the presence of iron citrate. In addition, the strain demonstrated the ability to metabolize D-tagatose, potassium 2-ketogluconate, and potassium 5-ketogluconate.

The subsequent increase in incubation time to 48 h resulted in an expansion of the spectrum of fermentable carbohydrates. *L. paragasseri* lg94 demonstrated the ability to utilize L-arabinose, D-melibiose, and gentiobiose. Further incubation for up to 72 h revealed inositol, D-lyxose, and potassium gluconate fermentation.

3.1.2 Determination of L-Lactic Acid Concentration

L. paragasseri lg94 exhibited intense L-lactic acid production, reaching a concentration of 6.41 g/L after 24 h of cultivation, indicating the high acid-forming capacity of this strain (Table 2).

Table 2. L-lactic acid concentration in *L. paragasseri* lg94 culture liquid.

Sample variant	E_{raw}	E_{L}	C, mmol/L	C, g/L	C, g/L
1:50	0.27	0.18	1.241	5.584	
1:100	0.19	0.10	0.69	6.21	6.41 ± 0.95
1:200	0.15	0.06	0.41	7.452	

Based on their acid-producing capacity, the strains were classified into two groups. Strains producing 1–2 g/L of acid in the culture medium were defined as moderate acid producers, whereas strains producing >2 g/L were classified as active acid producers. The optical density of the working solution (E_{ws}) was 0.09, while that of the positive control (E_{p}) was 0.29.

The optical density of the culture fluid was calculated as follows:

$$E_{\text{L}} = E_{\text{raw}} - E_{\text{ws}}$$

3.1.3 Titratable Acidity

Analysis of the titratable acidity of *L. paragasseri* lg94 grown on various substrates (glucose, lactose, and sterile milk) revealed the dependence of titratable acidity on the nature of the substrate used: on a medium with lactose, titratable acidity was 142 °T, which exceeded the value obtained during cultivation on a medium with glucose (100 °T); however, the level of titratable acidity on lactose was lower than on sterile milk (176 °T). A dense, uniform clot was observed during *L. paragasseri* lg94 cultivation on sterile milk. The formation of a characteristic aroma combining sweet and sour-milk notes was noted. The present results indicate the influence of substrate type on the metabolic activity and acid-forming capacity of the studied lactobacilli strains, as well as the formation of the product's organoleptic properties. Milk fermentation is a direct consequence of the strain's active acid formation. Lactic acid accumulation in milk leads to reduced pH, destabilizing casein micelles, and subsequent protein coagulation.

3.1.4 Viability of *L. paragasseri* lg94 Under Bile and Low pH Conditions

The results indicated that exposure to a universal buffer (pH 7.0) did not significantly affect the viability of the *L. paragasseri* lg94 strain. However, the strain demonstrated low resistance to acid stress; when exposed to a medium with pH 2.0, the CFU count was 0.5×10^3 CFU/mL, indicating a significant reduction in viability. In

Table 3. Antagonistic activity of *L. paragasseri* lg94 against test strains.

Test strain	Growth inhibition zone, mm (wells)	Activity level (wells)*	Growth inhibition zone, mm (streaks)	Activity level (streaks)**
<i>E. coli</i> ATCC 25922	13.6 ± 0.6	Weak	17.5 ± 0.7	High
<i>E. coli</i> 235 LMM	absent	–	16.5 ± 0.7	High
<i>P. aeruginosa</i> ATCC 27853	13.0 ± 1.0	Weak	18.5 ± 0.7	High
<i>P. aeruginosa</i> ODKB-58	absent	–	19.5 ± 0.7	High
<i>K. pneumoniae</i> ODKB-81	absent	–	15.0 ± 0.0	High
<i>K. pneumoniae</i> ODKB-82	absent	–	16.5 ± 0.7	High

*Well method: 10–15 mm—weak, 15–20 mm—moderate, >20 mm—high (diameter including well, ±1 mm); **Perpendicular streak method: 4–9 mm—weak, 9–14 mm—moderate, ≥14 mm—high.

contrast, *L. paragasseri* lg94 exhibited high resistance to 0.3% bile, as evidenced by a high CFU value of 1.3×10^8 CFU/mL. All measurements were compared with the control sample (strain culture in Britton-Robinson buffer), with the control sample showing a CFU count of 2×10^{10} CFU/mL. No changes in colony morphology were observed.

