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An investigation on probiotic properties, technological characteristics and safety evaluation of yoghurt bacteria

Haktan AKTAŞ[1] · **Bülent ÇETİN**[1]

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Abstract

Probiotics are highly important for human health and can be taken into the body through fermented products. However, many yoghurt bacteria are not probiotics. Moreover, the use of a limited number of bacteria in yoghurt production and the use of industrial yoghurts as starter cultures a cause decrease in the diversity of these bacteria. For these reasons, it is very important to isolate and protect probiotic yoghurt bacteria, and the present study is among the first important reports in which the probiotic properties of an enormous number of yoghurt bacteria have been extensively investigated. In this study, the probiotic properties of 439 yoghurt bacteria were investigated. Only nine isolates could tolerate bile salts, and the isolates were selected for further use as probiotic candidates. Analyses such as molecular and phenotypic identification, probiotic/technological property determination, and safety evaluation were performed on the 9 presumptive probiotic yoghurt isolates. *Lactobacillus delbrueckii* subsp. *bulgaricus* 239 M exhibited notable characteristics, such as tolerance to simulated gastric juice and strong auto/co-aggregation. Strain 239 M demonstrated a moderate hydrophobicity of 31.3%, which was higher than that of most isolates but still lower than the highest value observed (43.7%). Additionally, BSH activity and cholesterol assimilation were not detected in strain 239 M, indicating limitations in bile metabolism and cholesterol-lowering potential. While 239 M exhibits certain probiotic characteristics, it lacks functionality in key traits, such as BSH activity and cholesterol assimilation. Therefore, it represents a promising probiotic candidate, and further in vivo experiments are needed to determine its overall effectiveness.

Keywords *Lactobacillus delbrueckii* subsp. *bulgaricus* · Safety evaluation · *Streptococcus thermophilus* · Probiotic · Technological property

Abbreviations

ABTS	2-2'-Azino-di-3-ethylbenzthiazoline-6-sulphonic acid
ALUM	Research Laboratory Application and Research Centre
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BSH	Bile salt hydrolase
CFS	Cell free supernatant
CFU	Colony-forming unit
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EFSA	European Food Safety Authority
EPS	Exopolysaccharide
FAO	Food and Agriculture Organization
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
L. casei	*Lacticaseibacilluscasei*
L. delbrueckii subsp. *bulgaricus*	*Lactobacillus delbrueckii* Subsp. *bulgaricus*
L. monocytogenes	*Listeria monocytogenes*

✉ Bülent ÇETİN
bcetin@atauni.edu.tr

[1] Department of Food Engineering, Faculty of Agriculture, Ataturk University, Erzurum, Türkiye

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LA	Lactic acid
LAB	Lactic acid bacteria
MAR	Multiple antibiotic resistance
MEGA	Molecular Evolutionary Genetics Analysis
µg	Microgram
mL	Millilitre
µL	Microliter
mm	Millimetre
MRS	De Man, Rogosa and Sharpe
N	Normalite
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
nm	Nanometer
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal RNA
RSM	Reconstituted skim milk
S. aureus	*Staphylococcus aureus*
S. thermophilus	*Streptococcusthermophiles*
S. Typhimurium	*Salmonella* Typhimurium
WHO	World Health Organization

Introduction

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms that have beneficial health effects on the host. In accordance with the"Guidelines for the evaluation of probiotics in food", many tests, such as identification (molecular and phenotypic), safety evaluation (antibiotic resistance, virulence factor, haemolytic activity, etc.), functional characterisation (tolerance to bile salts and gastric juice, adhesion to gut epithelial cells, antagonistic activity, etc.), animal experiments, and human experiments, should be performed to determine the probiotic properties of microorganisms (FAO/WHO 2002). Since probiotics are important for human health, many commercial products containing probiotic microorganisms are available (Liang et al. 2023). On the other hand, probiotics can be taken into the body naturally through fermented foods. Moreover, fermented dairy products are an important probiotic source because starter and/or protective cultures are used in production (Kariyawasam et al. 2021).

Interest in plant-based fermented products has increased significantly in recent years because of factors such as greater awareness of dietary requirements, a focus on sustainability, and demand for functional dairy-free foods. These plant-based products, which are usually made from hazelnuts, almonds, cashews or walnuts, are fermented via lactic acid bacteria (LAB). They are currently the subject of active research into their probiotic potential and nutritional properties (Bernat et al. 2015; Meral-Aktaş et al. 2025). Despite interest in these alternatives, traditional cow milk yoghurt remains the most studied and widely consumed fermented product globally, primarily owing to its proven health benefits (Tamime and Robinson 2007). Yoghurt has positive effects on health, such as reducing allergies, increasing the synthesis of antimicrobial proteins in the body, controlling weight, and regulating blood pressure; *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delbrueckii* subsp. *bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*) are used as starter cultures (Fernandez et al. 2017). Unfortunately, the probiotic properties of yoghurt bacteria are weak (Sarkar 2019). For this reason, many studies and commercial products in which some probiotic LAB other than yoghurt bacteria are added to yoghurt exist (Li et al. 2022). On the other hand, the diversity of yoghurt bacteria is decreasing/disappearing day by day, mainly due to the repetitive use of a limited number of starter cultures (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*), which dominate the fermentation environment and suppress the growth of naturally occurring, potentially beneficial strains (Leroy and De Vuyst 2004). Furthermore, the use of commercial industrial yoghurt as a starter culture in home or artisanal production may lead to the proliferation of industrial strains, reducing diversity over time. This decrease/disappearance highlights the need for the isolation and preservation of probiotic yoghurt bacteria (Aktaş et al. 2022; Kayacan Çakmakoğlu et al. 2022).

The aim of this study was to investigate the probiotic properties of 439 yoghurt bacteria isolated from 119 yoghurt samples collected from 20 different provinces of Türkiye in a previous study by Aktaş and Çetin (2024). Analyses such as molecular (16S-rRNA PCR and species-specific primers) and phenotypic identification (growth at different temperatures, pH values and NaCl concentrations**,** arginine hydrolysis, CO_2 and H_2O_2 production), probiotic (tolerance to bile salts and simulated gastric juice, antagonistic activity, auto/co-aggregation, hydrophobicity, cholesterol assimilation and bile salt hydrolase activity) and technological properties (acidification, EPS production, proteolytic and lipolytic activity, lactic acid production), and safety evaluations (antibiotic resistance, virulence factor and haemolytic and DNase activity) were performed to determine the probiotic properties of the isolates. Thus, this study aimed to detect presumptive probiotic yoghurt bacteria that could be used in yoghurt production. Furthermore, this study is among the important reports in which the probiotic properties of an enormous number of yoghurt bacteria have been extensively investigated.

