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RESEARCH

In Vitro and In Silico Assessment of the Probiotic and Technological Potential of *Lacticaseibacillus rhamnosus* MW019593 Isolated from Algerian Cow's Milk

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Abstract

This study evaluated the in vitro and in silico probiotic potential and technological characteristics of *Lacticaseibacillus rhamnosus* MW019593 isolated from Algerian cow's milk. Safety was verified by the absence of hemolytic activity, biogenic amine production, and antibiotic sensitivity. Functional probiotic properties of *L. rhamnosus* MW019593 included moderate inhibition of gastrointestinal pathogens such as *Salmonella*, *Listeria*, *Clostridioides*, and *Escherichia coli*, tolerance to pH 2–3 and up to 1% bile salts, 46.4% hydrophobicity, 42.4% autoaggregation, and 12.9% adhesion to Caco-2/TC7 cells without cytotoxicity. The ability to form biofilms with a thickness of 23.7 µm, comparable to *L. rhamnosus* ATCC 53103, was observed using confocal microscopy. Technologically, *L. rhamnosus* MW019593 and ATCC 53103 showed similar growth profiles and biomass yields, reaching approximately 5×10^9 CFU/mL with a 63% yield after lyophilization. No significant difference in strain viability was noted at 4 °C and − 20 °C, which are suitable temperatures to maintain their viability for 3 months. Whole genome sequencing (WGS) was conducted on *L. rhamnosus* MW019593, followed by comprehensive in silico genomic analysis. Taxonomic validation using average nucleotide identity (ANI) and genome-to-genome distance hybridization (GGDH) confirmed the strain's affiliation to this species. Gene screening revealed the absence of virulence and antibiotic resistance genes, while beneficial genes, including those for bacteriocin production, were identified. These findings, supported by the demonstrated safety, functional, and technological properties of the strain, highlight its promising potential for food and therapeutic applications, pending in vivo validation.

Keywords *Lacticaseibacillus rhamnosus* · Gastrointestinal tolerance · Adhesion · Biofilms · Biomass yield · WGS

Introduction

Probiotics, defined as live microorganisms that confer health benefits when ingested in adequate amounts, have garnered significant interest in the food and medical fields [1]. Among them, the genus *Lactobacillus*, particularly the species *L. rhamnosus*, is recognized for its advantageous probiotic properties, notably in modulating gut flora and enhancing immune defenses [2, 3]. However, probiotic effects are often strain-specific, highlighting the importance of thoroughly characterizing promising new strains.

The genus *Lactobacillus* has recently undergone taxonomic revisions, leading to the reclassification of several species into new genera, such as the renaming of *Lactobacillus rhamnosus* to *Lacticaseibacillus rhamnosus* [4]. The continuous search for novel probiotic *Lacticaseibacillus* strains with enhanced functional properties and desirable

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technological traits has become a priority to meet consumer demands and expand applications [2, 5]. In this context, the *L. rhamnosus* species has gained significant interest due to its well-documented probiotic attributes in modulating gut health. *L. rhamnosus* strains are frequently incorporated into functional dairy foods and employed in food biopreservation strategies [3, 6]. Particularly, *L. rhamnosus* GG (ATCC 53103) is a validated probiotic strain that has been extensively investigated for its potential health benefits [6]. While numerous commercial probiotic strains originate from foreign sources, exploring local microbial biodiversity presents an opportunity to discover well-adapted, high-performing indigenous strains tailored to target populations [7, 8]. This study directly addresses this knowledge gap by providing a comprehensive evaluation of an Algerian *L. rhamnosus* strain, highlighting its unique attributes for local and global industrial applications. To date, few studies have focused on the search for probiotic strains from the Algerian microbial environment. Algerian cow's milk, characterized by its unique ecological and microbial composition, serves as an exceptional niche for identifying indigenous probiotic strains. Yet, the identification and characterization of such local strains would be a valuable asset for the development of Algerian probiotics. However, probiotic potential and technological performance can vary considerably among *L. rhamnosus* strains, necessitating comprehensive strain-specific characterization.

Key criteria for probiotic selection include safety aspects, tolerance to gastrointestinal conditions, antimicrobial activity, adhesion capabilities, biofilm formation ability, as well as growth characteristics, biomass yields, and stability of strain viability during storage at different temperatures. Therefore, an in-depth evaluation of these properties is crucial for identifying promising probiotic candidates suitable for industrial applications.

The primary objective of this study is to thoroughly evaluate the in vitro probiotic potential and technological traits of *L. rhamnosus* MW019593, a strain isolated from Algerian cow's milk. Specific aims include the following:

- Assessing safety parameters, gastrointestinal tolerance, and antibacterial activity
- Comparing growth profiles, biomass yields, and viability during storage under various conditions
- Using whole genome sequencing to confirm safety and probiotic potential

Through a systematic assessment of these parameters, the results were compared to those obtained with the reference strain *L. rhamnosus* ATCC 53103. In addition, in silico genomic analysis confirmed the safety of the strain with the absence of pathogenicity-coding genes (i.e., virulence and antibiotic resistance) and its probiotic potential with the presence of two bacteriocin-coding regions. These findings not only underline the relevance of exploring Algerian microbial biodiversity but also emphasize the industrial and therapeutic potential of this strain. This exhaustive characterization will highlight the probiotic assets and technological performance of the local *L. rhamnosus* MW019593 strain, paving the way for its future use in food and health applications. The findings from this study will contribute to the exploration of local microbial biodiversity and the selection of autochthonous strains for probiotic development and industrial production.

Material and Methods

Bacterial Strains and Cell Line Culture Conditions

The following strains were used in this study: *L. rhamnosus* MW019593, was first isolated from Algerian cow's milk during this study, and the reference strain *L. rhamnosus* ATCC 53103 was obtained from the American Type Culture Collection (USA). The initial identification of the strain MW019593 as *L. rhamnosus* was conducted using phenotypic characterization and 16S rRNA gene sequencing. Pathogenic strains included *Salmonella enterica* ATCC 10708, *Yersinia enterocolitica* ATCC 23715, *Clostridioides perfringens* ATCC 13124, *Listeria monocytogenes* ATCC 15313, *Vibrio parahaemolyticus* ATCC 17802, *Shigella sonnei* ATCC 25931, *Enterococcus faecium* ATCC 19433, *Pseudomonas aeruginosa* ATCC 27853, *Citrobacter freundii* ATCC 43864, *Escherichia coli* ATCC 25922, and *Aeromonas hydrophila* ATCC 7966. *Lacticaseibacillus* strains were routinely cultured in de Man, Rogosa, and Sharpe (MRS) broth or agar (Sigma-Aldrich, USA) at 37 °C for 24 to 48 h. Pathogenic strains were grown in Tryptic Soy Broth (TSB; Sigma-Aldrich, USA) at 37 °C for 24 h. The human colon adenocarcinoma cell line Caco-2/TC7 (RRID: CVCL_0233) (derived from ATCC HTB-37, USA) was originally developed by Chantret et al. [9] and was routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, USA) supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich, USA) and 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Sigma-Aldrich, USA) at 37 °C with 5% CO_2 and 95% humidity. For experimental assays (adhesion and cytotoxicity), the cells were seeded at a density of approximately 10^5 cells/cm^2 in 24-well tissue culture plates with the medium regularly changed and incubated to early confluence.

Figure 1 illustrates the experimental workflow employed in this study to characterize *L. rhamnosus* MW019593. It encompasses four main areas of investigation: safety assessment, evaluation of probiotic properties, genome analysis, and technological characterization.

