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Safety traits, genetic and technological characterization of *Lacticaseibacillus rhamnosus* strains

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ABSTRACT

Lacticaseibacillus rhamnosus is a species extensively studied and employed in food production for its widely recognized probiotic properties. This study aimed to enhance understanding of this species by characterizing a diverse range of strains sourced from international collections or isolated from various origins, such as dairy products, sourdoughs, wine or must, and human bodily excreta. Initially, 60 Lbs. *rhamnosus* strains underwent genotypic characterization utilizing Rep-PCR, RAPD, and Sau-PCR techniques to evaluate their genetic relationships and eliminate clones. Subsequently, a phenotypic characterization was conducted to assess strain resistance to different stressors such as pH, NaCl, and ethanol. Furthermore, particular attention was given to food safety aspects concerning the potential use of these strains as food starters and/or probiotics. This included investigating antibiotic resistance profiles, antimicrobial capabilities, biogenic amines production, presence of genes associated with ethyl carbamate production, and the occurrence of technologically relevant enzymes such as monoamine oxidase and diamine oxidase. The analyses revealed significant genetic variability among strains, with limited correlation to their sources of isolation. Additionally, strain-specific phenotypic traits were observed, emphasizing the importance of thorough strain characterization for food applications.

1. Introduction

The traditional application of Lactic Acid Bacteria (LAB) focuses on fermentations for extending the shelf life of foods, while enhancing their hygiene and safety standards. In recent years, there has been a surge in interest in the natural food preservation capabilities of these bacteria, largely driven by consumer preferences for reducing reliance on chemical preservatives (Voulgari et al., 2010). Among the LAB group, various bacterial species previously classified under the *Lactobacillus* genus, now reclassified into 25 genera, stand out for their technological attributes relevant to the food industry and production (Qiao et al., 2022; Zheng et al., 2020). Particularly noteworthy is the genus *Lacticaseibacillus*, encompassing several species long valued for their distinct traits, among which *Lacticaseibacillus rhamnosus* holds prominence. One of its most renowned strains is *Lbs. rhamnosus* GG, isolated in 1983 by Gorbach and Goldin, which has been extensively investigated in numerous clinical studies highlighting its beneficial effects on conditions such as antibiotic-associated diarrhoea, urinary tract infections, food allergies, and even inflammatory bowel diseases like ulcerative colitis and irritable bowel syndrome (Capurso, 2019; Segers & Lebeer, 2014). Consequently, *Lbs. rhamnosus* has emerged as one of the most extensively studied species due to its health-promoting properties (Hill et al., 2018).

Due to these factors and their technological characteristics, *Lbs. rhamnosus* strains are increasingly utilized in food production, preservation, and fermentation as starter cultures or probiotic microorganisms (Douillard et al., 2013). Hence, it is crucial to evaluate the resistance traits of these microorganisms to stressors present in various food matrices, such as water activity, ethanol concentration, pH, and oxygen levels, as these factors can significantly affect their survival. However, before direct application in food, each strain necessitates thorough characterization. Despite *Lactobacillus* spp. being generally recognized as safe (GRAS), all strains employed in the food industry must undergo taxonomic identification and genetic as well as phenotypic characterization, as mandated by the European Food Safety Authority (EFSA, 2007, 2021). Moreover, it is imperative to identify potential resistance and virulence factors that could pose health risks to susceptible

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individuals or genes transferable via horizontal transmission to other microorganisms (Colautti, Arnoldi, Comi, & Iacumin, 2021; Colautti, Rossi, Piazza, Comi, & Iacumin, 2023). Additionally, there are other potentially risky metabolic properties. Along the ADI pathway, lactic acid bacteria also produce carbamoyl phosphate and citrulline, which react with ethanol to produce ethyl carbamate, a potentially carcinogenic substance mainly found in wine and various fermented food products (Fang, Dong, Xu, He, & Chen, 2013; Tonon & Lonvaud-Funel, 2002). Biogenic amines (BAs), resulting from microbial decarboxylation of amino acids, pose a significant hazard as they often do not cause detectable qualitative food deterioration but can have toxicological effects on consumers (Costantini, Pietroniro, Doria, Pessione, & Garcia-Moruno, 2013; Coton & Coton, 2005; Ladero et al., 2011; Landete, Ferrer, & Pardo, 2007; Landete, de las Rivas, Marcobal, & Muñoz, 2007). Besides monitoring the production of these compounds, it is possible to select strains capable of reducing their concentration in the product through specific enzymes (Kongkiattikajorn, 2015).

Therefore, to comprehend the genetic and functional diversity of this microbial species in food applications, this study aimed to investigate the genetic traits of 60 strains of *Lbs. rhamnosus*. Their high heterogeneity in geographical origins and isolation matrices ensured a highly representative collection, providing a robust basis for assessing species characteristics. Such a comprehensive study can reveal strains with superior technological properties, optimized fermentative capacities, and resistance to environmental stresses, thereby enhancing the quality, safety, and shelf life of food products. Moreover, the diversity among strains facilitates the development of novel foods with distinctive, tailored attributes to meet the nutritional requirements of specific population groups, thereby making significant contributions to innovation and safety within the food sector. These strains, previously identified through species-specific PCRs and HRM analysis (Iacumin et al., 2015), underwent genetic analysis using Rep-PCR, RAPD, and Sau-PCR techniques. The presence of *arcABC* genes linked to ethyl carbamate, and other genes related to biogenic amines production were also investigated. Furthermore, the growth of *Lbs. rhamnosus* strains in different conditions, including varying levels of NaCl, ethanol, and pH, as well as their resistance to antibiotics and ability to produce antimicrobial compounds, was analysed. This thorough examination, conducted across numerous strains, is expected to enhance understanding of the genetic and phenotypic characteristics of this species, which is very significant for human health.

2. Materials and methods

2.1. Strains collection

For this study, 60 strains previously identified as *Lbs. rhamnosus* (Iacumin et al., 2015) were employed. The origin of the strains was heterogeneous, and they were sourced from various international collections as summarized in Table 1. To maintain their viability, strains were cryopreserved at −80 °C in cryovials containing DeMan, Rogosa, and Sharpe broth (MRS) (Oxoid, Waltham, USA) supplemented with 20 % glycerol. Before their use in subsequent analyses, the strains were revitalized on MRS Agar plates (Oxoid) and incubated under microaerophilic conditions at 30 °C for 48 h to ensure their purity.

2.2. DNA extraction from pure cultures

For DNA extraction, the revitalized pure cultures were grown in MRS broth (Oxoid). Two (2) mL of a 48-h culture were centrifuged at 10,000×*g* for 10 min at 4 °C to pellet the cells, which were then subjected to DNA extraction using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, USA) following the manufacturer's instructions.

Table 1
List of *Lbs. rhamnosus* strains employed in this work, with their isolation source.