Materials and methods

Strains and media

In a previous study by Aktaş and Çetin (2024), the acid-producing ability of 439 yoghurt bacteria from traditional and industrial yoghurt samples was investigated, and the techno-functional properties of the isolates with rapid acid-producing ability were examined. In the present study, the probiotic properties, technological characteristics and safety evaluation of isolates (presumptive probiotic strains) selected from a total of 439 yoghurt bacteria, including 223 *L. delbrueckii* subsp. *bulgaricus* and 216 *S. thermophilus*, according to their tolerance to bile salts were investigated to determine presumptive probiotic yoghurt strains. De Man-Rogosa-Sharpe (MRS, Merck, Darmstadt, Germany) and M17 media (Merck) were used for revival of the lactic bacilli and lactic cocci isolates stored in 40% glycerol at −80 ℃, respectively. Lactic bacilli isolates were incubated anaerobically at 37 ℃ for 48–72 h, and lactic cocci isolates were incubated aerobically at 37 ℃ for 24 h. *Lacticaseibacillus casei* (*L. casei*) ATCC 393 was used as a positive control for tolerance assays to bile salts and simulated gastric juice. *Enterococcus faecalis* (*E. faecalis*) ATCC 29212 was used as a positive control for bile salt hydrolase (BSH) activity. In addition, *Escherichia coli* (*E. coli*) O157:H7, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 9027, *Salmonella* Typhimurium (*S.* Typhimurium) RSHMB 95091, *Staphylococcus aureus* (*S. aureus*) ATCC 29213 and *Listeria monocytogenes* (*L. monocytogenes*) ATCC 7644 were used to determine the antagonistic activity and co-aggregation of the isolates. The pathogens were incubated on brain heart infusion agar (BHI Agar, Merck) at 37 ℃ for 24 h.

Tolerance to bile salts

A tolerance assay for bile salts was used as the first criterion for the selection of yoghurt bacteria. Therefore, all the isolates were subjected to analysis. Fresh cultures of the 439 isolates were suspended in phosphate buffered saline (PBS, pH 7.2) at a concentration of 10^8 CFU mL^{-1}. The bacterial suspensions (1%) were inoculated into MRS or M17 broth (Merck) with 0.3% bile salts (Merck), and percent survival was determined by viable cell count at 12 and 24 h on MRS or M17 agar (Merck) via the serial dilution method (Kumari et al. 2020). For growth abilities of the isolates in bile salts, the suspensions were inoculated into MRS or M17 broth (Merck) containing 0.3, 0.5 and 1% bile salts (Merck) and incubated at 37 ℃ for 24 h. Microbial growth was detected spectrophotometrically by a plate reader (Epoch, BioTek, Santa Clara, CA 95051, United States) at 600 nm. The growth of the isolates in bile salts was determined via the following formula (Salehizadeh et al. 2020):

$$C(\%) = \left[\frac{\triangle(T_{24} - T_0)_{Treatment}}{\triangle(T_{24} - T_0)_{Control}}\right] \times 100$$

C: Growth at bile salts.

$\Delta(T_{24}—T_0)_{Treatment}$: Difference between absorbance of isolate in media with bile salts and absorbance of the media without inoculation.

$\Delta(T_{24}—T_0)_{Control}$: Difference between absorbance of isolate in media without bile salts and absorbance of the media without inoculation.

Molecular identification

Twenty-seven F (27F) and 1541R primers were used for the identification of the yoghurt isolates (Table S1). DNA extraction was carried out according to the methods of Singh and Ramesh (Singh and Ramesh 2009). After 16S-rRNA PCR, sequencing was performed via an ABI 3130 XL genetic analyser (Applied Biosystems, Foster City, CA). DNA sequences were evaluated with the BLAST program in the National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). In addition, PCR with the species-specific primers, delF188-delR1042 for *L. delbrueckii* subsp. *bulgaricus* and P1-P2 for *S. thermophilus* (Table S1), was also performed on the isolates (Kayacan Çakmakoğlu et al. 2022).

Phenotypic characterisation

The growth of the yoghurt isolates at different temperatures (4, 10, 15 and 45 ℃), pH values (3.0, 4.0 and 5.0) and NaCl concentrations (1, 4, 6.5, and 10%) was detected spectrophotometrically by a plate reader (Epoch, BioTek) at 600 nm (Papamanoli et al. 2003). Arginine hydrolysis and CO_2 production by the isolates were determined according to the method of Drosinos et al. (2007) via Nessler's reagent (Merck) and Durham tubes, respectively. H_2O_2 production was investigated on ABTS agar (2–2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid). ABTS agar was incubated at 37 ℃ for 24 h. Then, the colonies were exposed to atmospheric oxygen for 24 h, and the purple zone around the colonies was evaluated as positive (Bhanwar et al. 2014).

Probiotic properties of strains

First, the tolerance of the yoghurt isolates to bile salts was investigated. According to the results, isolates that could

tolerate at least 0.3% bile salts were considered probiotic yoghurt bacteria. The following tests were performed on the genetically identified and phenotypically characterised presumptive probiotic yoghurt bacteria.

Tolerance to simulated gastric juice

PBS containing 0.3% pepsin and 0.5% NaCl adjusted to pH 3 with 0.1 N HCl (Merck) was used as the simulated gastric juice. The bacterial suspensions inoculated into the gastric juice were incubated at 37 ℃ for 3 h. Viable cell counts were determined via serial dilution method at the 2nd and 3rd h of incubation. The tolerance of the yoghurt isolates to simulated gastric juice was determined via the following formula (Talebi et al. 2018):

$$P(\%) = \left[\frac{log(NT)}{log(N_0)}\right] \times 100$$

P: Tolerance to simulated gastric juice.
NT: Viable cell counts at 2nd or 3rd h in simulated gastric juice.
N_0: Viable cell counts added to simulated gastric juice.