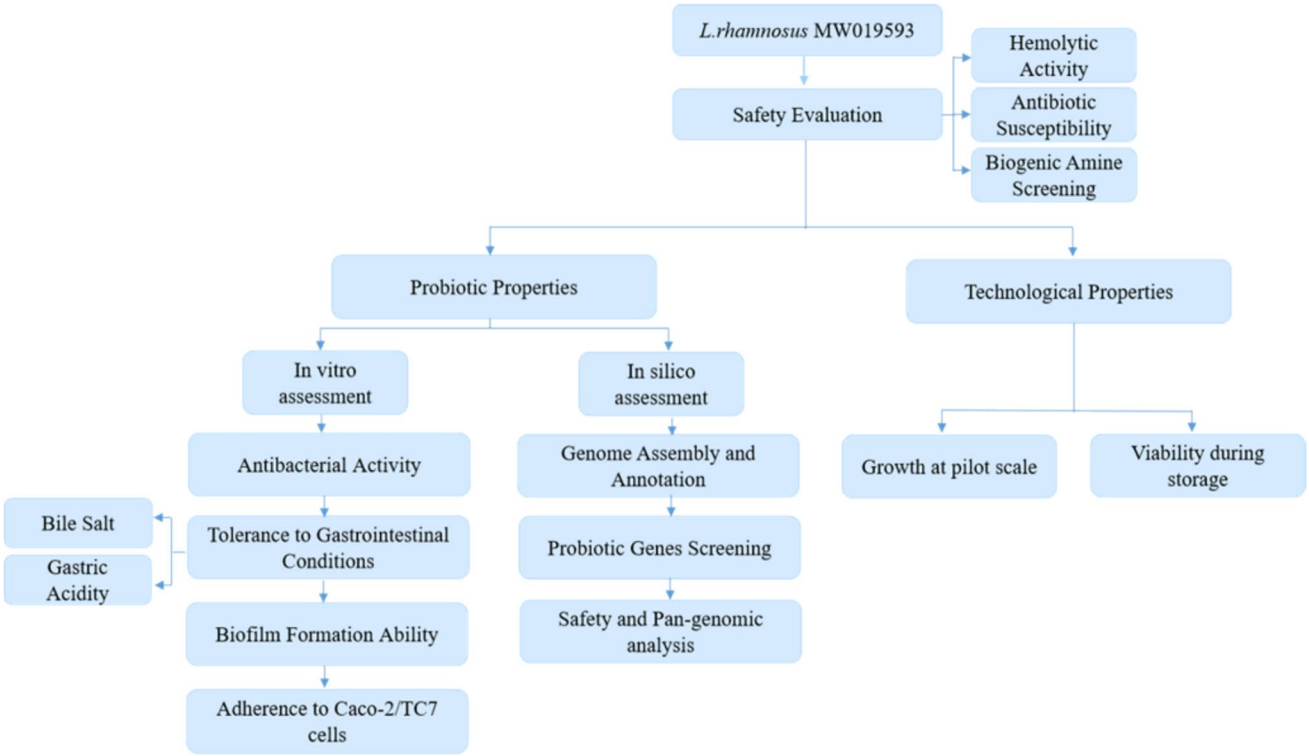

Fig. 1 Methodological steps for probiotic and technological evaluation of *Lacticaseibacillus rhamnosus* MW019593

Each component was systematically investigated using the methods detailed in the following sections.

Safety Aspects

Hemolytic Activity

The hemolytic activity of *L. rhamnosus* MW019593 was determined on MRS agar supplemented with 5% horse blood (Sigma-Aldrich, USA) according to Ghrairi et al. [10] with slight modifications. Following incubation at 37 °C for 24 h, the plates were examined for the presence of clear (β-hemolysis) or greenish (α-hemolysis) hemolytic zones or the absence of such zones (γ-hemolysis) around the colonies of *L. rhamnosus* MW019593.

Antibiotic Susceptibility

The antibiotic susceptibility test was performed following the disc diffusion method [11]. An inoculum of *L. rhamnosus* MW019593 at a concentration of 10^7 CFU/mL was seeded on MRS agar. The antibiotics used included penicillin (P, 10 µg/disc), amoxicillin (AMX, 25 µg/disc), cefazolin (CZ, 30 µg/disc), tetracycline (TE, 30 µg/disc), chloramphenicol (C, 30 µg/disc), vancomycin (VA, 5 µg/disc), azithromycin (AZM, 15 µg/disc), gentamicin (GN, 10 µg/disc), clindamycin (CD, 2 µg/disc), cotrimoxazole (COT, 25 µg/disc), and pefloxacin (PEF, 5 µg/disc). The diameter of the inhibition zones around the antibiotic discs were measured, and the interpretation in terms of susceptibility (S), intermediate (I), or resistance (R) was performed according to the criteria defined by CA-SFM [11].

Screening of Genes Involved in Biogenic Amine Production

To assess the potential of *L. rhamnosus* MW019593 to produce biogenic amines such as histamine, tyramine, and putrescine, a gene screening was performed using PCR. Total genomic DNA was extracted following the method of Leblond et al. [12], and specific primers were used to amplify the genes encoding the decarboxylase enzymes responsible for the production of these amines:

- Histidine decarboxylase (hd):
- F(5′AGATGGTATTGTTTCTTATG3′) and R(5′AGACCATACACCATAACCTT3′)
- Tyrosine decarboxylase (td):
- F(5′ACATAGTCAACCATRTTGAA3′) and R(5′CAAATGGAAGAAGAAGTAGG3′)
- Ornithine decarboxylase (od):
- F(5′TGCACTTCCATATCCTCCAG3′) and R(5′GAATTTCTGGACCAAATC3′)

The PCR products were then loaded (7 µL) onto a 1% agarose gel with a DNA ladder size marker (Invitrogen, USA). Electrophoresis was carried out in 0.5X TAE buffer at 120 V for 25 min.

Antibacterial Activity

The antibacterial activity of *L. rhamnosus* MW019593 was evaluated by the agar spot test on TSA as described by González et al. [13], against *Salmonella enterica* ATCC 10708, *Yersinia enterocolitica* ATCC 23715, *Clostridioides perfringens* ATCC 13124, *Listeria monocytogenes* ATCC 15313, *Vibrio parahaemolyticus* ATCC 17802, *Shigella sonnei* ATCC 25931, *Enterococcus faecium* ATCC 19433, *Pseudomonas aeruginosa* ATCC 27853, *Citrobacter freundii* ATCC 43864, *Escherichia coli* ATCC 25922, and *Aeromonas hydrophila* ATCC 7966. Standardized suspensions of 10^8 CFU/mL of *L. rhamnosus* from 24-h broth cultures in MRS were used. Volumes of 5 µL of these suspensions were spotted on TSA agar previously spread with 100 µL of a standardized 10^6 CFU/mL suspension of each target pathogen. Plates were incubated at 37 °C for 24 h. Diameters of inhibition zones around the spots were then measured to evaluate the antagonism of *L. rhamnosus*.

Tolerance to Simulated Gastrointestinal Conditions

Tolerance to Gastric Acidity

The acid tolerance of *L. rhamnosus* MW019593 and ATCC 53103 were evaluated in tubes containing 9 mL of MRS broth adjusted to different pH levels (1.5, 2, and 3) using a 5 mol/L hydrochloric acid (HCl; Sigma-Aldrich, USA) solution as described by Reale et al. [14] with some modifications. Tubes were then inoculated with *L. rhamnosus* at a concentration of 10^8 CFU/mL and incubated for 3 h at 37 °C. Samples were taken at different incubation times (0, 1, 2, and 3 h) and spread plated on MRS agar. After incubating the Petri dishes at 37 °C for 48 h, colony counts were performed.

Bile Salt Tolerance

The bile salt tolerance of *L. rhamnosus* MW019593 and ATCC 53103 was evaluated as described by Reale et al. [14]. The bacteria were cultured in MRS broth at pH 8 supplemented with different bile salt concentrations (Oxoid, Milan, Italy): 0.3%, 0.5%, and 1% (w/v). A control without bile salt was included. After 24-h incubation at 37 °C, bacterial growth was evaluated by plating on MRS agar and colony counting after 48-h incubation at 37 °C.

Hydrophobicity Test

The hydrophobicity of *L. rhamnosus* MW019593 and ATCC 53103 were evaluated as described by Kotzamanidis et al. [15]. An 18-h culture was centrifuged, washed in sterile phosphate buffered saline (PBS; Sigma-Aldrich, USA), and resuspended in sterile 0.1 M potassium nitrate (KNO3; Sigma-Aldrich, USA) to obtain an OD of $A0 = 0.5 \pm 0.02$ at 620 nm using a spectrophotometer (Thermo Scientific, USA). Three milliliters of the suspension was mixed with 1 mL xylene, incubated at room temperature for 10 min, vortexed for 2 min, and allowed to settle for 20 min. The aqueous phase OD (A1) was measured at 620 nm. Hydrophobicity percentage was calculated as:

$$H\% = (1 - A1/A0) \times 100.$$

Autoaggregation Test

The autoaggregation abilities of *L. rhamnosus* MW019593 and ATCC 53103 were evaluated. An 18-h culture was centrifuged, washed in sterile PBS, and resuspended in sterile PBS to obtain an OD of $A0 = 0.5 \pm 0.02$ at 620 nm. Two milliliters was vortexed for 10 s and incubated for 2 h at 37 °C [16]. The supernatant OD (A1) was measured at 620 nm. Autoaggregation percentage was determined using the following formula:

$$A\% = (1 - A1/A0) \times 100$$

Adherence of *L. rhamnosus* MW019593 to Caco-2/TC7 Cells

An in vitro adhesion assay was performed using the method described by Sugimura et al. [17] with some modifications. Prior to the adhesion assay, the culture medium of the Caco-2/TC7 cells was replaced with 500 µL of fresh DMEM without antibiotics. Inocula of *L. rhamnosus* MW019593 and ATCC 53103 were standardized to a concentration of 10^8 CFU/mL in DMEM medium. The well medium was aspirated, and 500 µL of bacterial strains were added, followed by incubation for 3 h at 37 °C, 5% CO_2. At the end of incubation, monolayers were washed with PBS to remove nonadherent bacteria and lysed by incubation for 15 min with 0.1% Triton X100. The lysates were then diluted (10^{-3} to 10^{-6}) and plated onto MRS agar to determine the number of adherent bacteria compared to the initial inoculum.

