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Isolation, characterisation and technological properties of raw donkey's milk isolate, *Lacticaseibacillus paracasei*, compared to raw goat's and cow's milk isolates

*Gabriela Greifová[1], Eva Drobná[1], Petra Olejníková[2], Gabriel Greif[3], Mária Greifová[3]**

[1]*Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University Bratislava, Bratislava, Slovak Republic*
[2]*Institute of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic*
[3]*Department of Food Technology, Institute of Food Science and Nutrition, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic*

**Corresponding author: maria.greifova@stuba.sk*

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Abstract: Donkey milk is a very promising matrix for the isolation of new potential starter cultures with probiotic properties. We isolated, identified, and compared the technological properties of the donkey milk isolate D23 with those of goat milk isolates G15 and G17 and bovine milk isolates C3 and C9. All isolates were identified as *Lacticaseibacillus paracasei* using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) and polymerase chain reaction (PCR) assays (determination of species-specific DNA fragments). Like the bovine and goat isolates, the donkey milk isolate D23 was able to grow in De Man–Rogosa–Sharpe (MRS) broth at various temperatures (10, 25, 30, 37, and 45 °C) and at different NaCl concentrations (0–6.5% w/v). Additionally, D23 showed notable proteolytic and autolytic activity, could grow in and acidifying ultra high temperature (UHT) bovine milk but exhibited very weak diacetyl production. None of the isolates displayed hemolytic activity, nor produced histamine, fenylethylamine and cadaverine. Finally, isolate D23 demonstrated interesting antibacterial and antifungal properties compared to the goat and bovine isolates, especially against *staphylococcus aureus* CCM 3953.

Keywords: lactobacilli; MALDI-TOF-MS; proteolysis and lipolysis; antimicrobial properties; safety

Donkey milk is renowned for its nutritional and health-promoting properties, especially when compared to more commonly consumed types of milk (Papademas et al. 2022). The chemical composition of donkey milk is more similar to human breast milk than to bovine, sheep or goat milk. More precisely, donkey milk has simillar amount of lactose (5.8–7.4% w/w), total protein content (1.5–1.8% w/w), whey protein content (0.49–0.80% w/w) and similar pH value (7.0–7.2), which tends to be neutral or slightly alkaline unlike bovine milk which is rather acidic (6.6–6.8). Since, protein portion of donkey milk consists of higher amount of whey protein than casein (52:37), it is easier to digest for human (Uniacke-Lowe et al. 2010; Aspri et al. 2017). Therefore,

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it is often consumed by individuals suffering from cow milk protein allergy (Papademas et al. 2022).

The microbial population of donkey milk is very low which could be due to the presence of natural antimicrobial components such as lysozyme, immunoglobulins, lactoferrin and lactoperoxidase. Total microbial content (TMC) of raw donkey milk is 2.40–5.87 log CFU·mL^{-1} (CFU – colony-forming unit). Lactic acid bacteria (LAB) are usually present with average relative abundance up to 4.2%. LAB species more frequently detected in donkey milk were *Lacticaseibacillus paracasei* (34.0%), *Lactococcus lactis* (29.9%) and *Carnobacterium maltaromaticum* (9.7%). Less abundant genera were *Leuconostoc*, *Enterococcus* and *Streptococcus* (Soto del Rio et al. 2016).

Lacticaseibacillus paracasei is most often identified as the main adventitious nonstarter lactic acid bacterium present and growing in ripening cheese (Minervini and Calasso 2022). Its abundance in donkey milk could be related to its important resistance to lysozyme as reported M'hamed et al. (2022). Many of isolated strains are classified as probiotics offering the various benefits (antimicrobial activity; immune system stimulation; anti-inflammatory, antioxidant, and the enhancement of intestinal bacterial microbiota) (Bengoa et al. 2021). The aim of this study was to characterize newly isolated and identified strains of *Lacticaseibacillus paracasei* originated from Croatian raw donkey milk samples and compared them with *L. paracasei* strains isolated from Slovak raw bovine and goat milk. A broader survey of *L. paracasei* strains naturally present in different raw milk samples might contribute to the detection of possible probiotic species with biotechnological potential.