Antagonistic activity

The antagonistic activities against *E. coli* O157:H7, *P. aeruginos* ATCC 9027, *S.* Typhimurium RSHMB 95091, *S. aureus* ATCC 29213, and *L. monocytogenes* ATCC 7644 were investigated via the well diffusion method. Moreover, pH adjustment (pH 6–7), enzyme (proteinase K, pepsin and trypsin), and temperature treatment (80 ℃ for 10 min) were performed on cell-free supernatant (CFS) to detect the source of antagonistic activity. The antagonistic activities of the isolates were determined according to the zone diameters around the wells (Gutiérrez-Cortés et al. 2018).

Auto/co-aggregation and hydrophobicity

The bacterial suspensions in PBS (10^7–10^8 CFU mL^{-1}) were vortexed and stored at 37 ℃ for the auto-aggregation experiments. At 2 and 4 h, the absorbance of the liquid phase, the upper part of the suspension, was detected by a plate reader (Epoch, BioTek) at 600 nm. Auto-aggregation was calculated via the following formula (Seddik et al. 2017):

$$Auto - aggregation(\%) = \left[1 - \left(\frac{A_t}{A_0}\right)\right] \times 100$$

A_t: Absorbance values for 2nd and 4th h.
A_0: Absorbance value for initial.

For co-aggregation of the isolates on *E. coli* O157:H7, *S.* Typhimurium RSHMB 95091, *S. aureus* ATCC 29213, and *L. monocytogenes* ATCC 7644, 2 mL each of pathogen and yoghurt isolate suspensions were mixed by vortexing for 30 s. The mixtures were incubated at 37 ℃ for 2 h. Then, the absorbance of the liquid phase, upper part of the suspension, was detected by a plate reader (Epoch, BioTek) at 600 nm. Co-aggregation was calculated via the following formula (Talebi et al. 2018):

$$Co - aggregation(\%) = \left[\frac{\frac{(Ax+Ay)}{2} - A(x+y)}{\frac{(Ax+Ay)}{2}}\right] \times 100$$

Ax and Ay: Absorbance value for initial (isolates and pathogens).

A(x + y): Absorbance value of the mixture after 2 h.

Three millilitres of the bacterial suspensions in PBS (10^7–10^8 CFU mL^{-1}) were mixed with 1 mL of xylene (Merck), and the mixture was homogenized by vortexing for 2 min for the hydrophobicity experiment. Then, it was held for phase separation at 25 ℃. The absorbance of the liquid phase was detected by a plate reader (Epoch, BioTek) at 600 nm. The hydrophobicity was calculated via the following formula (Seddik et al. 2017):

$$Hydrophobicity(\%) = \left[1 - \left(\frac{A_1}{A_0}\right)\right] \times 100$$

A_0: Absorbance value for initial.
A_1: Absorbance of liquid phase after phase separation.

Bile salt hydrolase activity

BSH activity was detected according to the methods of Byakika et al. (2020). Briefly, 10 µL of bacterial suspensions were spotted on Bile Esculine Agar (Condalab, Madrid, Spain) and incubated at 37 ℃ for 24 h. Dark brown colonies were evaluated as positive, and BSH activity was classified as low (≤ 10 mm), medium (11–15 mm) or high (≥ 16 mm). *E. faecalis* ATCC 29212 was used as a positive control.

Cholesterol assimilation

Cholesterol assimilation by the yoghurt isolates was determined colorimetrically via a plate reader (Epoch, BioTek) at 570 nm. The bacterial suspensions were inoculated into MRS or M17 broth (Merck) containing 0.2% bile salts and 1 µg cholesterol μL^{-1}. The mixtures were incubated at 37 ℃ for 24 h and centrifuged at 10,000 × g for 10 min. The amount of cholesterol assimilated by the isolates was detected via the supernatant with a cholesterol quantitation kit (Sigma Aldrich Co., St. Louis, MO, USA). MRS and M17 broth (Merck) were used as negative controls (Awasti et al. 2016).

Technological properties of strains

Acidification

Acidification of the yoghurt isolates was determined according to the methods of Tavsanli et al. (2021), with slight modifications. The bacterial suspension was inoculated at 1% to reconstituted skim milk (RSM), and the RSM was incubated at 42 ℃ for 8 h. The pH of the RSM was measured by a pH-meter (Hanna pH-211, Hanna Instruments, Inc., Woonsocket, RI, USA) at the 4th, 6th and 8th h of incubation.

Exopolysaccharide production

Exopolysaccharide (EPS) production genes in the isolates were investigated with epsA, epsB, epsD/E, and gtf primers (Table S1). DNA extraction was carried out according to the methods of Singh and Ramesh (2009), and the PCR mixture consisted of 12.5 µL of master mix (Ampliqon, Odense M, Danimarka), 1 µL of template DNA, 1 µL of primers, and 10.5 µL of nuclease-free water. The PCR conditions were as follows: initial denaturation at 95 ℃ for 15 min; 40 cycles of 95 ℃ for 30 s, at annealing for 30 s, and 72 ℃ for 30 s (Dertli et al. 2016).

Proteolytic and lipolytic activity

The proteolytic and lipolytic activities of the yoghurt isolates were determined via MRS or M17 agar (Merck) containing skim milk and tributyrin agar, respectively (Musikasang et al. 2009). After incubation at 37 ℃ for 24 h, zones larger than 1 mm were considered positive. Trypsin and *S. aureus* ATCC 29213 were used as positive controls for proteolytic and lipolytic activity, respectively.

Lactic acid production

Yoghurt isolates were incubated in MRS or M17 broth (Merck) at 37 ℃ for 36 h for lactic acid (LA) production. The mixture of broth and distilled water (1:1) was held at 30 ℃ for 30 min. After the mixture was centrifuged at 10,000 × g for 5 min, the supernatant was used for detection of LA via high-performance liquid chromatography (HPLC). Separation was carried out with metaphosphoric acid and acetonitrile (0.8 mL min^{-1}) by a C18 reversed phase column (250 × 4.6 mm, 5 mm in particle size) at 210 nm (Özcelik et al. 2016). The analysis was carried out at the Research Laboratory Application and Research Centre (ALUM), Iğdır University.