3.1.5 Antimicrobial Susceptibility

According to the disk diffusion assay, strain lg94 exhibited phenotypic susceptibility to ampicillin, amoxicillin, penicillin G, tetracycline, erythromycin, and chloramphenicol, as well as intermediate resistance to oxacillin. Phenotypic resistance to fosfomycin, gentamicin, and clindamycin was also observed.

3.1.6 Antagonistic Activity Study

Using the perpendicular streak method, high antagonist activity of *L. paragasseri* lg94 was detected against all six test cultures: the width of the inhibition zones ranging from 15.0 to 19.5 mm (Table 3). The largest zone (19.5 ± 0.7 mm) was recorded against the clinical isolate of *P. aeruginosa* ODKB-58, which may indicate increased sensitivity of this strain to the metabolites of *L. paragasseri* lg94.

However, the well method showed significantly weaker or no activity. Inhibition zones were detected only against the reference strains *E. coli* ATCC 25922 (13.6 ± 0.6 mm) and *P. aeruginosa* ATCC 27853 (13.0 ± 1.0 mm), indicating a weak level of antagonism. No growth inhibition zones were detected for all four clinical isolates (*E. coli* 235 LMM, *P. aeruginosa* ODKB-58, *K. pneumoniae* ODKB-81, and *K. pneumoniae* ODKB-82).

The resulting antagonism profile—strong activity in the perpendicular streak method with little or no effect in the well method—is typical of an acid-dependent inhibition mechanism and suggests that lactic acid is the primary antagonist of *L. paragasseri* lg94 under these culture conditions, which creates localized acid stress at the pathogen contact zone.

3.2 Genome Analysis of the Studied Strain

3.2.1 Genome Assembly

Assembly genome analysis yielded one chromosomal sequence and two plasmid sequences. The chromosome length was 1,894,585 bp, and the GC nucleotide percentage was 35.24. In the process of coding sequence labeling, 4001 sequences that potentially encode proteins, 85 tRNA sequences, 8 rRNA, 66 small miscRNA, and 3 tmRNA were identified.

3.2.2 Genetic Characteristics of the Strain

The BlastKOALA annotation revealed that the genome contains 2105 functional units (Fig. 1). The largest number of genes was classified as “Genetic information processing”. Using the KEGG Mapper application, it was found that *L. paragasseri* lg94 had the necessary pentose phosphate pathway enzyme complex and enzymes that ensure the transport and metabolism of pentasaccharides and hexasaccharides.

Biochemical testing of *L. paragasseri* lg94 revealed its ability to ferment various carbohydrates, including mono-, di-, and trisaccharides, sugar alcohols, α -glycosides and ketogluconate. Genomic analysis confirmed this metabolic potential by detecting genes encoding key glycolysis enzymes (*pgi*, *pfkA*, *fba*, *tpiA*, *gap*, *pgk*, *gpm*, *eno*, *eno2*, *pyk*), the pentose phosphate pathway (*gnd*, *rpe*, *rpi*, *tal*, *tkt*), and the gluconate dehydrogenase gene, indicating alternative pathways of glucose metabolism. The absence of the phosphoketolase gene (*xfp*) is a distinctive feature of the strain, allowing it to be classified as a homofermentative type. In addition, numerous *bgl* genes were found in the genome, which is consistent with the ability to hydrolyze β -glycosides revealed in biochemical tests, and the *lacS* gene (in two variants), indicating the presence of an effective lactose transport system. *L. paragasseri* lg94 has a high potential for lactic acid production (three *ldh* genes) by the homofermentative pathway (no *xfp* gene), which is confirmed by high titratable acidity in milk. The strain can also synthesize exopolysaccharides (*epsH* and *epsL* genes) and acetaldehyde (*aldC* and *adhE* genes).

Genes potentially responsible for the putative probiotic properties were identified in the genome. Genes or pro-