Origin	Strains
Raw and heat-treated milk, yogurt, milking machines	HA111[1], PRA152[2]
Green, creamy and seasoned cheeses (Italian cheeses: Scamorza, Parmigiano Reggiano, Grana Padano, Spressa, Asiago, Montasio, Canestrato di Moliterno, Morlacco, Bellunese, Pecorino, Caciocavallo, Provolone, Emmenthal, Raclette de Savoie; Chinese and Tunisian cheeses)	M15[3], O14[4], PRA 204[2], PRA232[2], PRA331[2], DBPZ0430[4], DBPZ0445[4], DBPZ0446[4], DBPZ0448[4], DBPZ0449[4], FSG01[4], CI230[5], CI4362[5], CF1350[5], CF377[5], D44[6], H25[6], 5A9T[3], 5D9T[3], L9[3], L47[3], LACcas13[7], DBPZ0428[4]
Fermented sausages	CTC1676[8], 2220[9]
Coffee	DIAL40[9]
Humans (saliva, dental caries, blood, urethra, feces of infants and adults)	TMW 1.1538[6], DBTA86[10], DBTC4[10], N171[11], N178[11], N715[11], N94[11], N95[11], N83[11], N201[11], N209[11], N2012[11], N132[11], N22[11], N26[11], N812[11], N173[11], N1110[11], N131[11], N21[11], N172[11], N2010[11], N2013[11], N202[11], N25[11], N176[11], N2011[11], Mo2[11], N1710[11], N175[11]
Unknown	NRRL B-176[12], DSMZ20021[13]

Legend: [1]Harmonium International Inc., Mirabel, Canada; [2]Dipartimento di Scienze Agrarie e degli Alimenti, Università degli Studi di Modena e Reggio Emilia, Italy; [3]Università degli Studi di Verona, Dipartimento di Biotecnologie, Strada le Grazie 15, Verona, Italy; [5]Dipartimento di Scienze e Tecnologie Agro-Alimentari, Unversità degli Studi di Bologna, Bologna, Italy; [6]Dipartimento di Scienze delle Produzioni Agrarie e Agroalimentari, Università degli Studi di Catania, Catania, Italy: [7]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Italy; [8]Institut de Recerca I Technologia Agroalimentaries (IRTA), Lleida, Spain; [9]Dipartimento di Scienze degli Alimenti, Università degli studi di Udine, Udine, Italy; [10]Dipartimento di Biotecnologie, Università degli Studi di Verona, Verona, Italy; [11]Dipartimento di Agricoltura, Ambiente e Alimenti, Unversità degli Studi del Molise, Campobasso, Italy; [12]ARS Culture (NRRL) Collection, United States Department of Agriculture, USA; [13]DSM: DSM, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany.

2.3. Rep-PCR, RAPD, and SAU-PCR analysis

The molecular characterization followed the protocol outlined in Iacumin et al., 2020; Iacumin et al., 2020). Rep-PCR was performed using the primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) (Vauterin & Vauterin, 1992). Reactions were conducted in a final volume of 25 μL using the following reaction mix: 100 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 μM primer $(GTG)_5$ and 1.25 UI *Taq*-polymerase (Applied Biosystems, Waltham, USA). Amplification consisted of 31 cycles with denaturation at 94 °C for 3 s, followed by annealing at 92 °C for 30 s, and extension at 65 °C for 8 min using a Euroclone Thermal Cycler (Celbio, Italy). The initial denaturation was performed at 95 °C for 2 min, followed by a final extension at 65 °C for 8 min.

RAPD analysis was performed using M13 primer (5′-GAGGGTGGCGGTTCT-3′) (Huey & Hall, 1989). Amplification was performed in a 25 μL reaction volume using the following reaction mix: 100 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 μM primer M13, 1.25 UI *Taq*-polymerase (Applied Biosystems, Waltham, USA). Reactions were conducted with a Euroclone Thermal Cycler (Celbio, Italy). The amplification cycle consisted in 35 repetitions of 94 °C for 1 min, 38 °C for 1 min, ramp to 72 °C at 0.6 °C/s, 72 °C for 2 min, performing an initial denaturation at 94 °C for 5 min, and a final extension at 72 °C for 5 min.

Sau-PCR analysis was performed using 1 μL of Sau3A, restriction endonuclease (10 U/μL) to digest 200 ng of DNA, in a final volume of 20 μL. Amplification reaction was performed in a 50 μL reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 2 μM primer SAG1 (5′-CCGCCGCGATCAG-3′) (Corich,

Mattiazzi, Soldati, Carraro, & Giacomini, 2005), 1.25 UI *Taq*-polymerase (Applied Biosystems, USA) and 1 μL of digested DNA. PCR reactions were carried out with a Euroclone Thermal Cycler (Celbio, Italy) using the following amplifications conditions: 25 °C for 5 min, ramp to 60 °C at 0.1 °C/s, 60 °C for 30 s, 2 cycles of 95 °C for 1 min, 50 °C for 15 s, ramp to 25 °C at 0.1 °C/s, ramp to 50 °C at 0.1 °C/s, 50 °C for 30 s, 35 cycles of 94 °C for 15 s, 46 °C for 1 min, 65 °C for 2 min, and the final extension at 65 °C for 2 min.

PCR products obtained from these three techniques were separated in a 1.5 % (w/v) agarose gel in 0.5X TBE at 120 V, for 6 h for RAPD and Rep-PCR and 4 h for Sau-PCR and stained for 30 min at the end of the electrophoretic run in 0.5X TBE buffer containing ethidium bromide 0.25 μL/mL (Merck, Darmstadt, Germany). The gels digitalized through the BioImaging System GeneGenius imaging software (Syngene, Bangalore, India) were analysed using the pattern analysis software package Gel Compare II Version 4.1 (Applied Maths, Sint-Martens-Latem, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithms (Vauterin & Vauterin, 1992).

2.4. Growth capabilities

To assess resistance to various stressors most commonly found in different food matrices (NaCl at concentrations of 2%, 4%, 6.5% w/v; ethanol at concentrations of 12%, 15% v/v; pH at values of 3.2, 3.8, 4.2, and 4.6), the growth of strains was evaluated in modified MRS broth. Initially, the strains were cultured in MRS broth for 24 h at 30 °C. The cultures were then centrifuged at 1500×*g* for 5 min, and the pellets were washed twice with maximum recovery diluent (0.1% w/v bacteriological peptone and 0.85% w/v NaCl at pH 7). Subsequently, the pellets were used to prepare standardized suspensions with an OD_{600} of 0.1 (corresponding to 10^7 CFU/mL). From these suspensions, 200 μL of modified MRS broth with different concentrations of NaCl (2%, 4%, 6.5% w/v), ethanol (12%, 15% v/v), or adjusted to different pH levels by HCl addition (pH 3.2, 3.8, 4.2, and 4.6) were inoculated in triplicate for each experiment at a final concentration of 10^2 CFU/mL. The growth of the strains was determined spectrophotometrically by measuring the optical density at 600 nm after 3, 24, and 48 h at 30 °C using a TECAN SUNRISE microplate reader (Tecan, Switzerland). Based on the change in optical density after 48 h of incubation, the strains were classified as sensitive (OD_{600} from 0 to 0.3), intermediate (OD_{600} from 0.3 to 0.9), or resistant (OD_{600} from 0.9 to 1.5) to the tested growth conditions.

2.5. Antibiotic resistance

The antibiotic susceptibility disk diffusion assay test was employed to evaluate the antibiotic resistance profiles. For this assessment, MRS agar plates were inoculated with 100 μL of bacterial suspensions with an OD_{600} of 0.1, prepared from MRS broth cultures incubated for 24 h at 37 °C (Belletti et al., 2009). Subsequently, different antibiotic disks were placed on the plates: cefoperazone 30 μg (CFP30), cefazolin 30 μg (KZ30), chloramphenicol 10 μg (C10), clindamycin 10 μg (DA10), erythromycin 30 μg (E30), kanamycin 30 μg (K30), ofloxacin 5 μg (OFX5), quinupristin/dalfopristin 15 μg (QD15), rifampicin 30 μg (RD30), streptomycin 25 μg (S25), tetracycline 10 μg (TE10), tobramycin 10 μg (TOB10), vancomycin 30 μg (VA30). Antibiotics for testing were selected based on a thorough literature review (Colautti et al., 2021). From the different concentrations mentioned in various studies, the highest concentrations were chosen to clearly indicate the potential resistance of the strain. Following microaerophilic incubation for 24 h at 37 °C, the diameters of inhibition zones were measured using callipers, and results were interpreted according to Charteris, Kelly, Morelli, & Collins, 1998; Charteris et al., 1998), categorizing the strains as sensitive (S, inhibition zone diameter 19–30 mm), intermediate (I, inhibition zone diameter 10–18 mm) or resistant (R, inhibition zone diameter 1–9 mm). *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains.