Safety evaluation of strains

Antibiotic resistance

Antibiotic resistance to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol, as recommended by the European Food Safety Authority (EFSA), was determined using the disc diffusion method. After incubation at 37 ℃ for 24 h, according to the zone diameter, the isolates were classified as resistant (≤ 15 mm), intermediate (16–20 mm), or susceptible (≥ 21 mm) (Sarkar et al. 2020). In addition, the multiple antibiotic resistance (MAR) index of the isolates was calculated via the following formula:

$$MAR = \frac{a}{b}$$

MAR: Multiple antibiotic resistance index.
a: The number of antibiotics to which the isolate is resistant.
b: The number of antibiotics used in this study.

Virulence factors

The virulence factors of the isolates were investigated via the use of the primers cylA, HYLn, ASA1, and ACE (Table S1) (Vankerckhoven et al. 2004; Martín-Platero et al. 2009; Padilla and Lobos 2013). DNA extraction was carried out according to the methods of Singh and Ramesh (2009). The PCR mixture and conditions were the same as those described in the "Exopolysaccharide production" section.

Haemolytic and DNase activity

Blood agar (Borah et al. 2016) and DNase agar (Merck) (Domingos-Lopes et al. 2017) were used for haemolytic and DNase activity, respectively. The isolates were spread on media, and petri dishes were incubated at 37 ℃ for 24 h for haemolytic activity and at 37 ℃ for 48 h for DNase activity. *S. aureus* ATCC 29213 was used as a positive control for activity.

Statistical analysis

Statistical analyses of the data were carried out via the SPSS version 20.0 package (SPSS Inc., Chicago, IL, USA). One-Way Analysis of variance (ANOVA) was performed for statistical comparisons of more than two groups, and significant differences were determined using Duncan test at $p < 0.05$ significance level. The phylogenetic tree was

created using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007).

Results and discussion

Tolerance to bile salts and simulated gastric juice

Bile salts and gastric juice are important for human digestion and may cause the death of microorganisms (de Melo Pereira et al. 2018). Importantly, probiotic microorganisms can tolerate bile salts and gastric juice, the two most important probiotic tests according to the FAO/WHO. In this study, only nine isolates were able to tolerate bile salts (Table S2 and Fig. 1). In addition, the 208 M and *L. casei* 393 strains tolerated bile salts more than the other isolates did at 12 h, whereas 560 M strain tolerated bile salts at 24 h ($p < 0.05$). Moreover, the 239 M isolate had the greatest growth after *L. casei* ATCC 393, the positive control, at 0.5% bile salts ($p < 0.05$). The 9 isolates could be probiotic candidates,

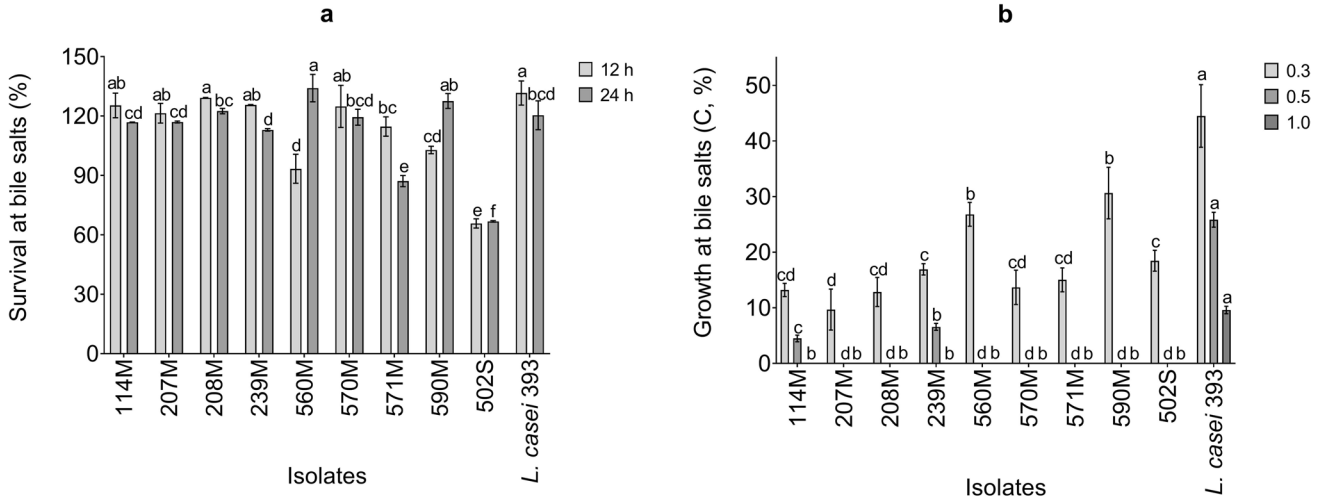


Fig. 1 Tolerance to 0.3% bile salts (**a**) and growth at 0.3, 0.5, and 1.0% bile salts (**b**) of yoghurt isolates (a-f shows statistical differences in the same incubation time or same bile salts concentration)