Cytotoxicity Test

The cytotoxicity of *L. rhamnosus* MW019593 and ATCC 53103 was assessed on the intestinal cell line Caco-2/

TC7. After 16-h incubation of the cells with the bacteria at 30 °C, the supernatant from each well was collected and the lactate dehydrogenase (LDH) rate was measured using a colorimetric kit (Pierce LDH Cytotoxicity Assay Kit, Thermo Scientific, USA). LDH activity, an indicator of plasma membrane integrity, allowed quantifying cell mortality. Control wells were prepared with Caco-2/TC7 cells alone (negative control) or with 1% Triton X-100 (positive control). The percentage of cytotoxicity was calculated using the following formula [18]:

$$\%\ \text{cytotoxicity} = (\text{Exp. OD} - \text{Spontaneous control OD})/(\text{Maximal control OD} - \text{Spontaneous control OD}) \times 100$$

broth and 400 µL of each bacterial strain. The plates were incubated at 37 °C for 72 h, with MRS medium changed every 24 h. The cultures (biofilms) were then stained with the nucleic acid dye SYTO™ 9 (0.5 µM) (Invitrogen, USA) in the dark for 20 min. The biofilms were observed using confocal laser scanning microscopy, with six acquisitions performed for each well. The biofilm images were processed using COMSTAT 2 software [19, 20], and the thicknesses of the biofilms formed by the two strains were measured.

Technological Characterization of *L. rhamnosus* MW019593

Evaluation of Growth Ability at Pilot Scale

The growth ability of *L. rhamnosus* MW019593 was evaluated in a 2-L pilot fermenter (Applikon, France) to compare its growth to the reference strain *L. rhamnosus* ATCC 53103. Precultures of each strain were prepared in 250-mL Erlenmeyer flasks containing 100 mL of sterilized MRS medium. Precultures were inoculated at 10^8 CFU/mL and then incubated for 18 h at 37 °C under anaerobic conditions. The 20-L fermenter containing 15 L of MRS was inoculated with 500 mL of preculture from each strain. The culture was grown at 37 °C, pH 6.5, under 90 rpm agitation for 18 h. At the end of fermentation, the culture was centrifuged (SorvallTM RC 6 Plus, France) at 600 rpm for 10 min at 8 °C in order to recover and weigh the biomass. Cryoprotectants (1% glycerol and 5% maltodextrin) (Sigma-Aldrich, France) were added to the centrifuged biomass before freezing it in aluminum trays at − 20 °C.

At the end of fermentation, samples were taken to check for contaminants on specific agar media (Kenner Fecal for enterococci, Mannitol-Yeast-Peptone for *Bacillus cereus* and Violet Red Bile Lactose for coliforms) (Sigma-Aldrich, USA). The viable cell count was determined by plating on MRS agar [21].

The recovered pellet was characterized by plate count and microbiological parameters. The biomass yield after centrifugation (R1) was calculated using the following formula:

$$R1(\%) = N1/N2 \times P1/V \times 100.$$

Where:

- N1 = Cell count in CFU/g of pellet
- N2 = Cell count in CFU/mL at the end of fermentation
- P1 = Mass of bacterial pellet after centrifugation
- V = Volume of culture medium used during fermentation

The frozen biomass was lyophilized (LYOVAC GT 70, Italy) for 2–3 days. The lyophilized powder was characterized by microbial count and microbiological analyses as described above. The biomass yield after lyophilization (R2) was calculated using the formula: $R2\ (\%) = N1/N2 \times P1/P2 \times 100$.

Where:

- N1 = Bacterial concentration of lyophilized biomass (CFU/g)
- N2 = Bacterial concentration after centrifugation (CFU/g)
- P1 = Mass of lyophilized pellet (g)
- P2 = Mass of pellet after centrifugation (g) [21]

Evaluation of Viability of Lyophilized Strains During Storage

The viability of lyophilized *L. rhamnosus* MW019593 and ATCC 53103 stored at − 20 °C, 4 °C, and 25 °C was determined monthly for 3 months by plating on MRS agar. A 1-g sample of lyophilized powder was diluted in 99 mL of peptone water (Sigma-Aldrich, USA). After homogenization, decimal dilutions were prepared. From the dilutions, 100 µL of each dilution (10^{-6} to 10^{-9}) was surface plated on MRS agar and incubated at 37 °C for 48 h. After incubation, colonies were counted [21].

Biofilm Formation by *L. rhamnosus* MW019593

The biofilm formation of *L. rhamnosus* MW019593 was compared to ATCC 53103 strain. For this experiment, the bacterial inocula were prepared in 0.9% physiological saline using a spectrophotometer (OD of 1 at a wavelength of 600 nm). A 24-well plate specifically designed for confocal microscopy (VivaScope 1500 Multilaser; Mavig, Mavig GmbH, Munich, Germany) was filled with 2 mL of MRS

Whole Genome Sequencing

A single colony was cultivated in MRS medium at 37 °C for 24 h. Genomic DNA was extracted using the GeneJet genomic DNA purification kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. DNA concentration and quality were assessed with the double-stranded DNA (dsDNA) high-sensitivity kit on a Qubit fluorometer (Thermo Fisher Scientific, USA). DNA integrity and purity were evaluated by 1% agarose gel electrophoresis. Library preparation was performed using the Illumina DNA prep kit (Illumina, USA), followed by sequencing with 2 × 250 bp paired-end reads on an Illumina Miseq system (Genomics and Bioinformatics service of the PS^{2}E platform, Rouen Normandy University, Evreux, France).

Bioinformatic Analyses

Genome Assembly and Annotation

Trimmomatic v.039 [22] was used for raw-reads trimming, Seqkit v.2.8.2 [23] to obtain general statistics from trimmed FastQ files, and Falco v.1.2.2 [24] for read-quality check. First taxonomic screening was obtained with Kraken2 v.2.1.3 [25]. Validated paired-end reads were assembled de novo with Unicycler v.0.5.0 [26], while contigs < 200 nt were removed using Seqkit, and assembly metrics calculated using QUAST v.5.0.2 [27]. Second taxonomic validation was achieved using FastANI v.1.34 [28] and Genome-to-Genome Distance Calculator 3.0 (at https://ggdc.dsmz.de/ggdc.php#). CheckM v.1.2.2 [29] was used to evaluate the percentage of completeness and contamination of the assembled draft genome, while bbmap v.39.06 [30] to evaluation genome coverage. Structural gene prediction and functional annotation were carried out using Bakta v.1.9.3 [31]. Functional sequence annotation to establish clusters of orthologous groups (COG) categories was achieved using EggNOG-mapper v.2.1.12 [32] where gene sequences were assigned gene ontology (GO) terms. Then, the distribution analysis of GO terms was performed and displayed using TBtools-II [33].

In Silico Safety Assessment

Multiple tools were used to achieve this assessment: PathogenFinder [34] was used with the "Firmicutes" model to predict the pathogenic potential, while abricate v.1.0.1 [35] was used to screen antibiotic resistance and virulence genes with default databases (i.e., ResFinder, CARD, ARGannot, and VFDB).

Screening Genes Related to Probiotic Features

A local database was created within Abricate to screen several gene-coding functions identified from the literature being associated with *Lactobacillus*. Gene sequences for each function were retrieved from GenBank: bile salt hydrolase, capsular polysaccharide biosynthesis, L-lactate dehydrogenase, S-layer protein, and S-ribosylhomocyrine lyase.

In addition, the genome sequence of *L. rhamnosus* MW019593 was mined through the BAGEL5 web-server (http://bagel5.molgenrug.nl) for potential bacteriocin gene clusters, as well as other bacterial ribosomaly synthesized and post-translationally modified peptides [36]. AntiSMASH v.7.1.0 [37] was used to retrieve gene clusters responsible for secondary metabolite biosynthesis.

Pan-genome Analysis

Whole genome sequences related to *L. rhamnosus* ATCC 53103 were retrieved from the NCBI Reference Sequence Database (RefSeq) and used as references for the pan-genome analysis using Roary v.3.13.0 [38]. Thus, corresponding FASTA files were annotated using Bakta as previously indicated. Then, the obtained general feature format (GFF3) files were used as inputs in Roary in order to identify core, accessory, and unique protein families.

Statistical Analysis

All experiments were conducted in triplicate and data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's *t*-test using STATISTICA 10 software. Differences were considered statistically significant at $p < 0.05$.

Results

Safety Aspects

L. rhamnosus MW019593 was found γ-hemolytic (absence of hemolytic zone around the colonies, Table 1), thus missing hemolytic activity. Additionally, the PCR analysis did not detect any genes encoding decarboxylase enzymes responsible for the production of biogenic amines, such as ornithine, histamine, and tyramine (Fig. 2).

The antibiotic resistance profile of *L. rhamnosus* MW019593 revealed susceptibility to 6 of the 11 antibiotics tested, including β-lactams (amoxicillin), aminoglycosides (gentamicin), and macrolides (azithromycin). However, it showed resistance to vancomycin, fluoroquinolones, and co-trimoxazole (Table 1).