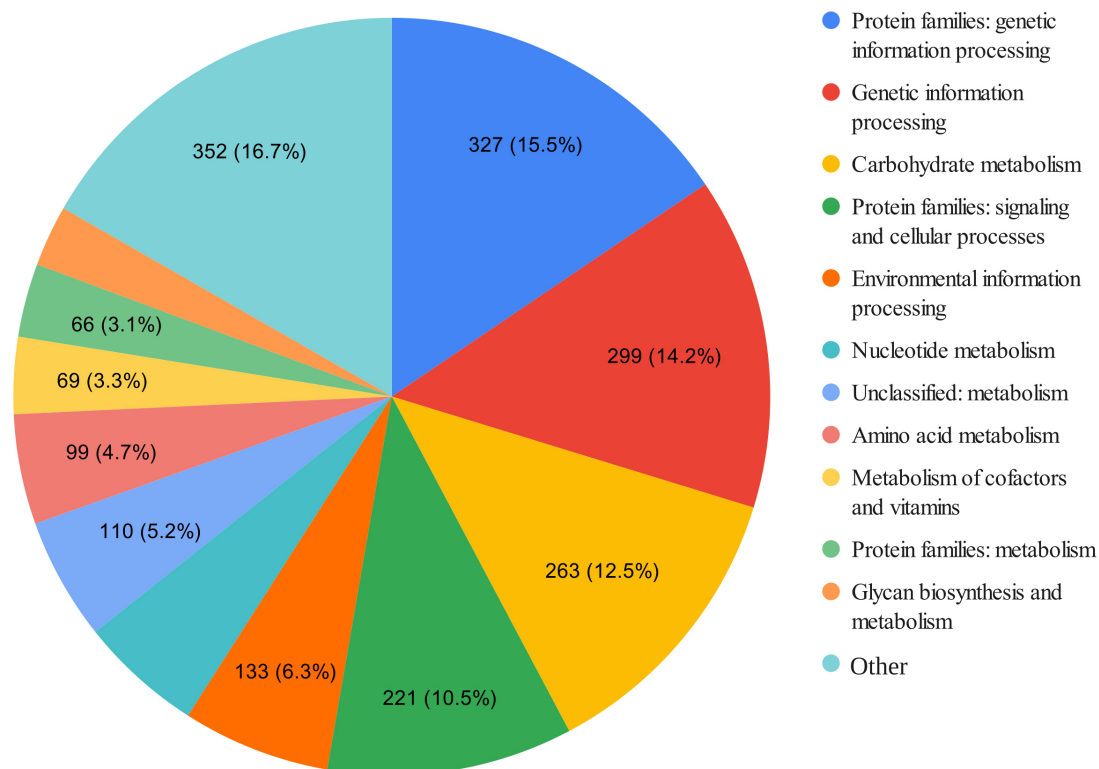


Fig. 1. Distribution of the number of genes in the genome of *L. paragasseri* Ig94 by functions of metabolic pathways. Different colors represent different groups of metabolic pathways, grouped according to the KEGG classification. KEGG, Kyoto Encyclopedia of Genes and Genomes.

teins involved in acid and bile tolerance, intestinal mucosal adhesion, temperature tolerance, host immunomodulation, antimicrobial activity, and internal defense were identified.

The genes *NhaC* (Na^+/H^+ antiporter *NhaC*) and F₀F₁ ATP synthase are involved in pH and gastric tolerance. Moreover, bile acid tolerance is mediated by *cbh* choloylglycine hydrolase and *ycaD*, an MFS transporter. The adhesion genes triose phosphate isomerase *tpiA* and elongation factor Tu *tuf* are responsible for the strain's adhesive properties. The molecular chaperone genes *DnaJ* and *DnaK* and the cold shock protein *cspA* mediate temperature tolerance. Proteins encoded by the LTA synthase family protein *ltaS1*, D-alanyl-lipoteichoic acid biosynthesis protein (*DltD*), glycosyltransferases (*mshA*, *epsF*, *ftsW*, *rodA*, *glyE*), and glucosaminefructose-6-phosphate aminotransferase (*GlmS*) genes participate in host immunomodulation processes.

The antimicrobial oxidase genes pyruvate oxidase *pox5*, which confer resistance to other bacteria, were also found in the genome.

3.2.3 Confirmation of Species Identification

The online tool MiGA was used to confirm the identification of the strain under study. The closest relatives of

the strain Ig94 found by MiGA in the database were *L. paragasseri* GCF 003584685 T (98.74% AAI) and *L. gasseri* GCF 000014425 T (96.64% AAI). The dataset most likely belongs to the same root GCF 003584685 1 (*p*-value: 0), probably belongs to the species *Lactobacillus paragasseri* (*p*-value: 0.012), and may even belong to the same dataset as GCF 003584685 (*p*-value: 0.37).

3.2.4 Pathogenicity, Toxicity, and Virulence Gene Screening

No antibiotic resistance, pathogenicity, or disinfectant resistance genes were found in the studied strain's genome. The key virulence genes (*gelE*, *hyl*, *asa1*, *cylA*, *esp*, *cytK*, *nhe*, *hbl*, and *sprE* [35]) were also absent.