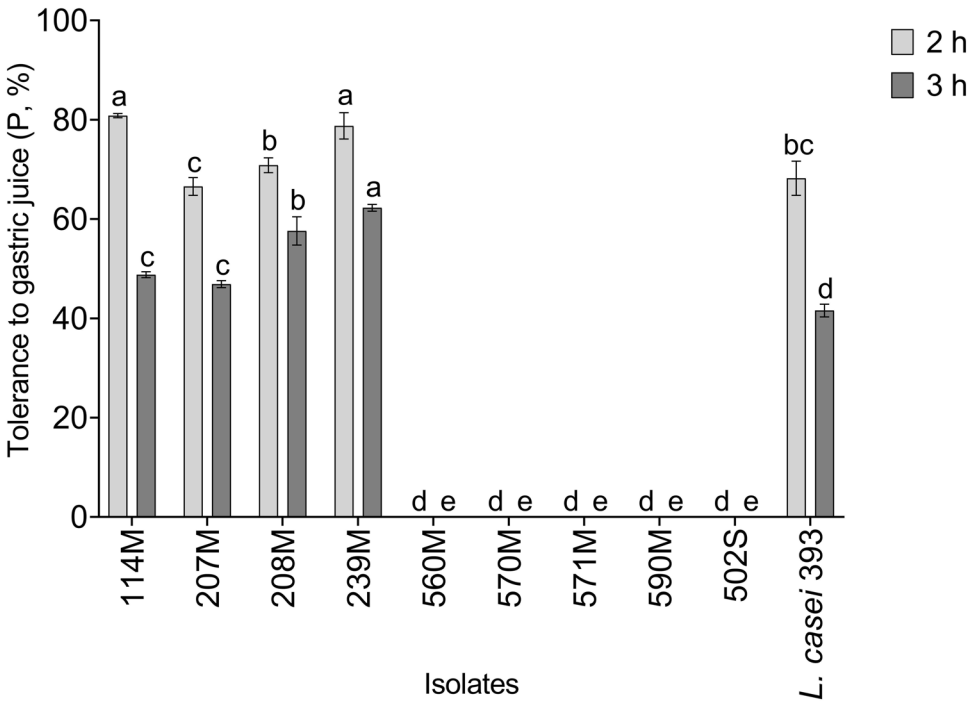


Fig. 2 Tolerance of presumptive probiotic yoghurt isolates to simulated gastric juice (a-e shows statistical differences in the same hour)

and all further analyses were performed on the 9 isolates. The tolerance of the 9 isolates to simulated gastric juice are given in Table S2 and Fig. 2. Among all the isolates, only the 114 M, 207 M, 208 M and 239 M isolates were able to survive in simulated gastric juice, and the 239 M isolate had the highest tolerance to simulated gastric juice ($p < 0.05$). In addition, these isolates survived longer than *L. casei* ATCC 393 did at 3 h in simulated gastric juice ($p < 0.05$). Similarly, yoghurt bacteria have weak probiotic properties (Sarkar 2019), and lactic bacilli are more resistant than lactic cocci are to gastrointestinal conditions (Pereira and Gibson 2002). Therefore, the results obtained from the present study were similar to those of other studies (Asan-Ozusaglam and Gunyakti 2022).

Molecular identification

Molecular identification revealed that the 114 M, 207 M, 208 M, 239 M, 560 M, 570 M, 571 M, and 590 M isolates were *L. delbrueckii* subsp. *bulgaricus*, with 98.26–100% similarity rates. On the other hand, the 502S isolate was identified as *S. thermophilus* with a 100% similarity rate (Table S3 and Fig. S1). In addition, PCR using species-specific primers confirmed these results (Fig. S2).

Phenotypic characterisation

The results of the phenotypic characterisation of the 9 presumptive probiotic yoghurt isolates are given in Table S4. All the isolates grew at 45 ℃, whereas no isolates grew at 4, 10, or 15 ℃ (with the exception of *S. thermophilus* 502S at 15 ℃). Since yoghurt bacteria are thermophilic (Hajimohammadi et al. 2016), all the isolates were expected to grow at 45 ℃, as in the study by Bhattarai et al. (2016). On the other hand, all the isolates grew at pH 5, but only the *L. delbrueckii* subsp. *bulgaricus* 207 M, 208 M, and 239 M isolates developed at pH 4. Bhattarai et al. (2016) reported that *L. delbrueckii* subsp. *bulgaricus* isolates developed at pH values of 4.50 and 6.50, whereas *S. thermophilus* isolates grew only at pH 6.50. Aktaş et al. (2022) reported that only 5 *L. delbrueckii* subsp. *bulgaricus* and 2 *S. thermophilus* strains grew at pH 3.90. Finally, Prabhurajeshwar and Chandrakanth (2019) stated that *Lactobacillus* spp. from yoghurt did not grow at pH 3 or 4. In this study, all the isolates grew only at a 1% NaCl concentration, and similar results were reported by Bhattarai et al. (2016) and Prabhurajeshwar and Chandrakanth (2019). No isolates hydrolysed arginine and produced CO_2, and these results are in agreement with other studies by Hajimohammadi Farimani et al. (2016) and Aktaş et al. (2022). On the other hand, *L. delbrueckii* subsp. *bulgaricus* 114 M, 207 M, 208 M, 239 M, 560 M, 571 M, and 590 M isolates produced H_2O_2. Similarly, some studies reported that *L. delbrueckii* subsp. *bulgaricus* is a good producer of H_2O_2, whereas *S. thermophilus* produces slight amounts of H_2O_2 or even no H_2O_2 at all (Beyatli et al. 2007).

Probiotic properties of strains

Antagonistic activity

The *L. delbrueckii* subsp. *bulgaricus* 114 M, 207 M, 208 M, 239 M, and 590 M isolates had antagonistic effects on some of the foodborne pathogens (Table 1). Moreover, *L. delbrueckii* subsp. *bulgaricus* 208 M and 239 M inhibited four different pathogens, including *E. coli* O157:H7, *P. aeruginosa* ATCC 9027, *S.* Typhimurium RSHMB 95091, and *S. aureus* ATCC 29213. In contrast, the *L. delbrueckii* subsp. *bulgaricus* 560 M, 570 M, 571 M, and *S. thermophilus* 502S isolates did not show antagonistic activity. Most of these antagonistic activities were due to pH. On the other hand, the antagonistic effects of *L. delbrueckii* subsp. *bulgaricus* 207 M on *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 29213 could be caused by bacteriocin (Fig. S3). Many studies have shown that *L. delbrueckii* subsp. *bulgaricus* can produce bacteriocin and that bacteriocin has antagonistic activity against pathogens such as *L. monocytogenes* and *S. aureus* (Radha and Padmavathi 2017). These isolates with antagonistic activity can be used as biopreservatives in the food industry to prevent spoilage and food-borne diseases. In addition, antimicrobial peptides produced by probiotics can make it difficult for pathogens to attach/adhere to epithelial cells in the human body (Servin 2004). For these reasons, the use of probiotic and/or starter cultures with antimicrobial activity is very important for both food preservation and human health.