Table 1 Hemolysis, biogenic amines, and antibiotic susceptibility of *L. rhamnosus* MW019593

L. rhamnosus MW019593	
Hemolytic activity	γ-hemolysis
Histidine decarboxylase (hd)	ABS
Tyrosine decarboxylase (td)	ABS
Ornithine decarboxylase (od)	ABS
Antibiotic susceptibility	
Antibiotics	Susceptibility profile
Cotrimoxazole	R
Clindamycin	S
Vancomycin	R
Pefloxacin	R
Gentamicin	S
Cefazolin	I
Amoxicillin	S
Penicillin	S
Tetracycline	S
Chloramphenicol	I
Azithromycin	S

ABS absence, *S* susceptible, *R* resistant, *I* intermediate

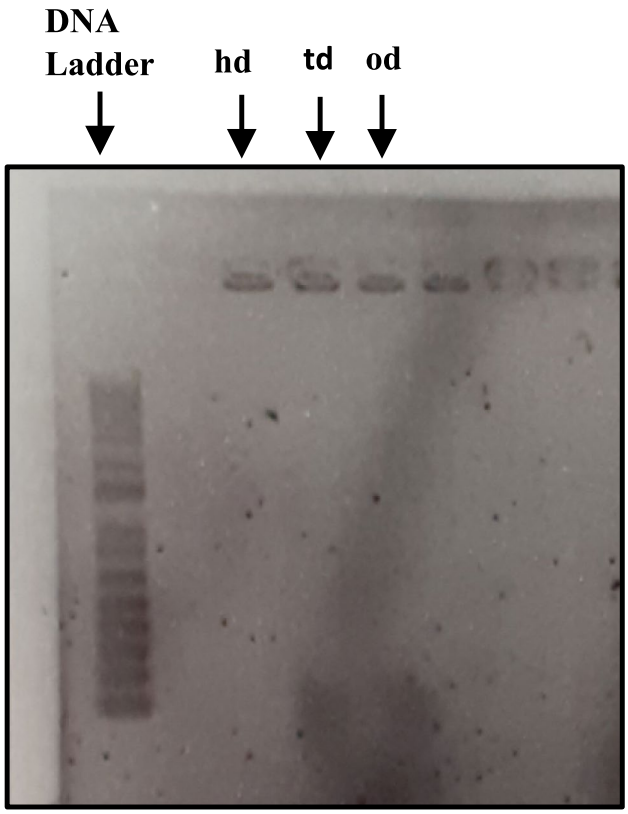

Fig. 2 Agarose gel electrophoresis for the detection of amplified genomic DNA encoding decarboxylase enzymes in *L. rhamnosus* MW019593. hd, histidine decarboxylase; td, tyrosine decarboxylase; od, ornithine decarboxylase

Antibacterial Activity

L. rhamnosus MW019593 showed inhibitory activity against various pathogens (Table 2), with inhibition zone diameters ranging from 9 to 15 mm, depending on the species. The most inhibited pathogens were *C. freundii* (15.5 ± 1.3 mm), *A. hydrophila* (15.2 ± 1.4 mm), *P. aeruginosa* (14.1 ± 1.2 mm), and *L. monocytogenes* (13.5 ± 1.2 mm).

Tolerance to Gastric Acidity

At pH 1.5 (Fig. 3A), both *L. rhamnosus* MW019593 and the reference strain ATCC 53103 exhibited a significant reduction in viability after 3 h of incubation, with a decrease of 5 to 6 log CFU/mL. In contrast, at pH 2 and 3 (Fig. 3B and C), the viability of both strains remained relatively stable over the 3-h incubation period, showing only a 1 to 2 log CFU/mL reduction.

Evaluation of Bile Salt Tolerance, Hydrophobicity, and Autoaggregation

The strains *L. rhamnosus* ATCC 53103 and MW019593 displayed similar tolerance to different bile salt concentrations (0.3%, 0.5%, and 1%) compared to the control (Table 3), thus retaining viability above 95% after 3 h of incubation ($p > 0.05$). The analysis of hydrophobicity and autoaggregation, as presented in Table 3, reveals no significant differences between the *L. rhamnosus* ATCC 53103 and MW019593 strains ($p > 0.05$).

Table 2 Inhibition zone diameters (mm) of pathogenic strains by *L. rhamnosus* MW019593

Pathogenic strain	Diameter (mm)
L. monocytogenes ATCC 15313	13.5 ± 1.2
S. enterica ATCC 10708	11.6 ± 0.9
E. faecium ATCC 19433	12 ± 1.1
C. freundii ATCC 43864	15.5 ± 1.3
S. sonnei ATCC 25931	11.6 ± 0.8
Y. enterocolitica ATCC 23715	9.1 ± 1.0
C. perfringens ATCC 13124	9 ± 0.7
V. parahaemolyticus ATCC 17802	10.2 ± 1.1
P. aeruginosa ATCC 27853	14.1 ± 1.2
A. hydrophila ATCC 7966	15.2 ± 1.4
E. coli ATCC 25922	13 ± 1.5

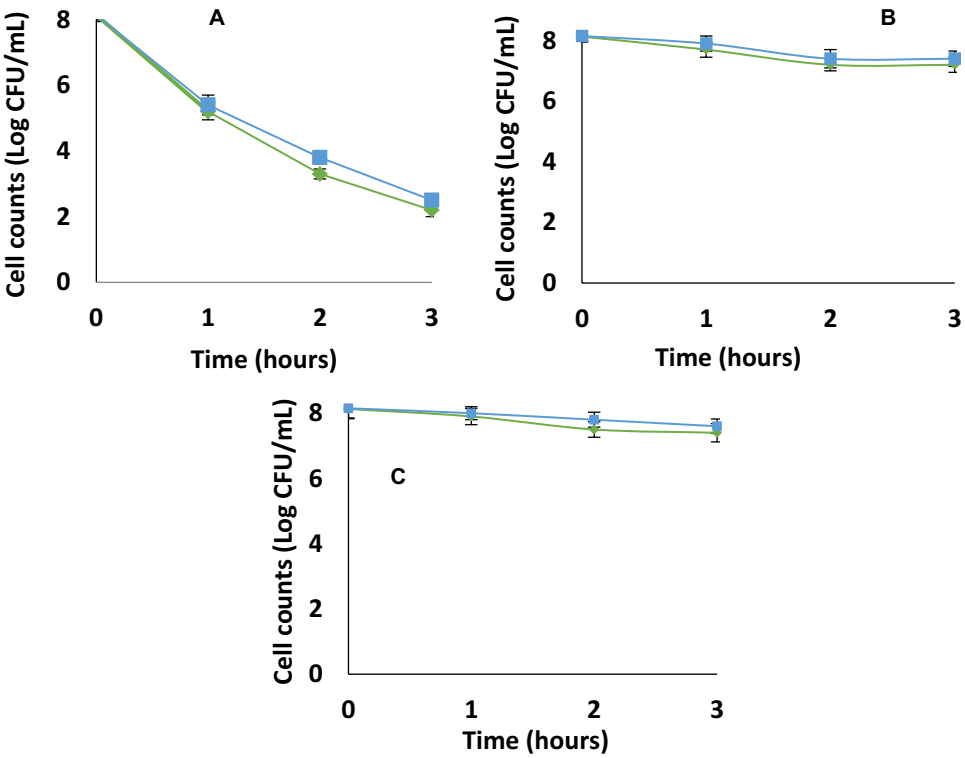

Fig. 3 Survival of *L. rhamnosus* MW019593 (◆) and *L. rhamnosus* ATCC 53103 (■) counts over time at different pH values: A, pH 1.5; B, pH 2; C, pH 3

Table 3 Evaluation of bile salt tolerance, hydrophobicity and autoaggregation of *L. rhamnosus* MW019593 and *L. rhamnosus* ATCC 53103

Strains	Strains cell count Log (CFU/mL) at different bile salt concentrations				Hydrophobicity (H%)	Autoaggregation (A%)
	0% (control)	0.3%	0.5%	1%		
L. rhamnosus ATCC53103	8.87 ± 0.16	8.85 ± 0.2	8.76 ± 0.31	8.67 ± 0.21	46.4 ± 0.3	42.4 ± 0.21
L. rhamnosus MW019593	8.88 ± 0.25	8.83 ± 0.16	8.74 ± 0.23	8.65 ± 0.31	46.2 ± 0.13	42.3 ± 0.19

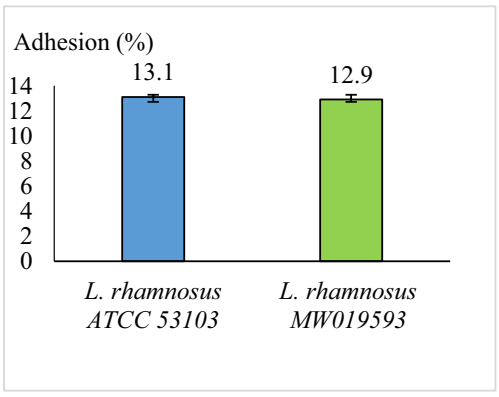

Fig. 4 Adhesion of *L. rhamnosus* MW019593 and ATCC 53103 to Caco-2/TC7 cells

Adhesion and Cytotoxicity of *L. rhamnosus* MW019593 to Caco-2/TC7 Cell Natural Mortality

The adhesion rate of *L. rhamnosus* MW019593 and ATCC 53103 strains to Caco-2/TC7 cells was 12.9% and 13.1%, respectively (Fig. 4), indicating similar values ($p > 0.05$). Similarly, the cytotoxicity tests reveal comparable mortality rates, with 17.8% for *L. rhamnosus* MW019593 and 17.2% for *L. rhamnosus* ATCC 53103, showing no significant difference compared to Caco-2/TC7 cells cultured alone (16.2%) (Fig. 5) ($p > 0.05$).