2.6. Antimicrobial capabilities (bacteriocin production)

The agar well diffusion assay, as described by Schillinger and Lucke (1989) was employed to evaluate the production of antimicrobial peptides. In brief, the strains were cultured overnight in MRS broth at 37 °C. The cell-free supernatant was obtained by centrifuging the culture broth for 10 min at 7000×*g* and further filtered through a 0.2 μm pore-size cellulose acetate membrane. The filtered supernatant was then divided into three aliquots: one aliquot was used without any modification, one aliquot was pH adjusted to 6.5 as a control to eliminate the antimicrobial effect caused by acidity, while the other aliquot was treated with catalase (1 mg/mL, Merck) to remove the hydrogen peroxide and with proteinase K (2 mg/mL, Merck) to deactivate any peptides with potential antimicrobial activity using *Latilactobacillus sakei* I151 as control.

The supernatants were evaluated in triplicate on Brain Heart Infusion (Oxoid) soft agar plates (1% agar) inoculated with different pathogens (*Listeria monocytogenes* ATCC7644, *Staphylococcus aureus* DSM 4910, *Escherichia coli* DSA, *Salmonella enteritidis* DSA). Four wells of 5 mm in diameter were made into each plate and filled with 100 μL of the overnight LAB cultures, the filtered supernatant, the pH 6.5 filtered supernatant, or the PK-treated supernatant. The plates were then incubated for 24 h at 37 °C, and inhibition zones were assessed.

2.7. Biogenic amines (BAs) production

The assessment of biogenic amine production was conducted according to the method described by Bover-Cid and Holzapfel (1999). To induce enzyme production, before the screening test, each strain underwent sequential overnight incubation at 30 °C for five cycles in MRS broth supplemented with 0.1% of each precursor amino acid (tyrosine free base, histidine monohydrochloride, ornithine monohydrochloride, arginine hydrochloride, and lysine hydrochloride, Merck) along with 0.005% of pyridoxal-5-phosphate (Merck). Subsequently, strains were inoculated onto Bover-Cid agar plates and broth decarboxylase media. A change in purple colour of bromocresol purple in response to a pH shift, dependent on the production of the more alkaline BA from the amino acids initially included in the medium, indicated a positive reaction in both agar and broth. Furthermore, a multiplex PCR assay was performed to detect the presence of *hdc*, *tyrdc*, *agdi* and *odc* genes, associated with the degradation of histidine, tyrosine, agmatine and ornithine respectively, following the protocols described by Coton et al. (2010). *Enterococcus faecalis* EF37 (for *tyrdc* and *agdi*), *Streptococcus thermophilus* PRI60 (for *hdc*) and *Lacticaseibacillus rhamnosus* N132 (for *odc*) were utilized as positive controls. To confirm the correct amplification, the amplicons obtained from the positive controls were cloned into pGEM-T Easy vector (Promega, Milan, Italy) and sent to Eurofins Genomics (Ebersberg, Germany) for sequencing.

2.8. arcABC genes presence

The presence of *arcABC* genes, encoding enzymes involved in the arginine dihydrolase system (ADI) pathway in *Lacticaseibacillus* spp., was assessed among the strains. To detect the presence of *arc* genes, degenerate primers *arcA* (arginine deiminase), *arcB* (ornithine transcarbamylase) and *arc* (carbamate kinase), which produce amplicons of 266, 181, and 343 bp, respectively, were used as described by Araque, Gil, Ramon, Bordons, & Reguant, 2009; Araque et al., 2009). *Lactiplantibacillus plantarum* strains 64 and 70 (strains collection of the Viticulture and Oenology Department, Stellenbosch University, South Africa) were used as positive controls. Following PCR, amplicons were separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5X TBE buffer supplemented with 0.025% (v/v) EtBr, and sent to Eurofins

Genomics (Ebersberg, Germany) for sequencing.

2.9. Diamminobenzidine (DAB) assay and Multi Copper Oxidase (MCO) detection

The Diamminobenzidine (DAB) assay and Multi Copper Oxidase (MCO) activity were assessed according to Callejón, Sendra, Ferrer, and Pardo (2014). Briefly, strains were cultured overnight at 37 °C on modified MRS supplemented with L-cysteine (0.5 g/L) and biogenic amines (putrescine, tyramine, histamine) at a concentration of 10 mg/L each. These cultures were centrifuged at 10,000×*g* for 10 min, and the resulting cell pellets were washed twice with 25 mL of a 50 mM solution of sodium phosphate buffer (pH 7.4). After the second wash, the cells were resuspended in 500 μL of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF).

The suspensions were then placed into 1.5 mL tubes along with 1 g of glass beads and agitated using a Qiagen Shaker for 10 cycles, each lasting 40 s. Every 5 cycles, the tubes were cooled on ice for 5 min. Cell extract was obtained by centrifuging the tubes at 10,000×*g* for 15 min. Subsequently, 25 μL of each sample were combined with gel loading buffer 2X (Merck), loaded onto a polyacrylamide gel (with a stacking concentration of 4% and non-denaturing concentration of 8%), and electrophoresed using a Miniprotean system (BioRad, Hercules, USA) at 30 mA for 1 h in Tris-glycine buffer (25 mM Tris base, 192 mM glycine).

Following electrophoresis, the gel was used to assess DAB and MCO activities. Amine-degrading activity was determined by immersing the gel in a sodium phosphate buffer (50 mM, pH 7.4) containing 1 mM histamine, tyramine, and putrescine for 15 min, followed by transfer to a solution of sodium phosphate buffer (50 mM, pH 7.4) supplemented with horseradish peroxidase (1000 U/L) and diaminobenzidine (DAB, 0.25 mM) for 3 h. Observation of brown colour on the active band indicated the presence of amine-degrading activity. MCO activity was evaluated by immersing the gels in a sodium acetate buffer (100 mM, pH 4) containing 10 mM 2,6-dimethoxyphenol (DMP) for 5 min, followed by transfer to the same buffer supplemented with 1 mM $CuSO_4$ for 10 min. The presence of an orange-yellow band indicated MCO activity. Each strain was assessed in triplicate.

2.10. Statistical analysis

All statistical analyses were conducted using the R software version 4.1.2. Specifically, to summarize the collected data and to understand how the different strains separate based on all the variables considered as a whole, while evaluating any correlations between the different factors, a heatmap was produced using ggplot2 (Wickham, 2016), where the normalized data values are represented by cells coloured according to the Z-values, which represent the observed values.