Screening of genes responsible for the production of biogenic amines was conducted. Lactic acid bacteria produce biogenic amines in fermented and spoiled food products [36]. Increased levels of biogenic amines can negatively affect the human body, causing hypertension, headache, increased heart rate, vomiting, or diarrhea [37, 38]. No genes associated with the production of histamine, putrescine, and tyramine (*hdcA*, *tyrRS*, *odc*, *aguA*, *potE*) were found in the genome of the *L. paragasseri* Ig94 strain, but the *hisS* gene (histidyl-tRNA synthetase) was found.

However, this gene is a housekeeping gene present in many bacteria.

3.2.5 Genome Stability: Plasmids and Mobile Genetic Elements

Two plasmids were found in the *L. paragasseri* lg94: AC196 and AE118 (Table 4). Plasmids did not carry pathogenicity or resistance determinants.

Table 4. Characteristics of plasmids found in the genome of *L. paragasseri* lg94.

Parameters	lg94-AE118	lg94-AC196
Contigs	7	1
Size	19,433	3239
GC content %	31.5	37.4
Replicone type	-	rep_cluster_707
Relaxase type	-	MOBQ
Predictable mobility	Non-mobile	Mobile
Cluster	AE118	AC196
Hypothetical host carrier	<i>Lactobacillus</i>	Lactobacillaceae

Plasmid AC196 contained only one contig with a length of 3239 bp. Six coding sequences were identified and annotated as proteins with unknown functions. Plasmid AE118 contained seven contigs with a length of 19,433 bp. A total of 22 coding sequences were identified, 14 of which were annotated as proteins with unknown functions. The remaining genes were represented by transposase, sporulation inhibitors, and various transport proteins.

No prophage sequences or CRISPR-Cas loci were detected in the studied genome.

3.2.6 Genes Involved in the Production of Secondary Metabolites

Clusters of genes producing enterolysin A, helveticin J and gassericin T were found for the strain lg94. A cluster of genes responsible for the production of gassericin T is shown in Fig. 2. We also identified the *ribB*, *ribT*, and *cobC* genes responsible for riboflavin and cobalamin synthesis.

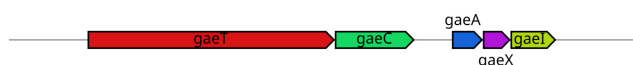


Fig. 2. Gassericin T production cluster in the genome of *L. paragasseri* lg94. Different genes of the gassericin production cluster are represented in different colors.

4. Discussion

Despite the growing interest in this species, information on its genetics, and particularly on the genetic basis of its probiotic potential, remains limited. At the same time, bacteriocins have been identified as one of the key

factors underlying probiotic activity; however, their detection using conventional *in vitro* methods is complex and labor-intensive. In this context, *in silico* genome analysis for the presence of bacteriocin operons is considered a promising and more efficient approach for bacteriocin screening. According to the literature, genomes of *L. paragasseri* harbor operons responsible for the production of various bacteriocins, including pediocin, gassericins A, T, and K7B, acidocin B, bacteriocin LS2 (chains a and b), bacteriolysin, helveticin-J, and enterolysin A, with genes encoding gassericin T, acidocin B, and gassericin A being the most prevalent (Table 5, Ref. [39–41]).

The UBLG-36 genome contains genes encoding proteins that may determine its putative probiotic properties. Genes or proteins involved in acid and bile tolerance, intestinal mucosal adhesion, temperature tolerance, host immunomodulation, antimicrobial activity, and endocrine defense have been identified [39]. Strain lg94 has the same spectrum of genes that provide probiotic potential, except for some adhesion genes (LPXTG cell wall anchor domain-containing protein, Membrane lipoprotein lipid attachment site-containing protein, CpsD/CapB family tyrosine-protein kinase and MucBP domain-containing protein) and resistance to temperature shock (Co-chaperone GroES, Chaperonin GroEL).

CRISPR-Cas adaptive immune systems have previously been described in a number of *L. paragasseri* and *L. gasseri* genomes and are considered an important factor in resistance to invasive DNA, including bacteriophages and plasmids, as well as a potential taxonomic marker for distinguishing closely related species. A published study by Zhou *et al.* [41] demonstrated the widespread distribution of the CRISPR-Cas subtype II-A in both species, along with species-specific differences in Cas protein composition and locus organization, as supported by phylogenetic analyses of Cas1, Cas2, and Cas9. However, in the present study, no components of the CRISPR-Cas system, including the *cas1*, *cas2*, and *cas9* genes, were detected in the analyzed *L. paragasseri* genomes. This discrepancy with previously reported data indicates pronounced intraspecies variability in *L. paragasseri* with respect to the presence of CRISPR-Cas systems and may reflect differences in the evolutionary histories of strains as well as adaptation to environments with reduced pressure from mobile genetic elements.