Biofilm Formation

Results presented in Fig. 6 show that both *L. rhamnosus* strains MW019593 and ATCC 53103 possess similar in vitro biofilm formation capacities, with thicknesses of 23.7 µm and 23.9 µm, respectively ($p > 0.05$).

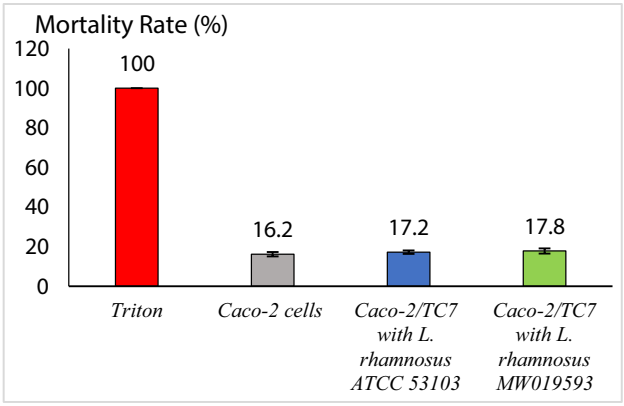

Fig. 5 Cytotoxic effects of *L. rhamnosus* MW019593 and ATCC 53103 on Caco-2/TC7 cells

Evaluation of Growth Ability at Pilot Scale

The results showed that the two strains *L. rhamnosus* ATCC 53103 and MW019593 exhibited similar growth profiles and biomass yields (Table 4) ($p > 0.05$). At the end of fermentation, both strains reached cell concentrations of approximately 5×10^9 CFU/mL ($p > 0.05$). After centrifugation, the concentrated bacterial pellets contained about 7×10^{10} CFU/g, with a biomass yield of around 50% ($p > 0.05$). Lyophilization yielded powders containing approximately 2.2×10^{11} CFU/g, representing a yield of 63% ($p > 0.05$). No contamination by pathogenic microorganisms (enterococci, *Bacillus cereus*, and coliforms) was detected, attesting to the proper conduct of the production processes.

Evaluation of Viability of Lyophilized Strains During Storage

The monitoring of viability during storage at different temperatures (25 °C, 4 °C, and − 20 °C) did not reveal any significant difference between the two strains of *L. rhamnosus* ATCC 53103 and MW019593 ($p > 0.05$) (Fig. 7). At 25 °C, a 3-log reduction was observed after 3 months. At 4 °C, bacterial populations remained stable for 2 months before decreasing by 1 log in the 3rd month. At − 20 °C, bacterial counts remained more stable over the 3 months.

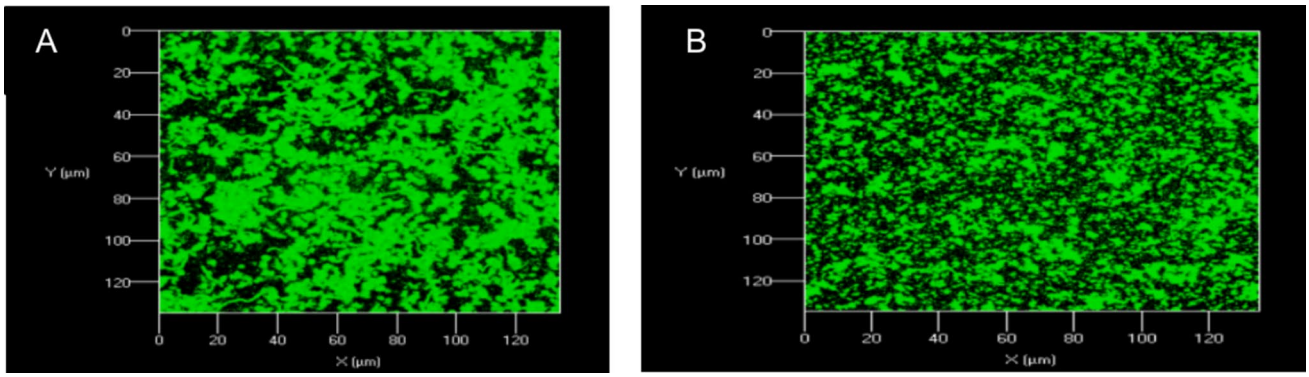

Fig. 6 Confocal microscopy observations of *L. rhamnosus* biofilm thickness (×100): **A** MW019593 (23.7 ± 0.3 µm), **B** ATCC 53103 (23.9 ± 0.35 µm)

Table 4 Evaluation of growth parameters of *L. rhamnosus* ATCC 53103 and MW019593 strains at pilot scale

Conditions	Microbiological parameters		Cell numbers		Biomass yield % (R1 and R2)	
	L. rhamnosus					
	ATCC53103	MW019593	ATCC53103	MW019593	ATCC53103	MW019593
At the end of fermentation	Absence of (enterococci, *Bacillus cereus* and coliforms)		$5.1 \times 10^9 \pm 0.18$ (CFU/mL)	$5.2 \times 10^9 \pm 0.21$ (CFU/mL)	N/A	N/A
After centrifugation	Absence of (enterococci, *Bacillus cereus* and coliforms)		$6.8 \times 10^{10} \pm 0.21$ (CFU/g)	$6.9 \times 10^{10} \pm 0.13$ (CFU/g)	48.5 ± 0.21	49.2 ± 0.31
After lyophilization	Absence of (enterococci, *Bacillus cereus* and coliforms)		$2.1 \times 10^{11} \pm 0.19$ (CFU/g)	$2.2 \times 10^{11} \pm 0.15$ (CFU/g)	62.4 ± 0.31	63.2 ± 0.23

N/A not applicable

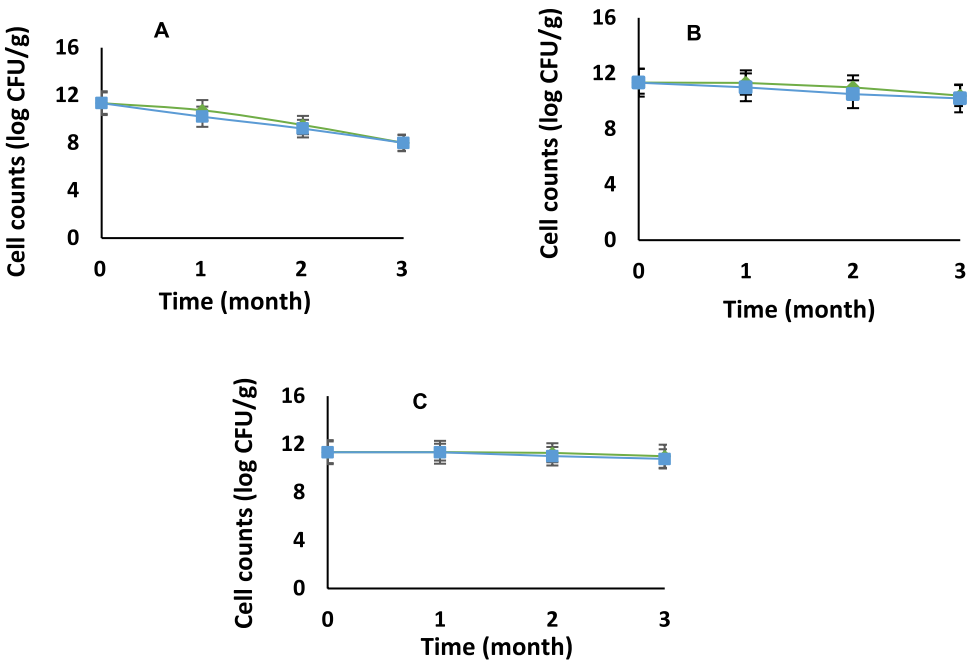

Fig. 7 Stability of *L. rhamnosus* MW019593 (◆) and *L. rhamnosus* ATCC 53103 (■) counts over month at different temperatures: A, 25 °C; B, 4 °C; C, − 20 °C

Genome Characteristics

The draft genome sequence of strain MW019593 was 3.09 Mbp size with 46.7% GC content and an N50 of 204 Kbp. It contained 2928 proteins coding DNA sequences (CDSs) representing a coding density of 85.7% (Fig. 8).