MATERIAL AND METHODS

Isolation of bacterial strains. Samples of local cow, goat, and donkey milk were collected aseptically from different areas of Slovakia (cow and goat) and Croatia (donkey) and frozen. 10 mL of each milk sample were homogenized with Peptone Salt Solution (0.1% peptone w/v (Merck, Germany), 0.9% NaCl w/v; pH 7.0 ± 0.2), followed by serial dilutions. From chosen dilutions, 0.2 mL was spread on De Man–Rogosa–Sharpe (MRS) agar (pH 5.7 ± 0.2; Merck, Germany) and LAMVAB selective medium (pH 5.0 ± 0.2, prepared according to Hartemink et al. (1997) and incubated anaerobically at 37 °C for 48 h. Well isolated colonies with typical lactobacilli characteristics were picked from each plate and transferred to MRS broth (pH 5.7 ± 0.2; Merck, Germany). Purified bacterial strains were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) and polymerase chain reaction (PCR) assay and characterised according to their morphological and biochemical properties (Gram staining, production of catalase, carbohydrate fermentation patterns and gas production). Cultures were stored in 40% glycerol (v/v) at –20 °C.

MALDI-TOF identification. Lactobacilli identification was conducted using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (AutoFlex I TOF-TOF apparatus, Bruker Daltonics Inc., Billerica, MA, USA Bruker, Germany) in linear positive-ion mode. Saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, USA) in 50% acetonitrile (v/v) and 2.5% trifluoroacetic acid (w/v) served as matrix solution. Each sample of isolated lactobacilli was prepared according to manufacturer's recommandation. Spectral data were analyzed with MALDI BioTyper software (RTF 3.0.0) using logarithmic score matching (0–3) in BioTyper Library v3.0 (Bruker Daltonics s.r.o., Brno, Czech Republic). A score above 1.9 confirmed bacterial species identification by comparing bacterial fingerprints to the reference database (Shulthess et al. 2013; Krahulcová et al. 2022).

Polymerase chain reaction assay identification

Isolation of genomic DNA. Lactobacilli were cultured in De Man–Rogosa–Sharpe (MRS) broth at 37 °C with 5% CO_2 for 24 h. Cells were collected by centrifuging at 10 000 revolutions per minute (rpm) for 1 min, washed with PBS, and resuspended in 180 µL lysis buffer (20 mM TRIS-HCl, 2 mM EDTA, 1.2% Triton X-100 (v/v), and 20 mg·mL^{-1} lysozyme) (Merck, Germany; Sigma Aldrich, USA). After adding 100 µL of 0.05 M EDTA, the mixture was vortexed and incubated at 37 °C for 1 h. Then, 50 µL of 10% SDS (w/v) (Serva, Estonia), 25 µL Proteinase K (Sigma Aldrich, USA), and 220 µL 0.05 M EDTA were added and incubated at 55 °C for 1 h. Next, 200 µL precipitation solution (6 mL of 5 M potassium acetate, 1.15% glacial acetic acid (v/v) and 2.85 mL of destilled H_2O) (CentralChem, Slovakia) was mixed in, incubated at –20 °C for 5 min, and centrifuged twice at 15 000 rpm at 4 °C. The supernatant was transferred to a new tube, and isopropanol (Serva, Germany) was added. After resting, the mixture was centrifuged, washed with 70% ethanol (v/v) (CentralChem, Slovakia), dried, and the DNA pellet was dissolved in 50 µL sterile water and stored at –20 °C. DNA concentration and purity were measured with a Microplate Reader (Epoch Biotek, Agilent Technologies, USA).

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PCR assay and electrophoresis. PCR was conducted in 0.2 µL microtubes with a 10 µL reaction mix, including 1.0 µL template DNA, 0.4 µL each of forward (5' CAA TGC CGT GGT TGT TGG AA-3') and reverse (5' GCC AAT CAC CGC ATT AAT CG-3') primers, 2.0 µL PCR master mix (5X HOT FIREPol® EvaGreen® qPCR Mix Plus, ROX, SolisBiodyne, Estonia), and 6.2 µL nuclease-free water (Acros Organics, Israel). The reaction protocol included initial heating at 95 °C for 12 min, followed by 30 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s, ending with a final extension at 72 °C for 5 min (QuantStudio 3, Applied Biosystems, USA). PCR products were run on a 2% agarose (w/v) gel (Wealtec, Taiwan) with a 100 bp DNA ladder (Serva, Germany) to confirm product size (Kim et al. 2020).

Carbohydrate metabolism and enzymatic activity profiles. The API 50 CH kit and the API-ZYM system (BioMérieux, Marcy-L'Etoile, France) were used to evaluate fermentation profile and enzymatic activity profile of lactobacilli isolates, respectively. Cell suspensions preparation, inoculation into the microtubes of API 50 CH and/or API-ZYM strips, incubation and evaluation of results were performed according to manufacturer' s instructions.