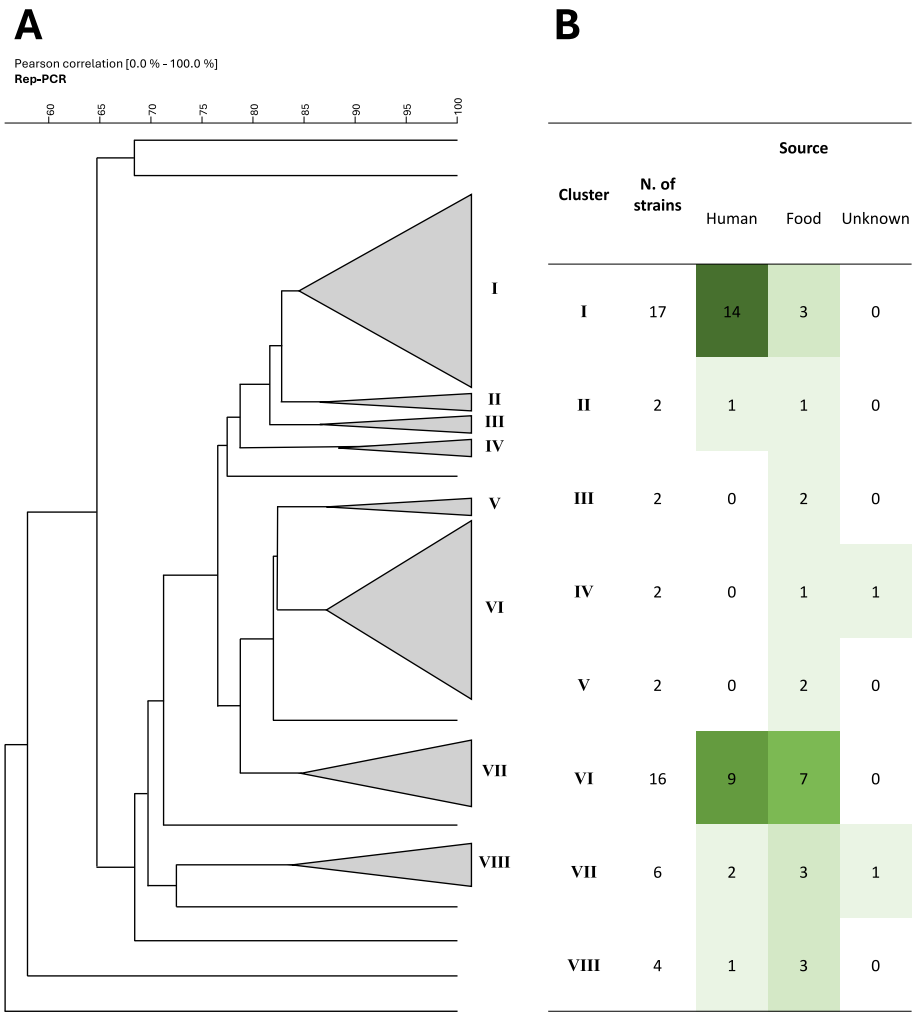


Fig. 1. (**A**) Cluster analysis using Pearson product-moment correlation coefficients and unweighted pair group method using an arithmetic average (UPGMA) of the profiles obtained by Rep-PCR (similarity coefficient 83%) analysis of the different tested strains. The similarity coefficient was arbitrarily chosen. Identified clusters are indicated with Roman numerals. (**B**) Composition of the clusters, concerning to their source of isolation for Rep-PCR.

3. Results and discussion

3.1. Genetic characterization

The three fingerprinting techniques employed addressed the genetic characteristics of the strains differently, resulting in distinct clustering patterns. Comparing the genetic profiles obtained with the Rep-PCR assay using a coefficient of similarity of 83%, a value reported to distinguish between different species of lactobacilli (Gevers, Huys, & Swings, 2001), the strains were grouped into 8 main clusters (CL), while 9 strains did not group with others (Fig. 1A). Analysing the composition of the clusters based on the isolation matrix (Fig. 1B), most of the strains of human origin clustered in CL I (14 strains) and CL VI (9 strains). However, clustering with other strains isolated from food occurred in both CL I (3 strains) and CL VI (7 strains), indicating the absence of a strict relationship to their source of isolation.

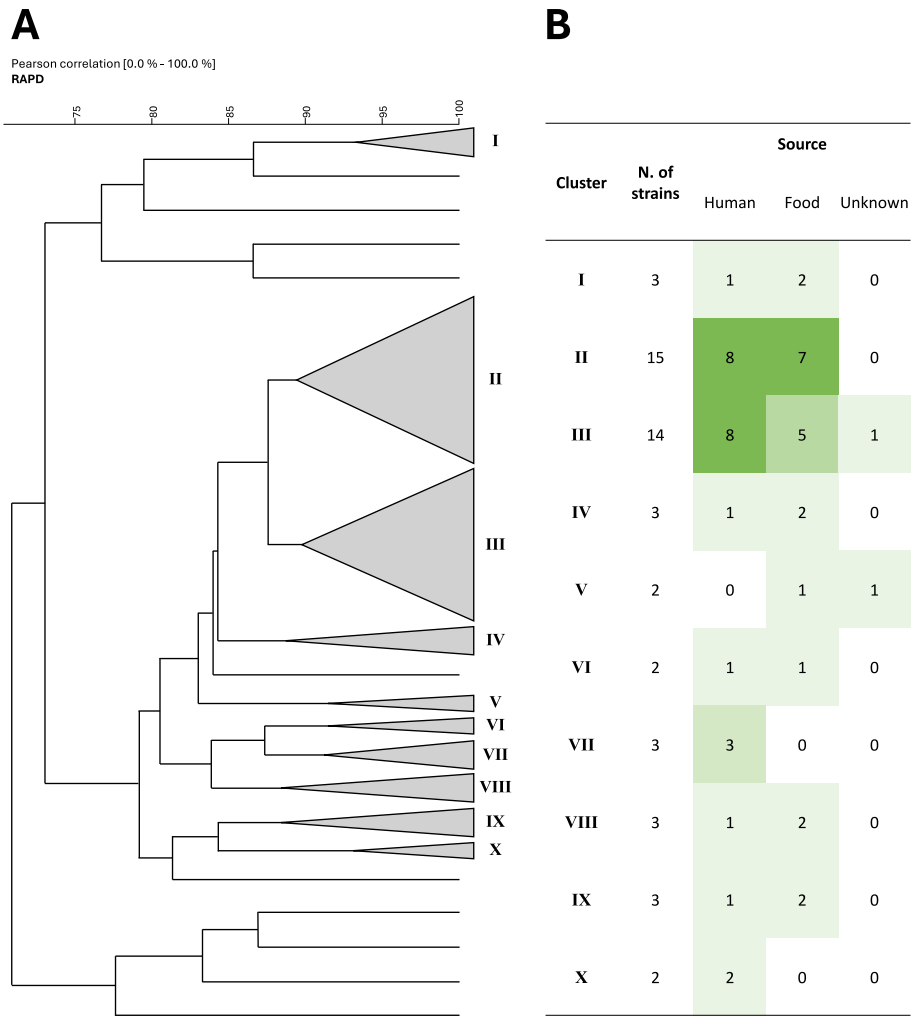
Using a more restrictive percentage of similarity of 88% in the case of RAPD, the strains were divided into 10 clusters, while 10 strains remained ungrouped (Fig. 2A), demonstrating a high genetic variability. Similarly to Rep-PCR, the majority of human-origin strains clustered in the two main clusters, but alongside strains isolated from food in both CL II (8 human strains, 7 food strains) and CL III (8 human strains, 5 food strains), again indicating unclear correlations between the strains and their source of isolation, except for CL VII and X, which comprised strains isolated solely from human sources (Fig. 2B). The high number of clusters and the presence of 10 isolated strains underscored the high genetic heterogeneity of the considered strains, as observed by other authors for this species (Turková, Rittich, & Španová, 2012).

Lastly, Sau-PCR fingerprints were also clustered using a similarity percentage of 88% (Fig. 3A). This resulted in 10 clusters and 13 individual strains. In this case, an even greater differentiation of the strains was observed, with a higher number of them remaining ungrouped. However, even in this case, the different clusters mostly comprised strains from diverse matrices, particularly the main CL II, IV, and VI (Fig. 3B).

From these analyses, a high genetic variability of the strain collection analysed in this study emerged, highlighting the significant intraspecific differences among *Lbs. rhamnosus* strains and suggesting the adequacy of this collection in effectively representing the differences observable in these microorganisms.

3.2. Stressors resistance

The assessment of resistance to different stresses induced by varying concentrations of NaCl, ethanol (EtOH), and pH revealed consistent resistance characteristics among all tested strains (Fig. 4). Concerning pH, the majority of strains (98%) demonstrated resistance at pH value of 4.6, with a significant percentage (68%) maintaining resistance at a pH of 3.8. Looking at pH values of 3.2, none of the strains demonstrated high resistance, and only10% showed intermediate tolerance. Regarding



Cluster	N. of strains	Source		
		Human	Food	Unknown
I	3	1	2	0
II	15	8	7	0
III	14	8	5	1
IV	3	1	2	0
V	2	0	1	1
VI	2	1	1	0
VII	3	3	0	0
VIII	3	1	2	0
IX	3	1	2	0
X	2	2	0	0

Fig. 2. (**A**) Cluster analysis using Pearson product-moment correlation coefficients and unweighted pair group method using an arithmetic average (UPGMA) of the profiles obtained by RAPD (similarity coefficient 88%) analysis of the different tested strains. The similarity coefficient was arbitrarily chosen. Identified clusters are indicated with Roman numerals. (**B**) Composition of the clusters, concerning to their source of isolation for RAPD.