Analysis of the genome revealed no acquired antibiotic resistance genes, focusing specifically on horizontally

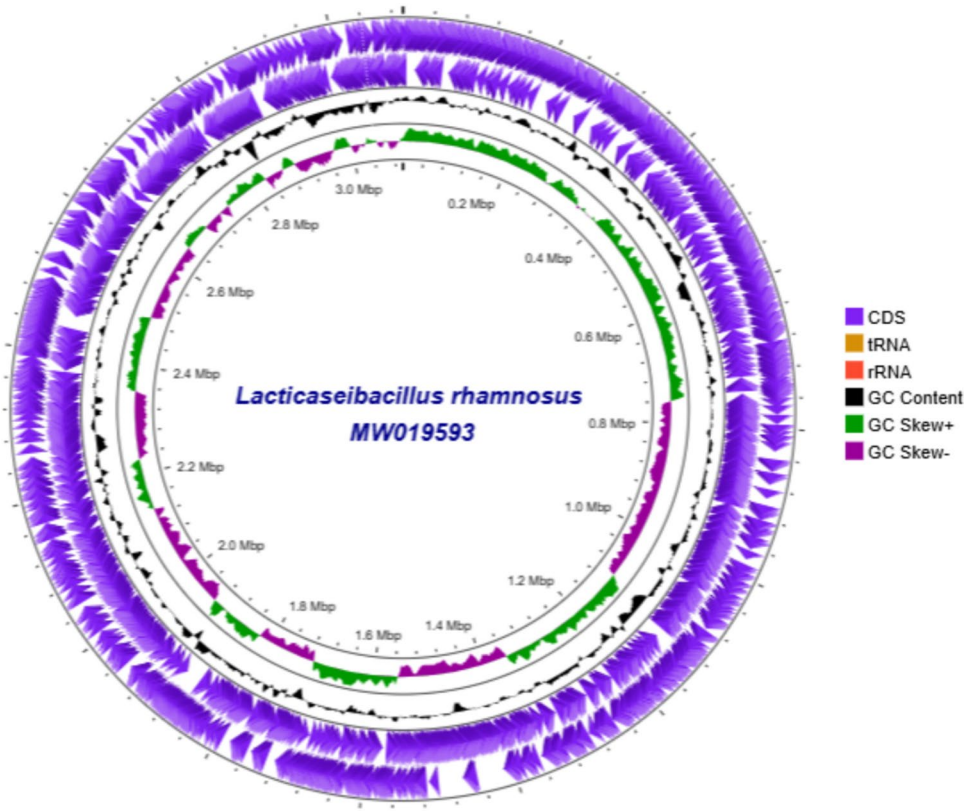

Fig. 8 Whole-genome map of *Lacticaseibacillus rhamnosus* MW019593 generated with Proksee [39]. The genome map consists of five different circles from the inner to the outer circle: (1) measuring scale, (2) GC skew, (3) G + C content, (5) forward CDS, and (6) reverse CDS

Table 5 Sequencing metric and genome characteristics of *Lacticaseibacillus rhamnosus* MW019593

Parameter	*Lacticaseibacillus rhamnosus* MW019593
Sequencing metrics	
No. of total reads	1,803,638
Mean coverage (×)	124
Accession no	
GenBank	JBEXCI000000000
SRA	SRR29705130
Genomic data	
Genome size (bp)	3,094,257
G + C content (%)	46.7
No. of contigs	83
N50 (bp)	204,177
No. of CDS	2928
No. of tRNAs	45
No. of rRNAs	2
Virulence factor(s)	*none*
Antibiotic resistance gene(s)	*none*
# of coding genes of interest for probiotic properties	
Bacteriocin	2
Secondary metabolite biosynthesis	2
Bile salt hydrolase	1
Capsular polysaccharide biosynthesis protein	1
L-lactate dehydrogenase	3
S-layer protein	1
S-ribosylhomocysteine lyase	1

transferred resistance determinants. Further information regarding the genomic statistics is presented in Table 5.

Taxonomic Validation

FastANI analysis revealed a high ANI of 99.5% with the type strain *L. rhamnosus* DSM20021^T, and the genome-to-genome distance calculator (GGDC) estimated an in silico DNA-DNA hybridization (*is*DDH) value of 97.55% (using Formula 2 as recommended), exceeding the 95 and 70% cut-offs for species designation, respectively. These genomic metrics provide strong evidence for the accurate classification of MW019593 within the *L. rhamnosus* species. Moreover, average nucleotide identity analysis showed that this strain shares 97.4% identity with the *L. rhamnosus* ATCC 53103 strain, which was employed as a reference for in vitro experiments in this study. The high degree of genomic similarity between MW019593 and the well-characterized ATCC 53103 strain further supports its taxonomic assignment and suggests potential functional similarities.

Functional Classification

The classifications of annotated ORFs based on the Eggnog-mapper and Clusters of Orthologous Groups database are presented in Fig. 9. The classification reveals 25 subsystems, with carbohydrates, protein metabolism, and amino acids and their derivatives being the most represented categories. Genes associated with carbohydrate metabolism include those involved in sugar transport,

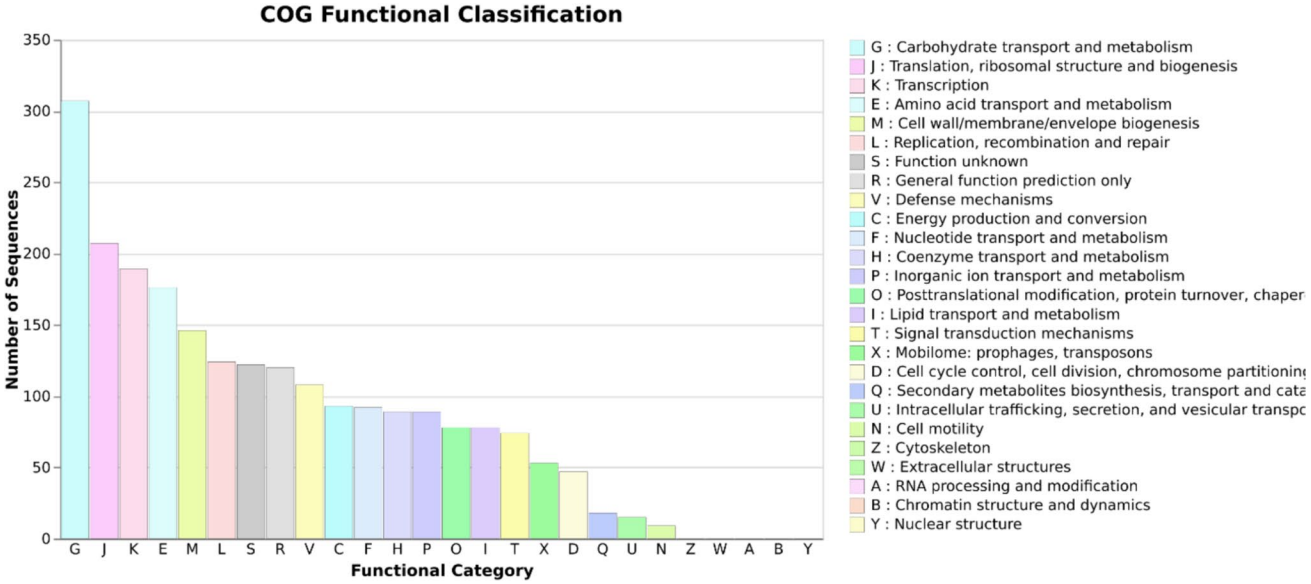

Fig. 9 Functional annotations of ORFs based on Eggnog-mapper and Clusters of Orthologous Groups database for the *L. rhamnosus* MW019593 genome

glycolysis, pentose phosphate pathway, and other sugar utilization pathways. Protein metabolism genes encompass various peptidases, proteases, and amino acid transporters. The amino acid metabolism category includes genes for biosynthesis, degradation, and interconversion pathways of multiple amino acids. The full list of genes is provided in Online Resource 1.

Mining for Bacteriocin Gene Clusters

Two putative bacteriocin clusters were identified in the genome, although additional experimental validation would be needed to confirm their functionality. BAGEL5 screening predicted two areas of interest (AOI) (Fig. 10). The first AOI contains genes with similarity to those encoding Enterolysin A, a class III bacteriocin. The second AOI shows sequence homology to genes involved in Carnocin_CP52 production, a class IIa bacteriocin.

Mining for Biosynthetic Gene Clusters

The antiSMASH analysis revealed two potential biosynthetic gene clusters. The first cluster was identified as a Type III Polyketide Synthase (T3PKS) cluster containing the hydroxymethylglutaryl-CoA synthase gene. The second cluster was classified as a RiPP-like cluster, featuring the lactococcin-G-processing and transport ATP-binding protein LagD gene.

Safety Assessment from Genomic Data

Genomic analysis revealed no detectable antimicrobial resistance (AMR) genes in the genome sequence. PathogenFinder analysis yielded a probability of 0.097 for human pathogenicity. No known virulence genes were identified in the genome sequence of *L. rhamnosus* MW019593.