Determination of technological characteristics

Growth at different temperatures and salt concentration. Overnight cultures of isolates were inoculated in MRS broth and incubated at five chosen temperatures (10, 25, 30, 37 and 45 °C) for 48 h and in MRS broth supplemented with NaCl at four chosen concentration (0.0, 2.0, 4.0 and 6.5% w/v) at 37 °C for 48 h. The degree of microbial growth was evaluated spectrophotometrically (Onda V-10 PLUS spectrofotometer, P.R.C EU) by measuring optical density (OD) at 600 nm. The tests were conducted in duplicate.

Proteolytic activity. Proteolytic activity of isolates was evaluated according to de Almeida Júnior et al. (2015). Colonies with a transparent halo with minimum diameter of 2 mm indicated positive proteolytic ability of isolates (Islam et al. 2021). Tests were conducted in duplicate.

Lipolytic activity. Lipolytic activity of isolates was determined by course of Nezhad et al. (2020). The presence of colonies surrounded by clear zones minimum 2–3 mm in diameter indicated lipolytic activity of isolates. All tests were performed in duplicate.

Autolytic activity. Autolytic activity of isolates was performed and the degree of autolysis (%) was calculated using the formula described by Nezhad et al. (2020). The tests were conducted in duplicate.

The acidification capacity. The acidification capacity of isolates was evaluated using slightly modified method described in Franciosi et al. (2009). Overnight lactobacilli culture was firstly centrifuged at 5 000 rpm for 5 min, then washed three times with 0.1% peptone water (w/v), and resuspended in the same volume. The isolates were than inoculated into 10 mL of ultra high temperature (UHT) low-fat milk and incubated at 37 °C for 72 h. After incubation, samples were stored in refrigerator at 4 °C for 14 days. During this period, spectrophotometric measurements were taken after 6, 24, 48 and 72 h, 7 and 14 days of incubation using a digital pH meter. The pH value of UHT milk at time 0 was 6.7 ± 0.1. At the same time, an appropriate portion of each sample measured under given time was diluted and the number of lactobacilli was quantified using the plate count method (1 mL of dilluted sample were mixed with 14 mL of MRS agar and cultivated under aerobic conditions at 37 °C for 48 h). The tests were conducted in duplicate.

Diacetyl production. The isolates were incubated in test tubes containing 1 mL of UHT milk (pH 6.7 ± 0.1) at 30 °C for 24 h. After this period, 0.5 mL of α-naphthol solution (Sigma, USA) (1% w/v) together with 0.5 mL of KOH solution (Alphatec, USA) (16% w/v) were added to these tubes and incubated for 10 min at 30 °C. Tubes with positive results showed the formation of a red ring at the top (Franciosi et al. 2009). The results were classified as weak, medium, or strong according to the intensity of the colour of the ring formed. The tests were conducted in duplicate.

Antimicrobial assay. The antimicrobial activity was studied by the dual culture overlay diffusion method (Smetanková et al. 2014). Briefly, lactobacilli (0.1 mL of overnight culture) were inoculated onto sterile disks (Rotilabo®-test flakes, ø 12 mm; Carl Roth, Germany) placed on MRS agar plates and incubated at 37 °C for 48 h under anaerobic conditions. The plates were then covered with soft BHI agar (broth + 0.8% agar w/v; Merck, Germany) consisting of 1% inoculum of indicator bacteria or with soft Sabouraud agar (broth + 0.8% agar w/v; Imuna, Slovakia) containing 10^4 mould spores per mL for indicator moulds and yeasts. Following aerobic incubation at 37 °C for 24 h for bacteria and at 25 °C for 48 h for moulds and yeasts, the diameters of the inhibition zones were measured. All inhibition tests were conducted in triplicate. The antimicrobial activity of the lactobacilli was evaluated against five indicator bacteria, four indicator moulds, and two indicator yeasts.

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Safety characteristics of isolates

Haemolytic activity. Overnight cultures were plated according to streak plate method on Columbia blood agar plates (pH 7.3 ± 0.2; MkB test a.s., Slovakia) to analyze hemolytic activity. Plates were incubated at 37 °C for 48 h. The appearance of clear, greenish or no zones around the bacterial colonies was indicated as α-, β-, or γ-hemolysis respectively (Islam et al. 2021).

Decarboxylase activity. The decarboxylase activity of the isolates was assessed following the method outlined by Dias et al. (2013). Incubation was carried out at 37 °C, and observations were made at 1, 4, 24, and 48 h. A violet color indicated a positive reaction, while a yellow color signified a negative result. A negative control tube containing base medium without amino acids was included in the analysis.