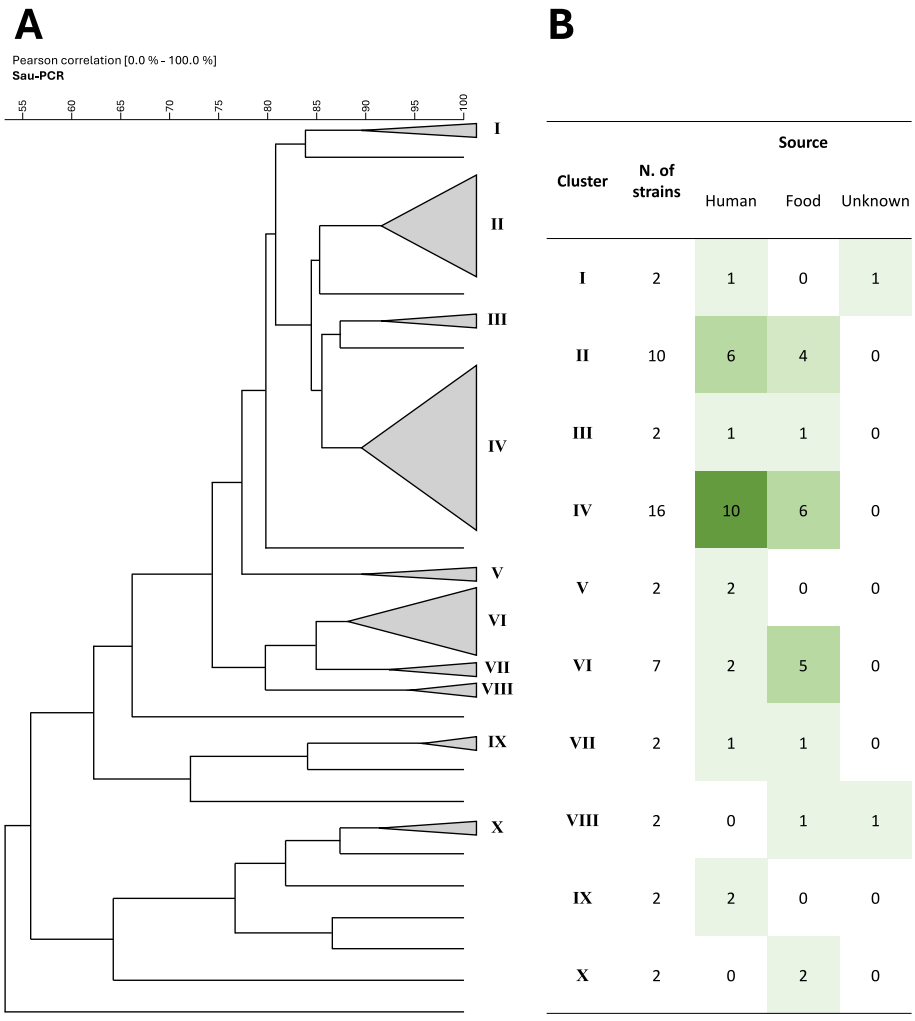


Fig. 3. (**A**) Cluster analysis using Pearson product-moment correlation coefficients and unweighted pair group method using an arithmetic average (UPGMA) of the profiles obtained by Sau-PCR (similarity coefficient 88%) analysis of the different tested strains. The similarity coefficient was arbitrarily chosen. Identified clusters are indicated with Roman numerals. (**B**) Composition of the clusters, concerning to their source of isolation for Sau-PCR.

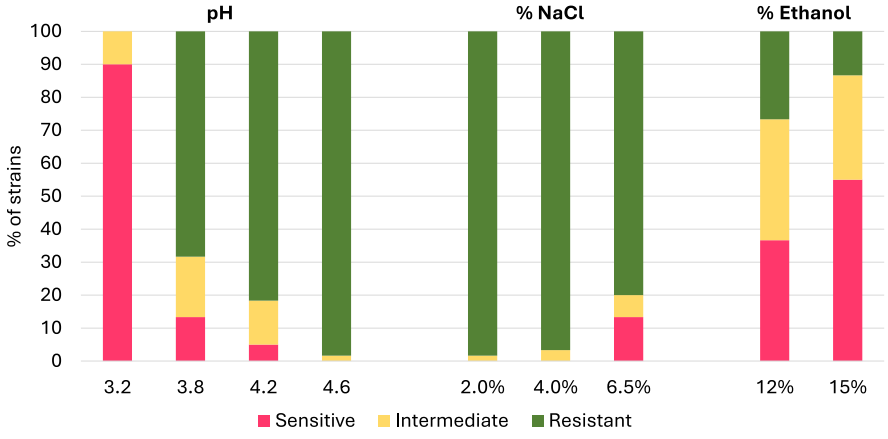


Fig. 4. Stress resistance results for pH, % NaCl and % Ethanol expressed as relative percentage of strains Resistant, Intermediate or Sensitive.

resistance to different concentrations of NaCl, a broad tolerance was observed. Only two strains were not resistant up to 4%, while 48 strains showed resistance up to 6.5%. Similarly, consistency in resistance levels among strains was noted across different EtOH concentrations, with only 22 strains (36.7%) sensitive at 12% concentrations and 33 strains (55.0%) sensitive at 15%. Among all the tested strains, *Lbs. rhamnosus* DBTC4 emerged as the most resilient under all conditions, displaying resistance to 6.5% of NaCl, 15% of EtOH, and intermediate resistance to pH value of 3.2. These results align with observations in the literature. Despite the well-known resistance of strains like *Lbs. rhamnosus* GG even

in the presence of gastric juices and environments with a pH of 2 (Corcoran, Stanton, Fitzgerald, & Ross, 2005), few lactobacilli exhibit pronounced resistance beyond certain very acidic pH values (Prasad, Gill, Smart, & Gopal, 1998). In many cases, a decrease in vitality has been observed at pH values of 3, with further reduction at 2.5–2 (Jacobsen et al., 1999; Vinderola & Reinheimer, 2003). Similarly, concerning osmotic stress, concentrations ranging from 2 to 6% NaCl were largely tolerated by *Lbs. rhamnosus* strains. The first negative effects on the vitality of this microorganism were reported with concentrations of 10% of NaCl, which significantly decreased the survivability of several strains of *Lbs. rhamnosus* (Liu, Li, Yang, Liang, & Wang, 2006; Pithva, Shekh, Dave, & Vyas, 2014; Prasad, McJarrow, & Gopal, 2003; Reale et al., 2015). Regarding ethanol resistance, there have been limited studies in the literature on the resilience of this species to this compound. In this sense, *Lbs. rhamnosus* strains were identified in a continuous wet mill fuel ethanol facility, demonstrating their capacity to tolerate alcoholic environments (Skinner & Leathers, 2004). This finding was further supported by other authors, who observed resistance of strains of this species to ethanol concentrations of up to 14% (Lee, Kim, Kim, & So, 2010). The results obtained in this study, given the number and variety of tested strains, corroborate the robust ethanol resistance of *Lbs. rhamnosus*, which can increase in the case of adaptation. Although not conventionally utilized in alcoholic fermentations, ethanol-resistant *Lbs. rhamnosus* strains could offer promising applications in the production of fermented probiotic foods. Co-inoculating these strains during fermentation could potentially lower the ethanol concentration in the final product. This application is particularly compelling as it meets consumer preferences for products with reduced ethanol content, while still harnessing the probiotic benefits of *Lbs. rhamnosus*.

This approach aligns with current trends promoting healthier and more balanced diets, thereby providing consumers with a broader array of choices in the fermented foods market (Chan, Toh, & Liu, 2021).