Genomic analysis of *L. paragasseri* lg94 allowed us to identify the genetic determinants of its metabolism and use potential. The strain has a complete set of genes encoding enzymes necessary for glycolysis and pentose phosphate pathways, ensuring efficient hexose and pentose metabolism. The genome lacks the gene encoding phosphoketolase, which allows us to classify *L. paragasseri* lg94 as a homofermentative strain that mainly produces lactic acid [41]. The presence of multiple *bgl* genes encoding β -glycosidases indicates a strong ability of the strain to hydrolyze various β -glycosidic bonds, cleaving, for example,

Table 5. Comparison of the genome of *L. paragasseri* lg94 with genomes of other strains belonging to the species *L. paragasseri*.

Strain	Reference	Bacteriocin gene cluster
lg94	this study	enterolysin A, helveticin J, gassericin T
UMB3776	Jackson <i>et al.</i> , 2024 [39]	gassericin T
UMB4347	Jackson <i>et al.</i> , 2024 [39]	gassericin T
UMB1634	Jackson <i>et al.</i> , 2024 [39]	gassericin T, A, S
UBLG-36	Mehra <i>et al.</i> , 2021 [40]	gassericin T, S, acidocin B
79 genomes	Zhou <i>et al.</i> , 2020 [41]	gassericin T, acidocin B, bacteriocin-LS2chain, helveticin J

amygdalin, arbutin, esculin, salicin, cellobiose, and gentiobiose, while the presence of multiple copies of the *bglF* and *bglH* genes may indicate increased activity or variability in substrates for these enzymes [42]. The detection of *bgl* genes is confirmed by the positive results of biochemical analysis demonstrating the ability of *L. paragasseri* lg94 to hydrolyze esculin. The presence of the gluconate dehydrogenase gene in the *L. paragasseri* lg94 genome suggests that the strain can metabolize glucose via an alternative pathway, indicating that gluconate can be used as an energy source under carbohydrate starvation conditions. The high titratable acidity of *L. paragasseri* lg94 in milk is likely due to the presence of two lactose permease gene variants, providing efficient lactose transport, and three lactate dehydrogenase gene variants, making the strain promising for milk fermentation.

In addition to key metabolic characteristics, *L. paragasseri* lg94 possesses genes that determine additional functional properties, making it an attractive candidate for improving fermented products and enhancing probiotic action. The discovery of a gene cluster associated with riboflavin (vitamin B2) synthesis and a gene involved in cobalamin (vitamin B12) suggests that *L. paragasseri* lg94 synthesizes these essential vitamins. The ability to synthesize B vitamins *in situ* is a significant advantage of using the strain as a probiotic because it allows the intestinal microbiota to be enriched with these important nutrients. This property can be used to enrich fermented products (e.g., yogurt or kefir) with vitamins, thereby increasing their nutritional value.

The presence of genes associated with the synthesis of exopolysaccharides indicates the potential ability of *L. paragasseri* lg94 to produce these polysaccharides. Exopolysaccharides are important components of the bacterial cell wall and are secreted into the environment. Exopolysaccharides produced by lactic acid bacteria have valuable properties that improve the texture and rheological characteristics of fermented products [43].

Lactobacilli inhabiting the intestinal biotope are gassericin producers [44]. According to Ene *et al.* [18], reported that all *L. paragasseri* strains with publicly available genomes had gassericin genes (T, S, or both). This suggests that the properties once attributed to *L. gasseri* may be more represented by *L. paragasseri* species [18]. The biologically active properties of gassericins have been described

in the literature. Liang *et al.* [45] showed that a modified gassericin could effectively inhibit the growth of *S. mutans in vitro* and the development of dental caries *in vivo*. These results reveal a new peptide-based agent as a topical treatment for dental caries, paving the way for clinical trials to explore its potential in caries prevention. Mahdavi *et al.* [46] demonstrated the effect of the bacteriocin gassericin A, which is produced by some intestinal bacteria, on an *in vivo* obesity model. Although gassericin A did not cause weight loss, it may alleviate obesity-related complications. There is evidence for the suitability of gassericin as a food preservative [47] and in veterinary medicine [48].