Auto/co-aggregation and hydrophobicity

The auto/co-aggregation and hydrophobicity results of the 9 presumptive probiotic yoghurt isolates are given in Table 2. Auto-aggregation of the isolates ranged 11.3–49.7% at 2 h and 17.5–70.9% at 4 h. *L. delbrueckii* subsp. *bulgaricus* 560 M had the highest auto-aggregation at 2 h, and *L. delbrueckii* subsp. *bulgaricus* 560 M and 239 M had the highest auto-aggregation at 4 h ($p < 0.05$). The isolates had co-aggregation ability in the range of 1.2–22.0% for *E. coli* BC12, 7.0–48.7% for *S.* Typhimurium RSHMB 95091, 1.8–39.1% for *S. aureus* ATCC 29213 and 6.7–36.9% for *L. monocytogenes* ATCC 7644. *L. delbrueckii* subsp. *bulgaricus* 114 M had the highest co-aggregation effect on *E. coli* BC12, *S.* Typhimurium RSHMB 95091 and *L. monocytogenes* ATCC 7644, and *L. delbrueckii* subsp. *bulgaricus* 239 M had the highest co-aggregation effect on *S. aureus* ATCC 29213 ($p < 0.05$). Similarly, Prabhurajeshwar and Chandrakanth (2019) reported that auto-aggregation of *Lactobacillus* isolates from yoghurt varied between 13.0 and 52.0%, and these bacteria can coaggregate with some pathogens.

Table 1 Antagonistic activity of presumptive probiotic yoghurt isolates against pathogens (zone diameter, mm)*

Isolates	Pathogens															
	E. coli O157:H7			*P. aeruginosa* ATCC 9027			*S.* Typhimurium RSHMB 95091			*S. aureus* ATCC 29213			*L. monocytogenes* ATCC 7644		
	P	E	H	P	E	H	P	E	H	P	E	H	P	E	H
L. bulgaricus 114 M	-	-	-	-	-	-	-	12.5 ± 0.6	10.4 ± 0.5	-	17.8 ± 0.9	15.6 ± 0.8	-	-	-
L. bulgaricus 207 M	-	7.5 ± 0.6	6.9 ± 0.3	-	-	-	-	-	-	9.3 ± 0.3	11.8 ± 0.5	13.3 ± 0.0	19.5 ± 0.6	-	19.1 ± 0.3
L. bulgaricus 208 M	-	8.8 ± 0.5	8.8 ± 0.3	-	17.9 ± 1.0	18.4 ± 0.3	-	7.0 ± 0.0	7.3 ± 0.5	-	10.1 ± 0.3	10.4 ± 0.5	-	-	-
L. bulgaricus 239 M	-	7.5 ± 0.6	7.5 ± 0.6	-	14.8 ± 0.5	15.3 ± 0.5	-	12.5 ± 0.6	11.8 ± 0.5	-	16.5 ± 0.6	15.8 ± 1.0	-	-	-
L. bulgaricus 560 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. bulgaricus 570 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. bulgaricus 571 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. bulgaricus 590 M	-	7.0 ± 0.0	6.8 ± 0.5	-	-	-	-	-	-	-	-	-	-	-	-
S. thermophilus 502S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*P: pH adjustment, E: Enzyme treatment, H: Temperature treatment

Auto-aggregation is related to the ability of microorganisms to aggregate and increase cell density in the gut. On the other hand, co-aggregation is considered an aggregation indicator of probiotic microorganisms with pathogens and thus prevents the localization of pathogens to the gut (de Melo Pereira et al. 2018). In this study, the *L. delbrueckii* subsp. *bulgaricus* 114 M, 239 M and 560 M isolates exhibited high auto/co-aggregation. As a result, these isolates may positively affect gut health.

According to the results, *L. delbrueckii* subsp. *bulgaricus* 239 M, 570 M and 502S were hydrophobic at rates of 31.3, 5.7 and 43.7%, respectively. Similarly, Abushelaibi et al. (2017) reported that the hydrophobicity of yoghurt isolates ranged 3.1–57.8%. Since hydrophobicity is an indicator of the ability of probiotic microorganisms to adhere to gut epithelial cells (Collado et al. 2007), these isolates may improve gut health.

Bile salts hydrolase activity

Bile salt deconjugation is highly important for human health because it lowers cholesterol levels (Shehata et al. 2016). Therefore, in this study, the BSH activity of 9 presumptive probiotic yoghurt isolates was examined. No isolates had BSH activity, whereas *E. faecalis* ATCC 29212, a positive control, had high activity (Fig. S4). Similarly, Tanaka et al. (1999) reported that none of 28 *L. delbrueckii* subsp. *bulgaricus* isolates had BSH activity. On the other hand, some studies reported that some probiotic LAB (Miremadi et al. 2014) can have BSH activity.

Cholesterol assimilation

Cholesterol is an important substance for diseases such as cardiovascular and colon cancer (Dilmi-Bouras 2006). Moreover, according to the"Guidelines for the evaluation of probiotics in food"by the FAO/WHO, probiotic properties are directly related to positive effects on health. In this study, the cholesterol assimilation ability of 9 presumptive probiotic yoghurt isolates was investigated to determine the possible positive effects of the isolates on health (Table S5). Only *L. delbrueckii* subsp. *bulgaricus* 560 M and 590 M assimilated cholesterol at rates of 6.24 and 5.23%, respectively. As a result, the cholesterol-lowering effects of these isolates are important for human health. Similarly, the ability of yoghurt isolates to assimilate cholesterol was reported by Dilmi-Bouras (2006).

Technological properties of strains

Acidification

All the isolates significantly decreased the pH of the RSM during the incubation period ($p < 0.05$) (Table 3). Similar

Table 2 Auto/co-aggregation and hydrophobicity of presumptive probiotic yoghurt isolates*