Statistical analysis

An analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed to evaluate the antimicrobial activity of the isolates. Statistical significance was set at $P \leq 0.05$. Each trial was conducted in triplicate. Results were reported as mean values with standard deviation (mean ± SD). All statistical analyses were performed using GraphPad Prism software (version 6.01).

RESULTS AND DISCUSSION

Isolation, identification, and characterisation of raw milk isolates

Lactobacilli were isolated from 3 various raw milk samples (donkey, cow and goat) asseptically collected. Among all isolates, we identified five of them as *Lacticaseibacillus paracasei* using two independent identification methods, MALDI-TOF mass spectrometry and PCR assay. MALDI-TOF mass spectrometry allowed identification of species of one order and two genera belonging to lactic acid bacteria (LAB) including order *Lactobacillales* and genera *Enterococcus* and *Leuconostoc* (Nacef et al. 2017; Normand et al. 2017). Identification scores of raw milk isolates are listed in Table 1. Any of listed scores of isolates did not reach the value of 2.300, therefore we proved the identification at the species level using PCR assay according to Kim et al. (2020), who had designed 37 species specific primers for lactobacilli identification, including unique gene encoding cation transport ATPase of *L. paracasei*. The product of PCR reaction possesses 106 bp and its presence in genomes of all 5 isolates is demonstrated in Figure 1.

Determination of basic phenotypic characteristics of isolates included Gram staining, catalase activity, gas production and fermentation and enzymatic activity profile has been performed. All five lactobacilli

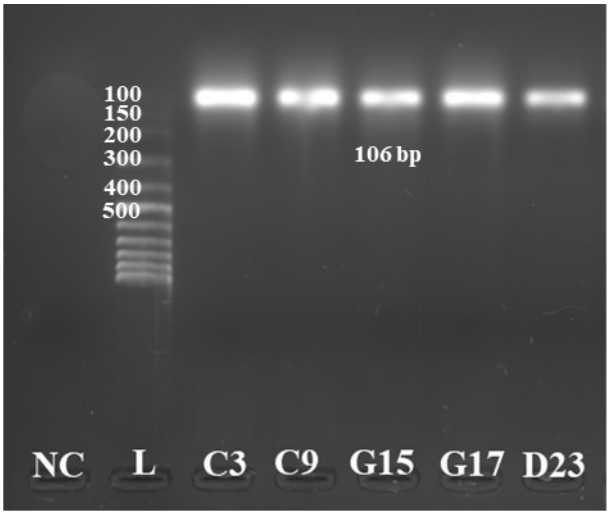

Figure 1. Results of PCR based identification

PCR – polymerase chain reaction; NC – negative control; L – 100 bp ladder (Serva, Germany); C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates; D23 – donkey milk isolate

Table 1. Identification of isolates using MALDI-TOF mass spectrometry

Isolate ID	Milk sample	Identified isolate	Score value
C3	cow	*Lacticaseibacillus paracasei*	2.265
C9	cow	*Lacticaseibacillus paracasei*	2.275
G15	goat	*Lacticaseibacillus paracasei*	2.087
G17	goat	*Lacticaseibacillus paracasei*	2.219
D23	donkey	*Lacticaseibacillus paracasei*	2.259

MALDI-TOF – matrix-assisted laser desorption ionization-time of flight; C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates; D23 – donkey milk isolate

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isolates were Gram positive, catalase negative, no gas producing rods, except for goat milk isolate G15 which produced gas after 48 h of incubation. Fermentation profile and enzymatic profile of isolates explored using API 50 CH test kit and API-ZYM test are listed in Table 2 and 3, respectively. We noticed basic differences in their ability to metabolize L-sorbose, D-sorbitol, D-melibiose, D-raffinose, amygdaline and arbutine and in enzymatic activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), cystein arylamidase, α-chymotrypsin, α-galactosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase. Those differences unabled us to distinguish between two goat milk isolates G15 and G17 (metabolism of arbutine; α-galactosidase, β-glucosidase activity) and between two bovine milk isolates C3 and C9 (cystein arylaminidase activity). Finally, non of isolates possessed β-glucuronidase, a bacterial carcinogenic enzyme negatively affecting the liver (M'hamed et al. 2022).

Technological properties of isolates

It is important to know the optimal growth temperature of isolates when implemented in manufacturing of fermented dairy products and cheeses. All tested isolates were able to growth at temperature from 10 °C to 45 °C (Figure 2). The optimal growth temperature for donkey milk isolate D23 was at 30 °C and 37 °C. Moreover, all tested isolates were able to growth in MRS medium supplemented with NaCl (0.0–6.5% w/v) (Figure 3). The importance of LAB salt tolerance is essential in the production of different types of cheese. Salting of cheeses serves mainly to improve flavour and promote syneresis of the curd, which can regulate the water content of the cheese. The addition of NaCl also has a positive effect on the activity of milk enzymes, starter and non-starter microorganisms, it can also reduce water activity, regulate the fermentation of residual lactose and therefore the pH value, the ripening process and the quality of ripened cheeses (Zimanová et al. 2016).