3.3. Antibiotics resistance

Understanding the resistance profiles of *Lbs. rhamnosus* strains is essential due to their widespread use as microbial starters and/or probiotics (Westerik, Kort, Sybesma, & Reid, 2018). Despite being Generally Recognized as Safe (GRAS), these strains have been linked to conditions such as peritonitis, urinary tract infections, endocarditis, or bacteraemia, particularly in vulnerable individuals. Therefore, a comprehensive evaluation of resistance factors in strains intended for human consumption is crucial, with a particular emphasis on transferable resistance factors (Colautti et al., 2021). While intrinsic resistance, such as vancomycin resistance in heterofermentative lactobacilli, cannot be horizontally transferred, resistance genes acquired through plasmids or transposons can be transmitted. Consequently, the European Commission has recommended prioritizing the detection of transferable antibiotic resistance markers in the safety assessment of microorganisms intended for human consumption (European Commission, 2004). Based on the phenotypic resistance analysis for the traits previously reported and examined in the literature (Charteris et al., 1998), the tested *Lbs. rhamnosus* strains demonstrated resistance to kanamycin 30 μg, ofloxacin 5 μg, streptomycin 25 μg, tobramycin 10 μg, and vancomycin 30 μg, while showing sensitivity to other tested antibiotics. These characteristics were consistent with observations made by other authors (Ammor, Belén Flórez, & Mayo, 2007; Belletti et al., 2009; Federici et al., 2014; Mathur & Singh, 2005). However, three strains exhibited resistance profiles deviating from these patterns. Specifically, *Lbs. rhamnosus* PRA331 showed intermediate resistance to kanamycin 30 μg, ofloxacin 5 μg, tetracycline 10 μg, tobramycin 10 μg, and vancomycin 30 μg, while being susceptible to streptomycin 25 μg. *Lbs. rhamnosus* CI230 was susceptible to ofloxacin 5 μg and vancomycin 30 μg, and showed intermediate resistance to kanamycin 30 μg, streptomycin 25 μg, and tetracycline 10 μg. *Lbs. rhamnosus* N2010 was sensitive to kanamycin 30 μg, streptomycin 25 μg, and vancomycin 30 μg, and exhibited intermediate resistance to ofloxacin 5 μg and tobramycin 10 μg (Table 2). These results contradict other studies that describe vancomycin resistance as an intrinsic characteristic of this species (Sharma, Tomar, Goswami, Sangwan, & Singh, 2014; Tynkkynen, Singh, & Varmanen, 1998).

3.4. Antimicrobial activity and bacteriocins production

LAB have garnered attention for their potential in combating the development and virulence of foodborne pathogens (Colautti, Orecchia, Comi, & Iacumin, 2022), as well as for their ability to produce bacteriocins, which could pave the way for innovative strategies to reduce the reliance on chemical preservatives in food, while ensuring product safety (Lahiri et al., 2022). Additionally, the production of lactic acid and hydrogen peroxide exhibits a bactericidal/bacteriostatic effect, attributed to cytoplasmic acidification and disruption of the proton motive force.

In this context, *Lbs. rhamnosus* tested strains did not demonstrate the production of potential bacteriocins; however, their untreated or pH adjusted supernatants exhibited a good ability to compete against pathogens. Indeed, most of the supernatants obtained from the strains were capable of directly inhibiting the growth of the tested pathogens (Fig. 5A). Similarly, inhibition was observed against *L. monocytogenes*, *S. enteritidis*, and *E. coli* due to the production of organic acids. However, no inhibition was observed against *S. aureus* (Fig. 5B). These results align with previous findings regarding the inhibition of these pathogens, although some studies have reported high antagonistic activity against *St. aureus* as well, reducing its adhesion thanks to an antibiofilm activity, reducing their cell viability and biomass, and the secretion of antimicrobial substances into the surrounding environment (Carvalho, Mergulhão, & Gomes, 2021; Georgieva et al., 2015; Prezzi et al., 2020).

3.5. Biogenic amines (BAs) production

Biogenic amines (BAs) are organic compounds characterized by aromatic, aliphatic, or heterocyclic structures, primarily formed through the microbial decarboxylation of amino acids. This decarboxylation process serves as an energy source for microorganisms in nutritionally deficient environments (Suzzi & Gardini, 2003). BAs can accumulate in various foods and beverages, including fish, cheeses, meat products, fermented vegetables, wine, and beer, due to microbial activity (Buňková et al., 2009; Silla Santos, 1996). While not always associated with a decline in sensory quality, the consumption of high levels of these amines can pose toxicological risks to consumers (Costantini et al., 2013; Coton & Coton, 2005; Ladero et al., 2011; Landete, de las Rivas, et al., 2007; Landete, de las Rivas, et al., 2007). Tyramine can cause symptoms such as vomiting, hypertension, and headaches, while histamine may lead to allergic reactions including low blood pressure, oedema, palpitations, vomiting, rash, and breathing difficulties. Putrescine and cadaverine, though not inherently toxic, can interfere with histamine and tyramine detoxification or exacerbate histamine toxicity, respectively (García-Ruiz, González-Rompinelli, Bartolomé, & Moreno-Arribas, 2011; Landete, de las Rivas, et al., 2007; Landete, de las Rivas, et al., 2007). Given these potential risks, it is to subject strains intended for food use to genetic and phenotypic characterisation to ensure the selection of starter cultures that do not produce biogenic amines (Suzzi & Gardini, 2003).

In this regard, out of the 60 tested strains of *Lbs. rhamnosus* using the screening method by Bover-Cid and Holzapfel (1999), 5 showed the ability to decarboxylate BAs precursor amino acids, while 10 strains harboured genes associated with these metabolites (Table 3). Specifically, strains D44, N178, N812, and L47 were found to decarboxylate tyrosine, while strain CTC1676 decarboxylated arginine and lysine. Conversely, the detection of genes related to biogenic amine production revealed the presence of the *odc* gene in strains N132, N21, N2013, and

Table 2
Differences in antibiotic resistance traits.

Strain	Origin	Antibiotics												
		CFP30	KZ30	C10	DA10	E30	K30	OFX5	QD15	RD30	S25	TE10	TOB10	VA30
Lbs. rhamnosus PRA331	Cheese	S	S	S	S	S	I	I	S	S	S	I	I	I
Lbs. rhamnosus CI230	Parmigiano Reggiano	S	S	S	S	S	I	S	S	S	I	I	R	S
Lbs. rhamnosus N2010	Body excreta	S	S	S	S	S	S	I	S	S	S	S	I	S

Antibiotic legend: CFP30, Cefoperazone 30 μg; KZ30, Cefazolin 30 μg; C10, Chloramphenicol 10 μg; DA10, Clindamycin 10 μg; E30, Erythromycin 30 μg; K30, Kanamycin 30 μg; OFX5, Ofloxacin 5 μg; QD15, Quinupristin/Dalfopristin 15 μg; RD30, Rifampicin 30 μg; S25, Streptomycin 25 μg; TE10, Tetracycline 10 μg; TOB10, Tobramycin 10 μg; VA30, Vancomycin 30 μg.
S: Sensitive, I: Intermediate and R: Resistant.

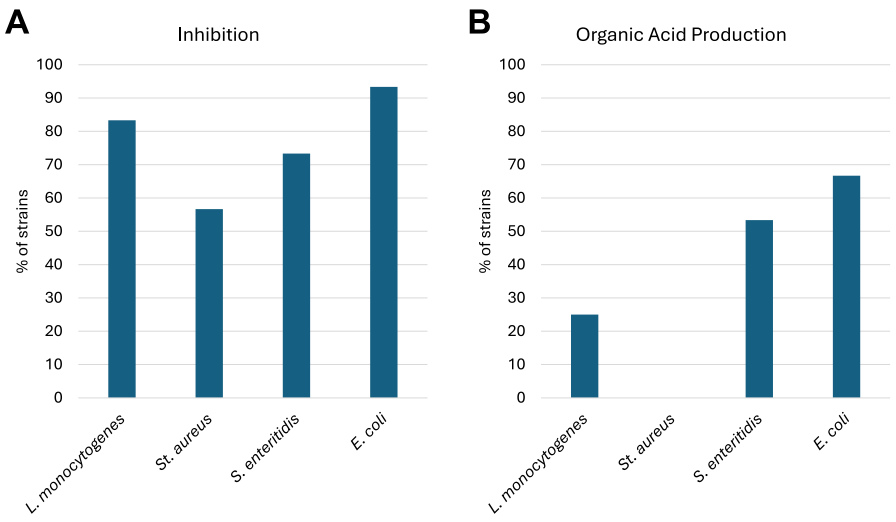


Fig. 5. Percentage of *Lbs. rhamnosus* untreated surnatants **(A)** and surnatants whose effect was attributable to the presence of organic acids **(B)** that showed inhibition capability against tested pathogens.