Isolates	Auto-aggregation (%)		Co-aggregation (%)				Hydrophobicity (%)
	2 h	4 h	*E. coli* O157:H7	*S.* Typhimurium RSHMB 95091	*S. aureus* ATCC 29213	*L. monocytogenes* ATCC 7644	
L. bulgaricus 114 M	35.2 ± 1.2^{b}	35.8 ± 1.4^{d}	22.0 ± 1.0^{a}	48.7 ± 0.6^{a}	32.9 ± 0.4^{b}	36.9 ± 2.2^{a}	0.0^{c}
L. bulgaricus 207 M	26.0 ± 1.6^{d}	31.2 ± 0.7^{e}	6.9 ± 0.8^{b}	14.5 ± 1.2^{d}	33.7 ± 2.7^{b}	22.4 ± 0.9^{c}	0.0^{c}
L. bulgaricus 208 M	30.9 ± 0.7^{c}	63.9 ± 2.2^{b}	20.3 ± 0.7^{a}	23.3 ± 1.5^{b}	18.3 ± 1.7^{c}	14.5 ± 0.6^{de}	0.0^{c}
L. bulgaricus 239 M	22.7 ± 1.5^{d}	70.9 ± 1.2^{a}	6.8 ± 1.3^{b}	19.7 ± 1.3^{c}	39.1 ± 1.0^{a}	8.7 ± 1.1^{f}	31.3 ± 8.0^{b}
L. bulgaricus 560 M	49.7 ± 1.8^{a}	70.8 ± 1.4^{a}	3.8 ± 0.4^{c}	7.0 ± 0.1^{e}	1.8 ± 0.1^{f}	8.0 ± 1.0^{f}	0.0^{c}
L. bulgaricus 570 M	11.3 ± 0.7^{f}	26.6 ± 1.7^{f}	8.3 ± 0.7^{b}	13.0 ± 0.7^{d}	13.7 ± 0.6^{d}	16.3 ± 0.8^{d}	5.7 ± 2.2^{c}
L. bulgaricus 571 M	15.2 ± 1.5^{e}	59.2 ± 1.1^{c}	20.7 ± 0.6^{a}	23.0 ± 1.6^{b}	12.8 ± 0.9^{d}	31.2 ± 0.6^{b}	0.0^{c}
L. bulgaricus 590 M	31.5 ± 3.3^{bc}	68.3 ± 1.3^{a}	21.5 ± 0.7^{a}	12.9 ± 1.2^{d}	11.0 ± 0.8^{d}	6.7 ± 0.1^{f}	0.0^{c}
S. thermophilus 502S	15.7 ± 0.8^{e}	17.5 ± 0.5^{g}	1.2 ± 0.5^{d}	15.4 ± 1.4^{d}	6.8 ± 0.5^{e}	13.3 ± 0.7^{e}	43.7 ± 6.7^{a}

*a-g shows statistical differences in the same column ($p < 0.05$)

Table 3 Technological properties of presumptive probiotic yoghurt isolates*

Isolates	Acidification (pH, mean ± SD)			EPS genes				Proteolytic activity	Lipolytic activity	LA production (ppm, mean ± SD)
	4 h	6 h	8 h	*epsA*	*epsB*	*p-gtf*	*gtf*			
L. bulgaricus 114 M	6.17 ± 0.00^{bA}	4.69 ± 0.16^{cB}	4.01 ± 0.01^{dC}	-	-	-	-	-	-	1747.89 ± 21.57^{bc}
L. bulgaricus 207 M	6.58 ± 0.01^{aA}	6.52 ± 0.00^{aA}	6.31 ± 0.08^{aB}	-	-	-	-	-	-	1877.22 ± 1.01^{a}
L. bulgaricus 208 M	6.57 ± 0.01^{aA}	6.48 ± 0.03^{aB}	6.30 ± 0.00^{aC}	-	-	-	-	-	-	1765.28 ± 7.53^{bc}
L. bulgaricus 239 M	6.52 ± 0.00^{aA}	6.27 ± 0.04^{bB}	5.90 ± 0.01^{bC}	-	-	-	-	-	-	1726.16 ± 3.65^{c}
L. bulgaricus 560 M	6.50 ± 0.01^{aA}	6.17 ± 0.09^{bB}	5.93 ± 0.00^{bC}	-	-	-	-	-	-	NA**
L. bulgaricus 570 M	6.58 ± 0.03^{aA}	6.26 ± 0.04^{bB}	5.93 ± 0.02^{bC}	-	-	-	-	-	-	NA**
L. bulgaricus 571 M	6.52 ± 0.01^{aA}	6.24 ± 0.04^{bB}	5.89 ± 0.00^{bC}	-	-	-	-	-	-	NA**
L. bulgaricus 590 M	6.57 ± 0.01^{aA}	6.50 ± 0.01^{aB}	6.29 ± 0.02^{aC}	-	-	-	-	-	-	NA**
S. thermophilus 502S	5.79 ± 0.33^{bA}	4.68 ± 0.09^{cB}	4.35 ± 0.02^{cB}	+	-	-	-	-	-	NA**

*LA: Lactic acid, SD: Standard deviation, NA**: not analysed because of industrial origin. a-d shows statistical differences in the same column ($p < 0.05$), A-C shows statistical differences in the same line ($p < 0.05$)

results were also reported by Tavsanli et al. (2021). *L. delbrueckii* subsp. *bulgaricus* 114 M and *S. thermophilus* 502S produced more acid than the other isolates did at 4 h and 6 h ($p < 0.05$). In addition, *L. delbrueckii* subsp. *bulgaricus* 114 M was the fastest acid-producing isolate at 8 h ($p < 0.05$). In addition, many studies have indicated that acidification is important for yoghurt production and that isolates are selected for production according to acidification (Xu et al. 2015). In this study, it was determined that isolates with rapid acidification could be good starter culture candidates for yoghurt production.

Exopolysaccharide production

EPS are substances that improves the textural properties of fermented products such as yoghurt and kefir. Moreover, EPS are non-digestible carbohydrates and have prebiotic properties (Dertli et al. 2016). In this study, only *S. thermophilus* 502S harboured the *epsA* gene (Table 3 and Fig. S5). Similarly, some researchers have reported that LAB can produce EPS (Dertli et al. 2016). Although the presence of *epsA* indicates the potential for EPS production, the quantity or composition of EPS was not determined in this study. Therefore, no conclusions can be drawn about its impact on product texture or health benefits. Therefore, further studies are needed to determine the EPS yield and structure produced by *S. thermophilus* 502S, as well as to evaluate its potential to improve product quality and promote health.

Proteolytic and lipolytic activity

In this study, no isolates had proteolytic and lipolytic activity (Table 3). Similarly, many researchers have reported that LAB have weak proteolytic and lipolytic activity (Vinderola

et al. 2004; Tavsanli et al. 2021). Therefore, the results from our study are in agreement with those of others. However, more sensitive assays should be performed to determine weak activities.