Proteolytic activity of isolate has been tested using skim milk agar and their lipolytic activity using cream fat agar. All tested isolates showed visible clarified zone of milk proteolysis, but none of them exhibited lipolytic activity. Bacterial lipolytic activity in dairy products such as milk, yogurt, and butter, is not desirable. However, in cheese manufacturing process, low lipo-

Table 2. Fermentation profile of isolates according to the API 50 CH kit

Active compound	Isolate ID									
	C3		C9		G15		G17		D23	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	–	–	–	–	–
Erythritol	–	–	–	–	–	–	–	–	–	–
D-arabinose	–	–	–	–	–	–	–	–	–	–
L-arabinose	–	–	–	–	–	–	–	–	–	–
D-ribose	+	+	+	+	+	+	+	+	+	+
D-xylose	–	–	–	–	–	–	–	–	–	–
L-xylose	–	–	–	–	–	–	–	–	–	–
D-adonitol	–	–	–	–	–	–	–	–	–	–
Methyl-bD-xylopyranoside	–	–	–	–	–	–	–	–	–	–
D-galactose	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+
L-sorbose	–	–	–	–	+	+	+	+	+	+
L-rhamnose	–	–	–	–	–	–	–	–	–	–
Dulcitol	–	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–	–
D-mannitol	+	+	+	+	+	+	+	+	+	+

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Table 2. To be continued

D-sorbitol	+	+	+	+	–	+	–	+	–	+
Methyl-aD-mannopyranoside	–	–	–	–	–	–	–	–	–	–
Methyl-aD-glucopyranoside	–	–	–	–	–	–	–	–	–	–
N-acetylglucosamine	+	+	+	+	+	+	+	+	+	+
Amygdaline	w	+	w	+	–	–	–	–	–	–
Arbutine	w	+	w	+	–	–	–	+	–	–
Esculine	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	+	w	+	+	+
D-maltose	w	+	w	+	+	+	+	+	+	+
D-lactose	+	+	+	+	+	+	+	+	+	+
D-melibiose	–	–	–	–	+	+	–	+	+	+
D-saccharose	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+
Inuline	+	+	+	+	+	+	+	+	–	–
D-melezitose	–	–	–	–	+	+	+	+	+	+
D-raffinose	–	–	–	–	+	+	–	+	+	+
Amidon	–	–	–	–	w	w	–	w	–	–
Glycogen	–	–	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–	–	–
Gentiobiose	w	+	w	+	–	w	w	w	–	w
D-turanose	+	+	+	+	+	+	+	+	+	+
D-lyxose	–	–	–	–	–	–	–	–	–	w
D-tagatose	+	+	+	+	w	w	+	+	+	+
D-fucose	–	–	–	–	–	–	–	–	–	–
L-fucose	–	–	–	–	–	–	–	–	–	–
D-arabitol	–	–	–	–	–	–	–	–	–	–
L-arabitol	–	–	–	–	–	–	–	–	–	–
Potassium gluconate	w	w	w	w	w	w	w	w	w	w
Potassium 2-ketogluconate	–	–	–	–	–	–	–	–	–	–
Potassium 5-ketogluconate	–	–	–	–	–	–	–	–	–	–

+ positive reaction; – negative reaction; w – weakly positive reaction; C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates; D23 – donkey milk isolate

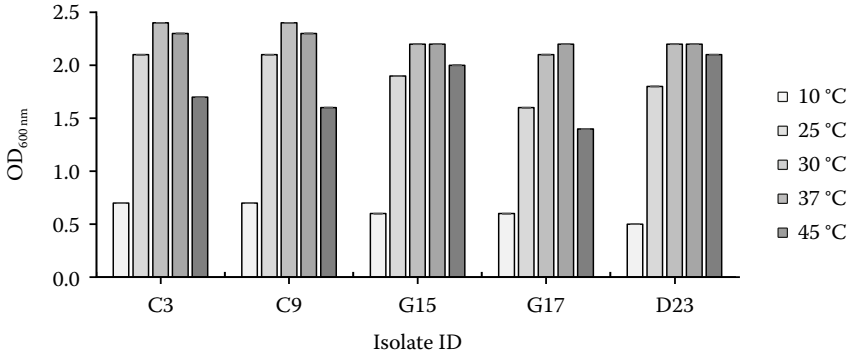

Figure 2. The growth of isolates in MRS broth at different concentration of NaCl (w/v)
MRS – De Man–Rogosa Sharpe; OD – optical density; C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates; D23 – donkey milk isolate