Table 3
Differences between detected genes and positive reaction in decarboxylase medium in *Lbs. paracasei* strains.

Strain	Presence/Absence of Genes tested				Decarboxylated amino acids				
	tyrdc	odc	agdi	hdc	Tyrosine	Histidine	Ornithine	Arginine	Lysine
D44	-	-	-	-	+	-	-	-	-
N178	-	-	-	-	+	-	-	-	-
N812	-	-	-	-	+	-	-	-	-
N132	-	+	-	-	-	-	-	-	-
N21	-	+	-	-	-	-	-	-	-
N2013	-	+	-	-	-	-	-	-	-
N202	-	+	-	-	-	-	-	-	-
L47	-	-	+	-	+	-	-	-	-
CTC1676	-	-	+	-	-	-	-	+	+
CI4362	-	+	-	+	-	-	-	-	-
N22	-	+	-	+	-	-	-	-	-
N26	-	+	-	+	-	-	-	-	-
N131	-	+	-	+	-	-	-	-	-

Legend: +, presence of the tested gene or decarboxylation activity; -, absence of the tested gene or decarboxylation activity.

N202, the *agdi* gene in strains L47 and CTC1676, and both the *odc* and *hdc* genes in strains CI4362, N22, N26, and N131.

When comparing the genetic and phenotypic results, it is notable that the *tyrdc* gene was absent in strains D44, N178, and N812, while strain L47 displayed the presence of the *agdj* gene instead of the expected *tyrdc* gene. Only strain CTC1676 consistently demonstrated putrescine production and the presence of the agmatine deiminase gene, *agdi*, aligning with both genotypic and phenotypic analyses. Discrepancies between the two techniques could stem from gene silencing or very low expression, making it challenging to detect products using conventional plate growth methods.

Strains positive for genes related to biogenic amine production that did not exhibit positive reactions in the medium likely failed to meet the prerequisites for biogenic amine formation, such as conditions conducive to bacterial growth, decarboxylase synthesis, and decarboxylase activity (Landete, Ferrer, & Pardo, 2005; Postupolski, Stasiak, Maćkiw, Kowalska, & Kucharek, 2021; Silla Santos, 1996). Conversely, false-positive reactions may arise due to the formation of alkaline-reacting chemicals, resulting in pH indicator colour changes in decarboxylase medium (Buňková et al., 2009). Therefore, PCR analysis could offer a more precise method for determining the potential of strains to produce biogenic amines, as it detects strains possessing genes

for the corresponding enzyme production. However, it is essential to acknowledge that the presence of a single gene does not necessarily indicate its expression, as environmental conditions profoundly influence microbial metabolism. Hence, it remains crucial to thoroughly assess both genotypic and phenotypic aspects of microorganisms.

3.6. *arcABC* genes presence

During the arginine deiminase (ADI) pathway in LAB, citrulline and carbamoyl phosphate are released and can naturally react with ethanol, leading to the formation of ethyl carbamate. This compound is a potential carcinogen found in various fermented foods, notably in wine (Fang et al., 2013; Tonon & Lonvaud-Funel, 2002). Certain heterofermentative LAB strains have demonstrated activity in degrading arginine (Liu, Pritchard, Hardman, & Pilone, 1994; Mira De Orduña, Liu, Patchett, & Pilone, 2000; Uthurry, Suárez Lepe, Lombardero, & García Del Hierro, 2006).

Only four strains presented at least one of the three genes encoding the main enzymes involved in this pathway: *arcA* (arginine deiminase), *arcB* (ornithine transcarbamylase), and *arcC* (carbamate kinase). *Lbs. rhamnosus* N1710 and N812, both isolated from human tissues/excreta tested positive for the *arcB* gene, while DBPZ0446 and L47, both isolated from cheeses, tested positive for *arcC*. Comparing these findings with existing literature reveals limited research on this topic concerning the genus *Lacticaseibacillus*, particularly *Lbs. rhamnosus*.

Studies on other facultative or obligate heterofermentative LAB, such as *Lentilactobacillus hilgardii, Lpb. plantarum*, *Lentilactobacillus buchneri*, and *Oenococcus oeni*, have highlighted their ability to degrade arginine and excrete citrulline (du Toit, Engelbrecht, Lerm, & Krieger-Weber, 2011; Mira De Orduña et al., 2000). The presence of the *arcABC* genes has been observed in the genomes of various LAB strains, including *Levilactobacillus brevis*, *Llb. hilgardii*, *Fructilactobacillus florum, Llb. buchneri*, *Lpb. plantarum*, *O. oeni*, *Pediococcus pentosaceus* and *Leuconostoc mesenteroides* (Araque et al., 2009; Divol, Tonon, Morichon, Gindreau, & Lonvaud-Funel, 2003; Mtshali, Divol, & Du Toit, 2012; Spano, Massa, Arena, & Manca De Nadra, 2007; Thierry Tonon, Bourdineaud, & Lonvaud-Funel, 2001).

3.7. Biogenic amines (BAs) degrading activity: diaminobenzidine (DAB) assay and Multi Copper Oxidase (MCO) detection

Given the potential presence of BAs, especially in fermented foods (Li & Lu, 2020), it is crucial to devise effective strategies to reduce or eliminate their occurrence not only to enhance the sensory qualities of the food but also to mitigate health risks to consumers (Tabanelli, 2020). Therefore, the selection and use of microbial starters that do not produce these metabolites becomes imperative. Moreover, it is worth considering the use of starters that also exert a bioprotective effect by inhibiting the growth of other microorganisms capable of producing BAs, and that possess the ability to degrade existing biogenic amines through specialized enzymes (Kongkiattikajorn, 2015). Such microorganisms can decrease the need for additives and preservatives by producing their own enzymes, such as monoamine oxidase (MAO) and diamine oxidase (DAO) (Callejón et al., 2014; Naila, Flint, Fletcher, Bremer, & Meerdink, 2010; Suzzi & Gardini, 2003).

Lbs. rhamnosus strains tested for these attributes revealed that 18 strains isolated from various dairy matrices and human tissues/excreta exhibited Monoamine Oxidase (MCO) activity, while no Amine Oxidase (AO) activity was detected using the Diaminobenzidine (DAB) assay (Table 4). These findings confirm the ability of several *Lbs. rhamnosus* strains to metabolize histamine, tyramine, putrescine, and 2,6-dimethoxyphenol (DMP) as substrates, leading to the oxidation of these compounds. Similar capabilities were also observed in related species such as *Lbs. casei* and *Lbs. paracasei* strains, as demonstrated by previous studies (Callejón et al., 2014; Fadda, Vignolo, & Oliver, 2001).

Table 4
MCO positive strains.