Pan-genome Analysis

Comparative genomic analysis of seven *L. rhamnosus* strains, including MW019593, DSM20021, and five genomes related to the GG/ATCC 53103 isolate, identified 3498 genes in the pangenome. Of these, 2292 genes (65.5%) constitute the core genome. The shell genome comprises 834 genes (23.8%), present in 15–95% of the strains. Additionally, 372 cloud genes (10.6%) were

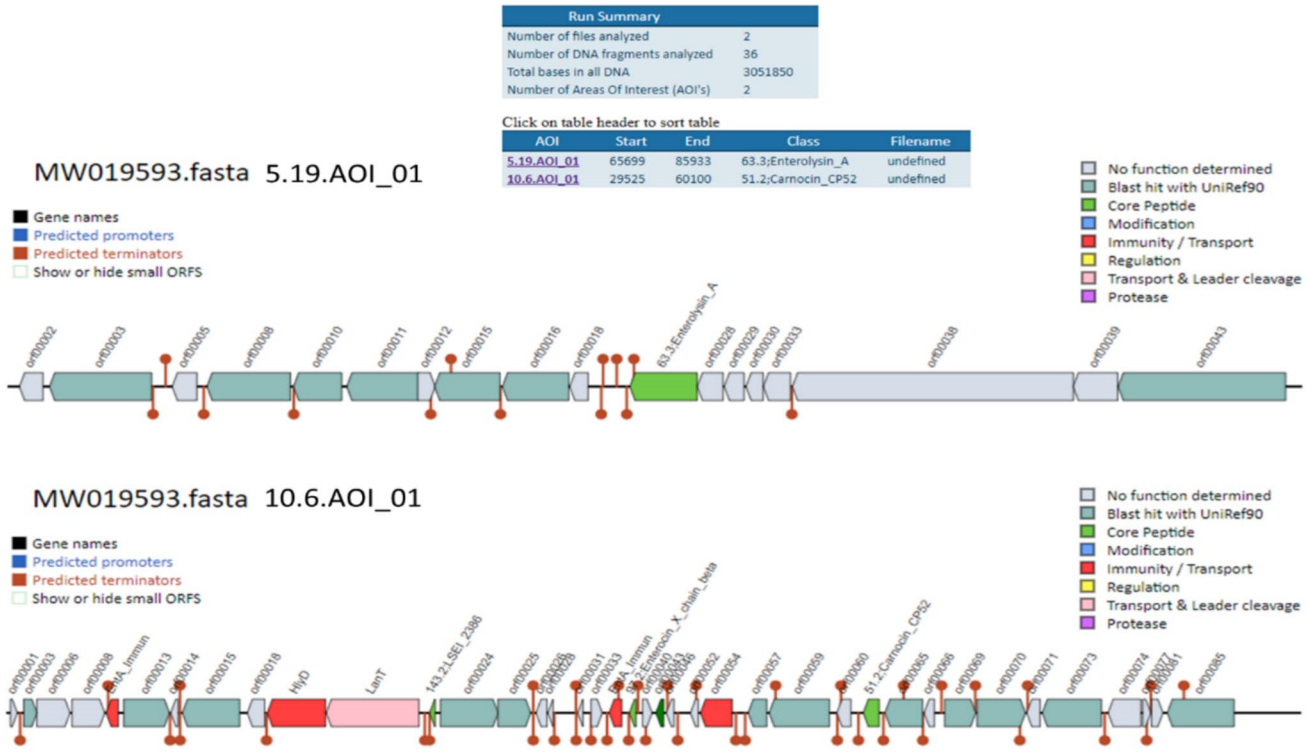

Fig.10 Schematic representation of the two genomic areas of interest (AOI) mined in *L. rhamnosus* MW019593 whole genome sequence using BAGEL5 web-server. These regions were predicted to possess putative bacteriocin gene clusters with Enterolysin A and Carnocin_CP52, respectively

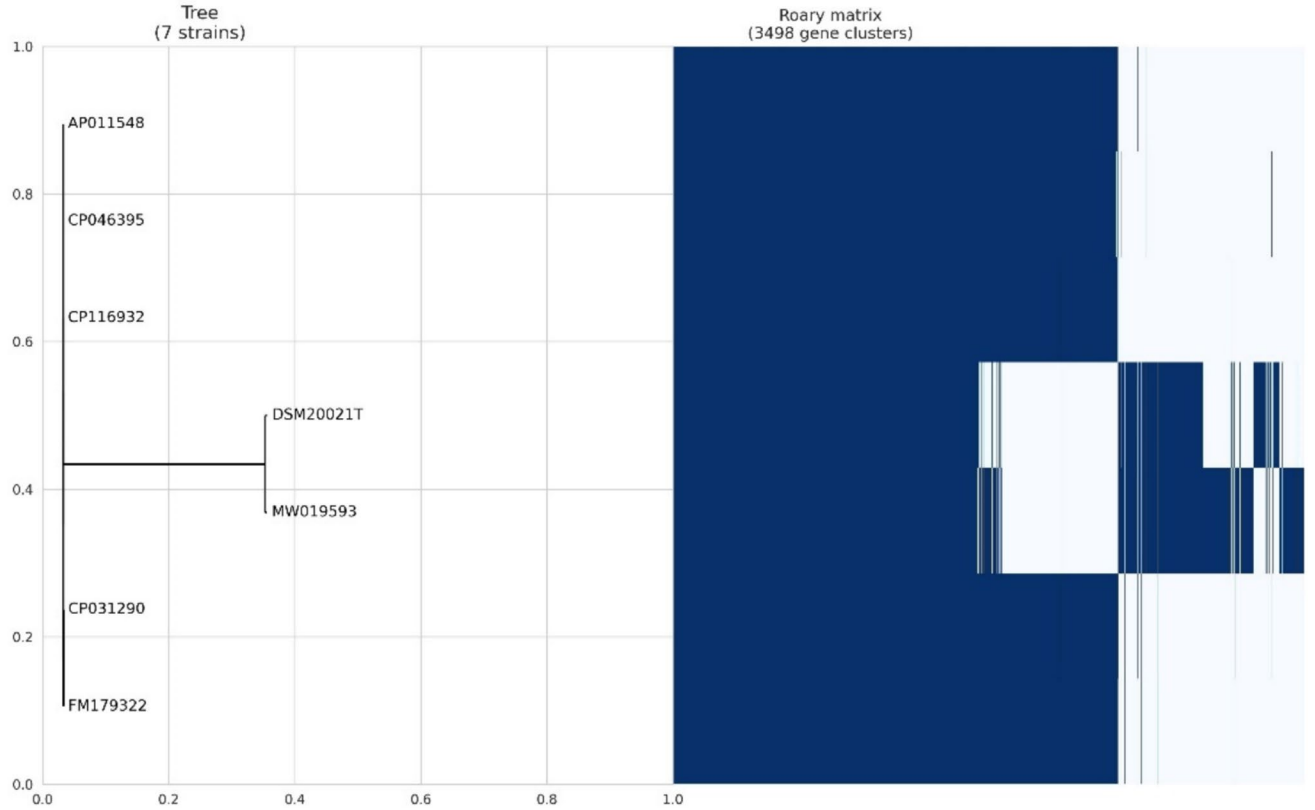

Fig. 11 Pan-genome analysis of seven *Lacticaseibacillus rhamnosus* strains including MW019593, DSM20021, and ATCC 53103-related accessions. Blue blocks indicate shared genes while white ones for absent loci

identified, present in less than 15% of the strains. No soft core genes were detected in this analysis (Fig. 11).

Discussion

Safety Aspects

The γ-hemolytic nature is essential for reducing hemolytic risks associated with the use of *L. rhamnosus* MW019593. The absence of detected genes encoding decarboxylase enzymes suggests a lack of biogenic amine production, supporting the strain's potential safety and confirming previous findings [40, 41]. However, future studies should validate these findings by confirming the absence of these genes in the annotated genome and assessing phenotypic production under specific growth conditions. The susceptibility to clinically relevant antibiotics such as β-lactams, aminoglycosides, and macrolides is a characteristic commonly found in *L. rhamnosus* [42]. Intrinsic resistance to vancomycin, fluoroquinolones, and co-trimoxazole are often reported [43]. This combined resistance presents no risk of gene transfer to pathogenic bacteria in the gut [44].

Antibacterial Activity

The moderate inhibition of various gastrointestinal pathogens, including *Salmonella*, *Listeria*, *Clostridioides*, and *E. coli*, by *L. rhamnosus* MW019593 suggests its probiotic potential as a barrier agent [45, 46]. The variations in inhibition levels are likely influenced by the specific antibacterial mechanisms employed by *L. rhamnosus*, such as the production of inhibitory metabolites, as well as the variable sensitivity of the targeted pathogens [47].

Tolerance to Simulated Gastrointestinal Conditions

The tolerance of *L. rhamnosus* MW019593 to simulated gastrointestinal conditions, including its survival at pH values of 2 and 3 and in the presence of bile salts, reflects its probiotic potential. The ability to withstand gastric acid stress, especially at $pH \geq 2$, is crucial for ensuring the arrival of viable probiotics in the intestine [48]. The acid tolerance of *L. rhamnosus* can be attributed to various physiological and biochemical mechanisms that enable its adaptation and survival under acidic gastric conditions [49]. Similarly, bile salt tolerance is considered a necessary property for the survival

of lactic acid bacteria in the duodenum and small intestine [50]. Recent studies have shown that various *L. rhamnosus* strains can retain high viability in the presence of bile salts, corroborating previous findings that bile salt resistance is a strain-specific trait in *Lactobacillus* [51, 52]. The observed acid and bile salt tolerance of *L. rhamnosus* MW019593, consistent with other *L. rhamnosus* strains reported in previous studies [14, 53, 54], suggests its potential for oral administration and survival in the gastrointestinal tract.