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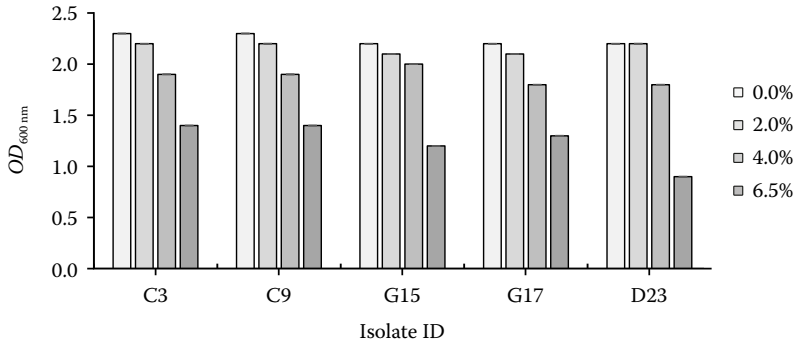

Figure 3. The growth of isolates in MRS broth at different concentration of NaCl (w/v)
MRS – De Man–Rogosa–Sharpe; OD – optical density; C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates; D23 – donkey milk isolate

lytic activity contributes to the development of specific desirable aroma and cheese teixture (Monfredini et al. 2012). Stefanovic et al. (2018) reported that the addition of chosen *L. paracasei* adjuncts cultures did not affect the gross composition or levels of lipolysis of the short-aged cheddar cheeses, but significantly increased proteolysis during ripening.

It has been shown that starters with increased autolytic abilities can accelerate proteolysis. In the manufacture of rennet-coagulated cheese, autolysis is a rate-limiting step for ripening (Collins et al. 2003; Zhao et al. 2023). Nezhad et al. (2020) described that the strains with autolysis rates between 25% and 65% had good autolytic properties. In our study, donkey milk isolate D23 showed interesting autolytic activity (>25%), but the highest one was observed for bovine milk isolates C3 and C9 (Table 3).

The acidifying activity of isolates was performed during 14-day incubation of isolates in UHT bovine milk. Despite different growth rates of isolates in bovine milk, as is designed in Figure 4A, their acidifying capacity was approximately the same (Figure 4B). Even donkey milk isolate D23 was able to growth and acidify UHT bovine milk. Rapid acidification potential is im-

Table 3. Enzymatic activity profile of isolates according to the API-ZYM system kit

Enzyme	Isolate ID				
	C3	C9	G15	G17	D23
Control	0	0	0	0	0
Alkaline phosphatase	2	2	2	3	2
Esterase (C4)	3	3	3	2	3
Esterase lipase (C8)	1	1	3	2	3
Lipase (C14)	0	0	0	0	0
Leu-arylamidase	5	5	5	5	5
Val-arylamidase	5	5	5	5	5
Cys-arylamidase	3	2	4	3	2
Trypsin	1	1	0	0	0
α-chymotrypsin	4	4	1	3	0
Acid phosphatase	5	5	4	5	4
Naphthol-A5-BI-phosphohydrolase	5	5	3	5	4
α-galactosidase	0	0	5	0	4
β-galactosidase	5	5	5	5	5
β-glucuronidase	0	0	0	0	0
α-glucosidase	5	5	4	5	3
β-glucosidase	4	4	1	3	0
N-acetyl-β-glucosaminidase	3	3	0	2	0
α-mannosidase	0	0	0	0	0
α-fucosidase	0	0	0	1	0

0, 1, 2 – negative reaction; 3, 4, 5 – positive reaction; C3, C9 – bovine milk isolates; G15, G17 – goat milk isolates;

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portant in the production of fermented foods, where lower pH values are desired not only for enhancing organoleptic properties but also to prevent the growth of pathogenic microorganisms (Salvucci et al. 2016).

Diacetyl production is an important attribute of starter cultures because it creates the desired flavor and buttery aroma in fermented dairy products (Ferrari et al. 2016). The most pronounced diacetyl production, as assessed through the intensity of the red ring formed after cultivation in milk, was demonstrated by isolate G17 followed by isolates C3, C9, all three with moderate intensity of ring staining. Fine red ring formation and thus weaker diacetyl production was observed in samples G15 and D23 (Table 4).