Strain	Source of isolation	Strain	Source of isolation
DBPZ0446	Caciocavallo Di Leo	N21	Body excreta
NRRL B-176	Unknown	N176	Body excreta
PRA331	Dairy industry	N2011	Body excreta
L9	Asiago cheese	DSMZ20021	Unknow
L47	Asiago cheese	Mo2	Unknow
N171	Body excreta	LACcas13	Grana Padano
N2012	Body excreta	HA111	Probiotic starter
N812	Body excreta	2220	Unknow
N1110	Body excreta	TMW 1.1538	Feces

3.8. Heatmap

Summarizing all the characteristics (reported in detail in Supplementary Table 1) of the strains analysed in this study through a heatmap (Fig. 6), it was thus possible to observe how the different strains differentiated and grouped together considering all these features simultaneously. The features were distinguishable in 3 groups based on their observed frequency and capacity to discriminate the strains (cluster I: kanamycin 30 μg, ofloxacin 5 μg, streptomycin 25 μg, tobramycin 10 μg and vancomycin 30 μg, pH 3.8, 4.2, 4.6, NaCl 2%, 4% 6.5%; cluster II: cefoperazone 30 μg, cefazolin 30 μg, chloramphenicol 10 μg, clindamycin 10 μg, erythromycin 30 μg, quinupristin/dalfopristin 15 μg, tetracycline 10 μg, pH 3.2, ethanol 12%, ethanol 15%, MCO production, histamine, tyramine, putrescine or agmatine, and cadaverine production, *odc*, *agdi*, *hdc*, *arcB*, and *arcC* genes; cluster III: rifampicin 30 μg, tyrdc, *arcA* gene).

Strain CI4362 was the most distinct from the others (branch A), particularly in terms of resistance to various antibiotics (kanamycin, ofloxacin, streptomycin, tobramycin, and vancomycin) and growth stressors (pH, NaCl). Notably, this strain also had genes associated with BA production (*odc* and *hdc*). Branch B grouped 8 strains isolated from different sources (4 human, 1 milk, 1 cheese, 1 sausage, 1 unknown), which differed in terms of sensitivity to pH and NaCl. Close to them, branch C clustered 22 strains, also heterogeneous based on the isolation matrix (9 cheese, 9 human, 1 sausage, 1 milk, 1 coffee), and differed mostly for the cluster I features. Among this cluster, strain L47 stood out for its pH resistance, histamine production, and presence of the *agdi* and *arcC* genes, while the two observable sub-clusters differed mainly for MCO production. Branch D included the single strain N202, while branch E consisted of 28 strains (11 cheese, 1 unknown, 16 human), differing from other clusters for the resistance to stressors comprised in cluster I. It can therefore be noted that the features resulted strain-specific as observed in other studies (Verdenelli et al., 2009). Also, strains did not segregate based on the isolation matrix. According to the literature, other studies have observed that although the core genome is conserved, different strains of lactobacilli exhibit specific traits based on the ecological niches they occupy (Cen et al., 2020). However, in agreement with the results of this study, other works found no significant difference in the overall profile of metabolism-related genes between dairy and gut-associated genomes (You et al., 2023). This could be because strains isolated from the human body could derive from ingested foods and survive (De Boeck et al., 2020), resisting and remaining viable to digestive processes (Pitino et al., 2012).

4. Conclusions

Genotyping assays revealed significant divergence and variability among the tested strains, with no discernible relationships based on their respective sources of isolation. Significant variability in safety traits and technological properties was also observed, indicating strain-specific capabilities. Notably, strains CI230 and N2010 were susceptible to vancomycin, challenging the notion that vancomycin resistance is intrinsic to this species.

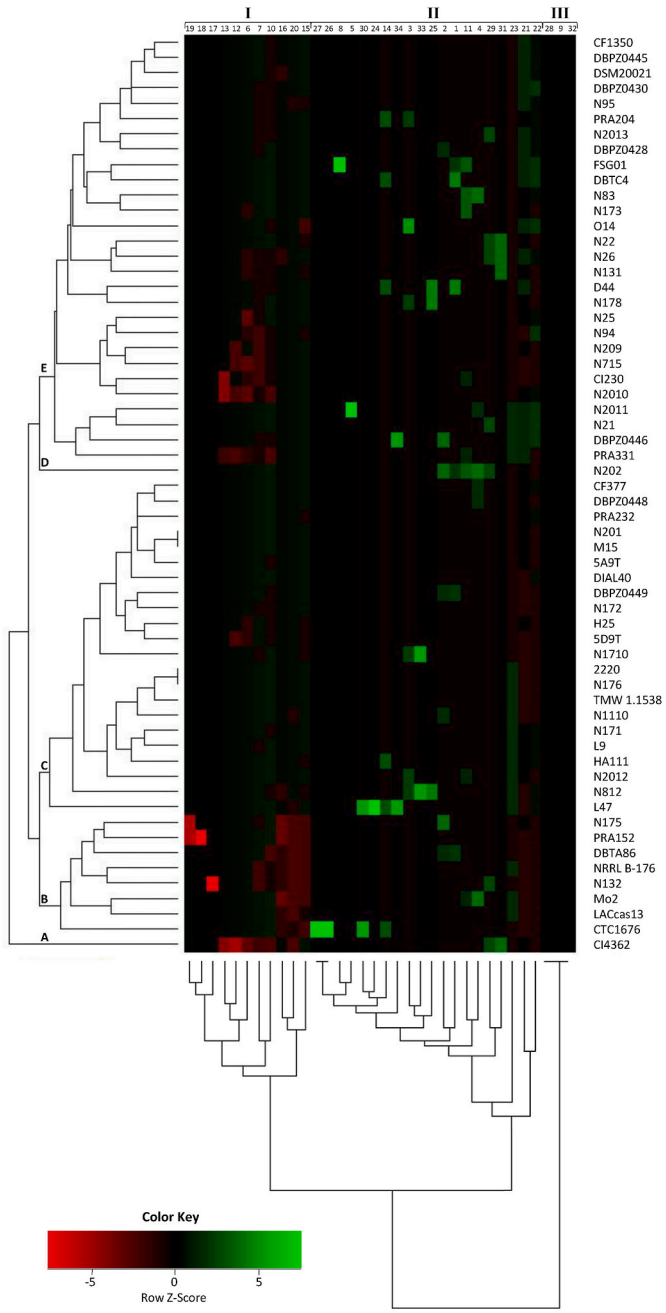


Fig. 6. Heat map of *Lbs. rhamnosus* strains obtained by the comparison of the characteristics studied. Each number indicate a characteristic: 1 to 13: antibiotic resistance (cefoperazone 30 μg, cefazolin 30 μg, chloramphenicol 10 μg, clindamycin 10 μg, erythromycin 30 μg, kanamycin 30 μg, ofloxacin 5 μg, quinupristin/dalfopristin 15 μg, rifampicin 30 μg, streptomycin 25 μg, tetracycline 10 μg, tobramycin 10 μg and vancomycin 30 μg); 14 to 22 growth abilities (pH 3.2, 3.8, 4.2; 4.6; NaCl 2%, 4%, 6.5% and ethanol 12%, 15%); 23 = MCO production; 24 to 27 BAs production (amino acids decarboxylation: histidine, tyrosine, arginine, lysine); 28 to 31 genes involved in BAs production (*tyrdc, odc, agdi, hdc* genes) and 32 to 34 genes involved in ethyl carbamate production (*arcA, arcB, arcC* genes).

Strain-specific characteristics included low antibiotic resistance and MCO presence in strain PRA331, possession of *arcC* and *agdi* genes of strain L47, and *arcB* gene and MCO activities without BA-related genes for strain N812. Despite phenotypic and genotypic segregation into different clusters, no strong correlations with ecological niches of isolation were observed.

In this sense, the study of these microorganisms and their metabolisms shows promise for reducing reliance on food additives. Research emphasizes their bioprotective efficacy, offering potential for substituting or supplementing antimicrobials and antioxidants, benefiting consumer health. The diversity of microbial strains demonstrated in this study, underscores their potential applications in functional foods and probiotics, essential for tailored product development. For instance, formulations for individuals with heightened susceptibility could integrate antibiotic-sensitive strains, while strains with non-transferable resistances could enhance certain antibiotic therapies to mitigate adverse effects.

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CRediT authorship contribution statement

Andrea Colautti: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Lucia Camprini:** Formal analysis. **Federica Ginaldi:** Formal analysis. **Giuseppe Comi:** Resources, Investigation. **Anna Reale:** Resources. **Francesca Coppola:** Resources. **Lucilla Iacumin:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

All authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116578.

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