Lactic acid production

LA is the most important organic acid produced by LAB in fermented products in D (-) and/or L (+) forms. While D (-) LA cannot be fully metabolised in the body and can even be harmful to human health, L (+) LA can be fully metabolised. Isolates producing high amounts of L (+) LA are important (Vodnar et al. 2010). In this study, four isolates produced LA (1726.16–1877.22 ppm) (Table 3). *L. delbrueckii* subsp. *bulgaricus* 207 M had the highest LA production ($p < 0.05$). These isolates may be used as starter cultures in yoghurt production with increased beneficial effects on health.

Safety evaluation of strains

Antibiotic resistance

Currently, uncontrolled antibiotic usage is an important global health problem. The transfer of antibiotic resistance genes to pathogens can lead to adverse effects such as more severe and prolonged diseases (Frieri et al. 2017). The antibiotic resistance profiles of the 9 presumptive probiotic yoghurt isolates are given in Table 4 and Table S6. The *L. delbrueckii* subsp. *bulgaricus* 560 M, 570 M, 571 M and 590 M strains of industrial origin were not resistant to the antibiotics used in this study. Other isolates were resistant to only gentamicin, kanamycin, and streptomycin, and their MAR index ranged 0.22–0.33. Furthermore, the *L. delbrueckii* subsp. *bulgaricus* 239 M strain has attracted attention among the isolates of traditional origin because it is resistant to only gentamicin and kanamycin. Similarly, some researchers have reported that LAB are resistant to kanamycin (Hajimohammadi Farimani et al. 2016). In addition, Wu et al. (2021) found that 17 LAB were resistant to gentamicin, kanamycin, and streptomycin. Owing to these similar results, it is thought that LAB are naturally resistant to gentamicin, kanamycin, and streptomycin. However, according to the EFSA guidelines, phenotypic antibiotic resistance testing alone is not sufficient for the safety assessment of probiotics (EFSA 2018). Therefore, to complement disk diffusion results, the genetic basis of resistance, particularly the presence of transferable antibiotic resistance genes such as those present on plasmids or transposons, should also be investigated. Furthermore, the potential for horizontal gene transfer should be assessed by identifying mobile genetic elements via molecular or whole-genome-based approaches.

Table 4 Antibiotic resistance profiles and MAR index of presumptive probiotic yoghurt isolates*

Isolates	Antibiotic resistance profile	MAR index
L. delbrueckii subsp. *bulgaricus* 114 M	CnKanStr	0.33
L. delbrueckii subsp. *bulgaricus* 207 M	CnKanStr	0.33
L. delbrueckii subsp. *bulgaricus* 208 M	CnKanStr	0.33
L. delbrueckii subsp. *bulgaricus* 239 M	CnKan	0.22
L. delbrueckii subsp. *bulgaricus* 560 M	-	0.00
L. delbrueckii subsp. *bulgaricus* 570 M	-	0.00
L. delbrueckii subsp. *bulgaricus* 571 M	-	0.00
L. delbrueckii subsp. *bulgaricus* 590 M	-	0.00
S. thermophilus 502S	CnKan	0.22

*MAR: Multiple antibiotic resistance, *Cn*: Gentamicin, *Kan*: Kanamycin, *Str*: Streptomycin

Virulence factor

Since virulence genes enable bacteria to overcome host defence barriers, this is an undesirable property for both probiotic and starter cultures (van Reenen and Dicks 2011). Moreover, the FAO/WHO stated in the"Guidelines for the evaluation of probiotics in food"that virulence factors are among the most important criteria for the evaluation of probiotic microorganisms. In this study, no isolates presented virulence genes (Fig. S6), and similar results were also found in a study by Pinto et al. (2020). On the other hand, Colombo et al. (2020) reported that some LAB from dairy products could have virulence factors. The isolates in this study were revealed not to pose any risk in terms of virulence genes. However, the presence of other virulence genes should also be analysed and the isolates should be revealed to be completely safe.

Haemolytic and DNase activity

Haemolytic and DNase activities are considered undesirable traits in probiotic and starter microorganisms because they are commonly associated with virulence factors and the potential for pathogenicity (Çetin and Aktaş 2024). In the present study, none of the isolates exhibited haemolytic or DNase activity. Similarly, many researchers have reported that LAB do not have haemolytic and DNase activities (de Amorim Trindade et al. 2022; Lee et al. 2022). On the other hand, while Jaouani et al. (2015) and Gök et al. (2020) stated that a small number of LAB exhibited haemolytic activity, Çetin and Aktaş (2024) reported that this characteristic was not present in LAB. This finding shows that characteristics such as haemolytic and DNase activity can vary between strains. The results of this study showed that the tested isolates do not pose a health risk and can be used as starter cultures for yoghurt production.

Conclusion

This study is one of the first reports in which the probiotic properties of such a high number of yoghurt bacteria have been extensively investigated. The results revealed that only four (0.91%) of the 439 yoghurt bacteria, including *L. delbrueckii* subsp. *bulgaricus* 114 M, 207 M, 208 M, and 239 M, presented potential probiotic characteristics. Furthermore, *L. delbrueckii* subsp. *bulgaricus* 239 M was identified as the best presumptive probiotic yoghurt bacterium because of its tolerance to bile salts and simulated gastric juice, antagonistic activity, high auto/co-aggregation and hydrophobicity ability, and resistance to few antibiotics compared with other strains. The results of all the analyses revealed that these potential probiotic isolates can be used as starter cultures in yoghurt production with enhanced positive effects on health. However, this study did not evaluate the co-culture compatibility of the probiotic candidates with traditional yoghurt starter cultures, especially *S. thermophilus*. As *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* are known to have a symbiotic relationship that is important for yoghurt fermentation, future studies should investigate their interactions and growth dynamics in co-culture systems. This will confirm whether these isolates are suitable for use as starter cultures. Finally, the weaknesses of yoghurt bacteria in terms of probiotic characteristics have been revealed in the present study and remind us that further studies on their isolation, characterisation and preservation are needed.

While this study provides valuable information on the probiotic potential of yoghurt bacteria, it is important to acknowledge several limitations. First, EPS production was investigated in the presence of genes, without any quantification or structural analysis. Second, antibiotic resistance was assessed by phenotypic disk diffusion, without genetic characterisation or risk assessment for horizontal gene transfer. Third, co-culture compatibility tests with traditional yoghurt bacteria were not performed, which limits the application of the identified strains as starter cultures. Future studies should address these issues to strengthen functionality and safety assessments.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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