Antimicrobial properties of isolates

Antibacterial and antifungal activity of isolates was performed by using disc difusion method on the basis of capacity of isolates to suppress the growth of indicator bacteria such as *Staphylococcus aureus* CCM 3953, *Escherichia coli* CCM 3988, *Listeria monocytogenes* NCTC 4886, *Enterococcus hirae* CCM 2423 and *Pseudomonas aeruginosa* CCM 3955 and indicator yeasts (*Candida albicans* CCM 8186 and *C. parapsilosis* CCM 8260) and microscopic fungi (*Aspergillus flavus* CCM F-171, *Mucor racemosus* CCM 8190, *Rhisopus oryzae* CCM F-8284 and *Penicillium purpurogenum* CCM F-257). The degree of growth supression (expressed as the mean diameter of microbial growth inhibition zone ± standard deviation) of indicator bacteria and indicator yeasts / microscopic fungy is listed in Table 5 and 6, respectively. Donkey' s milk isolate D23 showed the greatest antibacterial potential against tested indicator bacteria, especially against *S. aureus* CCM 3953 and *E. coli* CCM 3988 and against microscopic fungi *M. racemosus* CCM 8190. On the other hand, goat' s milk isolate G15 showed the greatest antifungal potential against the rest 5 indicator yeasts / microscopic fungi.

Antimicrobial properties of lactobacilli are, in general, attributed to their metabolic production concerning lactic and phenyllactic acid and bacteriocins (Ye et al. 2021). Our isolates seem therefore very interesting in terms of food safety because of their ability to preclude the growth of food-borne pathogens.

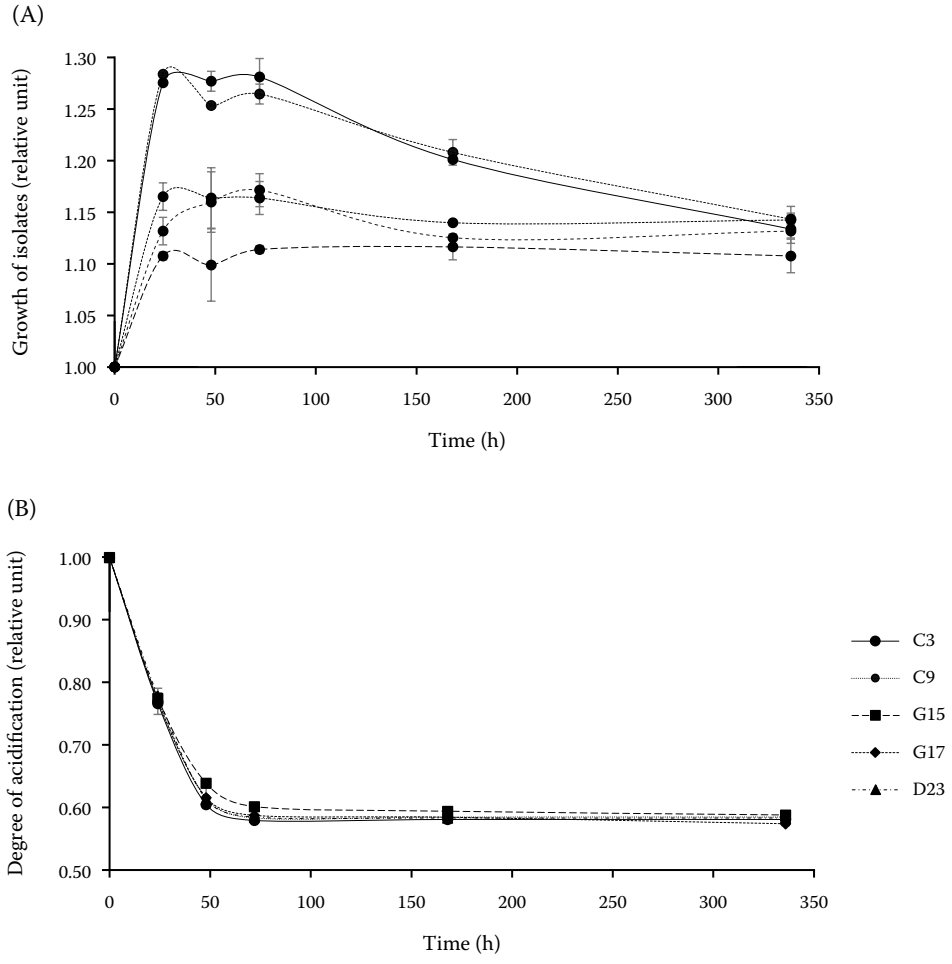

Figure 4. (A) The growth of isolates in UHT milk and (B) the degree of acidification expressed in relative unites extrapolated to values measuret at $t = 0$; UHT – ultra high temperature