5. Limitations

Several limitations of this study should be considered. First, the investigation was based on a single strain, *L. paragasseri* lg94, which limits the generalizability of the findings at the species level. Given the known strain-specific nature of probiotic properties, further comparative analyses involving multiple *L. paragasseri* strains are required.

Second, the functional properties of the strain were primarily evaluated using *in vitro* assays and *in silico* genome analysis. Although these approaches provide valuable preliminary insights into the potential of probiotics, they do not fully reflect the gastrointestinal environment's complex conditions. The observed low resistance to highly acidic conditions (pH 2.0) may have different implications *in vivo*, where buffering effects of food and host factors are present.

Third, the antagonistic activity results suggest an acid-dependent inhibition mechanism; however, the contribution of specific antimicrobial compounds (e.g., bacteriocins such as gassericin T, enterolysin A, and helveticin J) was not experimentally validated. Additional targeted studies are needed to confirm their expression and biological activity.

Fourth, although whole-genome sequencing did not reveal genes associated with virulence, pathogenicity, or antibiotic resistance, genomic predictions alone cannot fully exclude potential safety risks. A comprehensive safety assessment, including *in vivo* toxicity and long-term colonization studies, is required.

Finally, this study did not include *in vivo* experiments or clinical validation, which are essential to confirm the

strain's probiotic efficacy, safety, and functional effects in the host organism.

6. Conclusions

The main characteristics of the *L. paragasseri* lg94 strain were established. The biochemical profile results indicate that *L. paragasseri* lg94 has several saccharolytic activities. The diversity of fermentable carbohydrates may be an important factor determining the adaptation of the strain to various ecological niches and its potential use in the food industry or as a probiotic. *L. paragasseri* lg94 has high L-lactic acid production and the ability to ferment milk, making it potentially useful for the production of fermented products (e.g., yogurt, cheese, sauerkraut, etc.) or as a biopreservative. The high acid-forming activity of *L. paragasseri* lg94 can be used to suppress the growth of unwanted microorganisms and increase the shelf life of food products. The absence of antibiotic resistance, pathogenicity, and virulence determinants, as well as prophage sequences and CRISPR-Cas loci in the genome, was confirmed. The ability of *L. paragasseri* lg94 to synthesize exopolysaccharides can be used to improve the quality of fermented dairy products. *L. paragasseri* lg94, which encodes genes for acetaldehyde metabolism, has the potential to produce this aromatic compound. The cultivation conditions must be optimized to control the acetaldehyde concentration to ensure a pleasant aroma. This strain is capable of gassericin, riboflavin, and cobalamin biosynthesis. Thus, whole-genome sequencing confirmed the safety and probiotic potential of *L. paragasseri* lg94. The next stage of the work is recommended to study its antagonistic activity and metabolite production.

Abbreviations

AAI, average amino acid identity; CFU, colony-forming units; Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; DNA, deoxyribonucleic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; MALDI, matrix-assisted laser desorption ionization; OD, optical density; RNA, ribonucleic acid.

Availability of Data and Materials

The genome sequencing results are submitted to the NCBI database under PRJNA1248600 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1248600>) as accession SAMN47864912 (https://www.ncbi.nlm.nih.gov/sra?LinkName=biosample_sra&from_uid=47864912). The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NB, NSe and LR designed the research study. EK, AG, NSm, RZ, AM and NG performed the research. EK, AG and NSm analyzed the data. EK, NB, AG, and NSm wrote the manuscript, EK wrote the first draft. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

When working with the study participants, the ethical principles set forth in the Helsinki Declaration of the WMA (2013 edition) and the Order of the Russian Ministry of Health dated April 1, 2016, No. 200n "On Approval of the Rules of Good Clinical Practice" were observed. The study was approved by the Biomedical Ethics Committee of the Federal State Budgetary Scientific Institution 'Scientific Center for Family Health and Human Reproduction Problems' (Protocol No. 5 dated May 16, 2016) and was conducted with the informed consent of adolescents over 15 years of age or parents/legal guardians of children under 15 years of age.

Acknowledgment

The study was conducted using equipment from the Center for the Development of Progressive Personalized Health Technologies and the Irkutsk Region Human Microbiota Collection (Irkutsk). We thank the reviewers of this publication for their opinions and suggestions.

Funding

The study was carried out within the framework of state topic "Metabolic potential of microbial consortia as a basis for a technological approach to the development of new probiotic preparations" (No. 125011600372-4).

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work the authors used TrinkaAI in order to check spell and grammar. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBE47162>.

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