Adhesion and Cytotoxicity

The *L. rhamnosus* MW019593 strain exhibits promising surface properties for potential probiotic profiling. According to Abdulla et al. [55], *Lactobacillus* strains with hydrophobicity rates above 40% are considered hydrophobic, an important probiotic characteristic promoting adhesion to intestinal epithelial cells and persistence in the gastrointestinal tract [56]. Additionally, the autoaggregation ability observed at 42.3% for *L. rhamnosus* MW019593, similar to other studies on *L. rhamnosus* [57], is also a probiotic selection criterion enabling the formation of microcolonies that can more readily adhere to epithelial cells [58]. The 12.9% adhesion rate of *L. rhamnosus* MW019593 to Caco-2/TC7 cells is consistent with the findings of Zawistowska-Rojek et al. [59] on other strains, with adhesion to intestinal cells being a key criterion for persistence and host interaction [60]. Furthermore, the lack of significant cytotoxicity on Caco-2/TC7 cells compared to controls, in line with Zheng et al. [61], suggests that MW019593 is not toxic to intestinal cells and could potentially persist in the gastrointestinal tract without adverse effects. However, further in vivo studies are needed to confirm the probiotic potential of this strain.

Biofilm Formation

Ramírez et al. [62] indicated that the biofilm production capacity varies depending on the bacterial strain. Rezaei et al. [63] demonstrated that both *L. rhamnosus* and *L. plantarum* form robust and highly desirable biofilms. Their analysis of gastrointestinal digestion further revealed that biofilm formation positively influences the viability of probiotic cells. Consequently, they concluded that biofilm formation plays a crucial protective role in the survival of these bacteria within probiotic yogurt throughout processing, storage, and gastrointestinal conditions. Biofilm formation by probiotic bacteria, such as *Lacticaseibacillus* spp., is considered a beneficial property because it could promote colonization and longer permanence in the mucosa of the host, and biofilms prevent colonization by pathogenic bacteria. The capacity of *Lactobacillus* to form biofilms on abiotic surfaces (glass or polystyrene) has been studied, and the results indicate that only some strains have this property. It has also been demonstrated that the exopolysaccharides (EPS) produced by some biofilm-forming strains can inhibit the formation of biofilms by certain pathogenic bacteria [64].

Technological Characterization of *L. rhamnosus* MW019593

The results obtained demonstrate the potential of *L. rhamnosus* MW019593 for industrial production, as it achieves the required cell concentrations and biomass yields $\geq 10^{10}$ CFU/g in powder after lyophilization according to de Roissart and Luquet [65]. A high biomass yield is essential when selecting lactic acid bacteria (LAB) strains for industrial applications. Indeed, even a technologically promising strain loses appeal by industry scientists if it cannot be produced in sufficient quantities. Growth rates and yields vary according to species, strains, environmental conditions, and culture media [65, 66].

Although the current conditions seem favorable, further investigations would allow optimization of the production parameters for this strain. It would be advisable to evaluate the influence of medium composition, pH, temperature, aeration, as well as production stability on a larger scale. Such optimization would maximize yields and ensure the industrial viability of this promising strain.

Storage conditions also influence long-term viability. In accordance with the literature, storage at low temperature (≤5 °C, or even − 18 °C) better preserves the viability of lyophilized LAB [66]. The study by Gardiner et al. [67] on two lyophilized strains stored for 2 months at 4 °C showed stability for *L. paracasei* NFBC 338 but a 1-log decrease for *L. salivarius* UCC 118.

In summary, refrigeration (4 °C) or freezing (− 20 °C) seem most appropriate to maintain high viability levels for *L. rhamnosus* MW019593 for at least 3 months. This characteristic favors potential industrial production and commercialization as a probiotic. However, optimization of other preservation parameters, such as cryoprotectants, water content/water activity, and oxygen level, is essential to increase viability during storage.

Bioinformatic Analyses

The whole genome sequencing and bioinformatic analyses of *L. rhamnosus* MW019593 reveal valuable insights into its genetic potential as a probiotic. The genome size (3.09 Mbp) and GC content (46.7%) are found to be similar to other *L. rhamnosus* strains [68]. ANI and GGDH analyses confirm the strain's taxonomic classification within the *L. rhamnosus* species.

The abundance of carbohydrate metabolism genes suggests versatility in sugar utilization, potentially contributing

to the strain's adaptability in fermentation environments. The extensive protein metabolism genes indicate significant proteolytic capabilities, which could be beneficial in dairy fermentations. As mentioned by Cui and Qu [69], the stronger ability in the utilization of carbohydrates reflects the adaptation ability of strains in different environments with various carbohydrates. The strain's capacity to metabolize a wide variety of carbohydrates relies on their various sugar transport systems and metabolic pathways.

The presence of diverse amino acid metabolism pathways suggests potential nutritional self-sufficiency and capacity for producing important metabolites.

The identification of putative bacteriocin clusters and biosynthetic gene clusters suggests potential antimicrobial capabilities, although experimental validation would be necessary to confirm their functionality and role in the observed antimicrobial activities [5]. The T3PKS and RiPP-like clusters further indicate potential for producing bioactive compounds, which may contribute to the strain's competitive abilities in complex microbial environments [70]. T3PKS genes have been found in several *Lactobacillus* species, such as *Lactiplantibacillus plantarum* [58] and *L. rhamnosus* [59, 71], which indicates their capacity to synthesize various secondary metabolites. These secondary metabolites are involved, among other things, in antimicrobial activity and signaling [71].

The absence of detectable AMR genes, virulence factors, and low pathogenicity of probability supports the strain's safety profile and indicates that it is safe for use as a probiotic. With the need to identify novel safe and effective probiotics for human use, the absence of AMR genes in this strain is a positive indication, as AMR is an important public health concern [71].

Additionally, the genome analysis revealed genes encoding bile salt hydrolase, complete operons for capsular polysaccharide biosynthesis, and S-layer proteins, which align with our experimental observations of gastrointestinal tolerance and adhesion properties, though the specific contribution of each genetic element would require further investigation [72]. The pan-genome analysis reveals substantial genetic conservation among *L. rhamnosus* strains while highlighting strain-specific adaptations that may contribute to unique phenotypic traits.

Conclusion

This study comprehensively evaluates the probiotic characteristics of the *L. rhamnosus* MW019593 strain isolated from Algerian cow's milk. The key findings demonstrate the strain's promising safety profile, with no hemolytic activity or biogenic amine production, along with sensitivity to clinically relevant antibiotics. The strain exhibits promising functional properties, including moderate antibacterial activity against various gastrointestinal pathogens and notable tolerance to simulated gastrointestinal conditions, maintaining viability at pH 2–3 and in bile salt concentrations of up to 1%.

A significant advantage is the strain's ability to form biofilms and to adhere to Caco-2/TC7 intestinal epithelial cells (12.9%) without associated cytotoxicity. From an industrial perspective, *L. rhamnosus* MW019593 demonstrated growth profiles and biomass yields comparable to the reference ATCC 53103 strain, with viable preservation for at least 3 months under refrigeration (4 °C) or freezing (− 20 °C).

The whole genome analysis provides supportive insights into the phenotypic findings, revealing genes associated with gastrointestinal survival and two potential bacteriocin clusters. The genome analysis also confirms the strain's safety by showing no antibiotic resistance genes or virulence factors.

Although these characteristics are promising, further research is needed to validate the strain's potential applications. Several possible uses can be explored:

- Incorporation into fermented dairy products such as yogurt, cheese, and fermented milk drinks, leveraging its dairy origin;
- Development of dietary supplements in formulations such as capsules, powders, or liquid products;
- Investigating its potential for therapeutic applications targeting gastrointestinal disorders, provided its survival in the gastrointestinal tract is further validated;
- Inclusion in functional foods and beverages, particularly those requiring refrigeration;
- Development of synbiotic products combining *L. rhamnosus* MW019593 with suitable prebiotics.

Future directions should focus on the following:

- Expanding the pan-genome analysis to include a larger number of *L. rhamnosus* strains for a more comprehensive understanding of strain-specific adaptations;
- Validating the promising in vitro results through studies on animal models and human clinical trials;
- Exploring the molecular mechanisms underlying the strain's adhesion, biofilm formation, and pathogen inhibition properties;
- Optimizing industrial-scale production processes and storage conditions to enhance the strain viability and its potential application.

This study successfully showcases the probiotic attributes of an indigenous Algerian *L. rhamnosus* strain, emphasizing the value of exploring local microbial biodiversity for probiotic development. The findings lay a strong foundation for future research and potential applications, while underscoring the need for continued studies to fully highlight the strain's potential in commercial and therapeutic contexts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-025-10508-3.

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Author Contributions Conceptualization: N.M. and N.C. Data curation, formal analysis, statistics and writing – Original draft: N.M., L.M., N.C. and A.M.B. Methodology: N.M., N.C., A.I., A.M., A.M.B and A.S. Supervision: N.M., N.C. and A.M.B.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Approval No ethical approval was required as no live animals were used in this study.

Competing Interests The authors declare no competing interests.

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