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Table 5. Antibacterial activity of isolates

Isolate ID	Inhibition zone (mm)				
	Escherichia coli CCM 3988	*Pseudonomas aeruginosa* CCM 3955	*Enterococcus hirae* CCM 2423	*Listeria monocytogenes* NCTC 4886	*Staphylococcus aureus* CCM 3953
C3	32.7 ± 2.2^{b}	32.2 ± 2.5^{ab}	32.5 ± 2.1^{a}	20.8 ± 1.5^{a}	33.8 ± 2.8^{bc}
C9	32.7 ± 0.9^{b}	29.7 ± 1.3^{a}	31.8 ± 1.3^{a}	22.3 ± 0.9^{a}	31.5 ± 1.7^{b}
G15	26.5 ± 1.3^{a}	35.0 ± 1.4^{b}	32.8 ± 2.2^{a}	18.5 ± 1.3^{a}	22.7 ± 1.7^{a}
G17	30.3 ± 1.9^{ab}	35.0 ± 3.2^{b}	33.5 ± 1.3^{a}	19.5 ± 0.6^{a}	36.5 ± 1.3^{c}
D23	40.5 ± 2.4^{c}	35.8 ± 0.9^{b}	33.0 ± 2.2^{a}	29.5 ± 1.7^{b}	47.8 ± 3.5^{d}

$^{a–d}$Mean values superscribed with different letters in the same column are significantly different ($P < 0.05$); data are expressed as mean ± SD (standard deviation)

Safety characteristics of isolates

Hemolytic activity is considered as virulence factor of pathogenic bacteria. Non of tested isolates exhibited hemolytic activity while cultivated on Columbia blood agar (5% sheep blood v/v). These findings indicated that all isolates were safe. Some *L. paracasei* isolates can exhibit α-hemolytic activity (green-hued zones around colonies), which might pose an opportunistic pathogenic risk (M'hamed et al. 2022).

Decarboxylase activity together with deaminase activity of lactic acid bacteria represent very effective strategy to survive the strong selective pressure of the acidic environment during cheese ripening. It is very important technological property during cheese repening, however, it can result in production of various unfavourable toxic product, such as biogenic amine histamine, tyramine, cadaverine, putrescin (Zuljan et al. 2016). Non of tested isolates showed the ability to produce histamine, cadaverine and fenylethylamine. Only donkey' s milk isolate D23 together with goat's milk isolates G15 and G17 were able to decarboxylate amine acid ornithine. This decarboxylation pathway often leads to putrescine and carbon dioxide production (Zuljan et al. 2016).

CONCLUSION

Raw donkey milk isolate D23, identified as *L. paracasei*, showed very interesting technological and antimicrobial properties compairing to bovine and goat milk isolates. These factors allow us to postulate isolate D23 as a potential starter culture for fermented dairy products, and its antimicrobial properties could be used to improve the safety of the final products.

Table 6. Screening of antifungal assays

Isolate ID	Inhibition zone (mm)					
	Candida albicans CCM 8186	*Candida parapsilosis* CCM 8260	*Aspergillus flavus* CCM F-171	*Mucor racemosus* CCM 8190	*Penicillium purpurogenum* CCM F-257	*Rhisopus oryzae* CCM F-8284
C3	31.5 ± 1.3^{b}	49.5 ± 1.0^{b}	23.0 ± 1.8^{ac}	29.0 ± 5.4^{bcd}	31.8 ± 2.4^{b}	24.3 ± 5.1^{a}
C9	34.5 ± 3.1^{b}	50.0 ± 0.0^{bc}	23.0 ± 0.8^{ab}	21.3 ± 1.5^{a}	26.0 ± 2.5^{a}	27.5 ± 1.3^{a}
G15	40.0 ± 1.4^{c}	55.0 ± 0.0^{cd}	29.0 ± 3.6^{d}	31.8 ± 0.5^{cd}	38.8 ± 1.0^{c}	47.0 ± 0.8^{c}
G17	23.0 ± 1.4^{a}	37.5 ± 2.9^{a}	22.0 ± 1.8^{a}	25.5 ± 0.6^{ab}	33.5 ± 2.4^{bc}	36.8 ± 1.3^{b}
D23	33.8 ± 1.3^{b}	54.0 ± 1.2^{bd}	27.5 ± 5.3^{bcd}	32.3 ± 1.3^{d}	35.8 ± 1.0^{bc}	41.3 ± 2.1^{b}

$^{a–d}$Mean values superscribed with different letters in the same column are significantly different ($P < 0.05$); data are expressed as mean ± SD (standard deviation)

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