



In vitro evaluation of probiotic and antioxidant potential of *Lacticaseibacillus paracasei* ED25

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Abstract

This study aimed to assess the in vitro probiotic and antioxidant potential of lactic acid bacteria (LAB) isolated from different white cheeses, also known as “Beyaz Peynir” in Turkey. A total of 58 bacterial strains were isolated from 11 different white cheeses obtained from small-scale dairies. According to some preselection criteria (having the distinctive features of LAB, exhibiting non-haemolytic property, and resisting the simulated gastrointestinal conditions such as low pH, pepsin, pancreatin and bile salt tolerance), four (ED13, ED20, ED25 and ED36) out of 58 isolates were selected for the subsequent experiments. Among the four isolates, ED25 exhibited the maximum lactase production and cholesterol removal potential, the highest biological activity (antimicrobial and antioxidant activity) and the lowest antibiotic resistance. In addition, the second highest B12-producing capacity were measured for ED25. The isolate ED25 was found to possess antimicrobial effectiveness against all tested microorganisms (*S. aureus*, *E. coli*, *S. Typhimurium*, *L. monocytogenes* and *C. albicans*) according to the agar well diffusion method. In vitro antioxidant activity assay demonstrated that the culture supernatant of the ED25 had the ability to scavenge DPPH (49%), ABTS (37%), OH• (51%) and O₂^{•-} (38%) radicals. According to the sequences analysis of the 16 S rRNA gene, the isolate ED25 was identified as *Lacticaseibacillus paracasei* (GenBank accession number: OP036674.1). Due to strong biological activities, *L. paracasei* ED25 may be used as a probiotic agent against gastrointestinal disorders, infections and oxidative stress-mediated diseases.

Keywords Antimicrobial · Cholesterol removal · Lactic acid bacteria · Radical scavenging

Abbreviations

ABTS 2	2': azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	GRAS	Generally Recognized As Safe
BLAST	Basic Local Alignment Search Tool	HPLC	High-performance liquid chromatography
CFU	Colony Forming Unit	LAB	Lactic acid bacteria
DPPH 1	1-diphenyl-2-picrylhydrazyl	MRS	agar deMan Rogosa Sharpe agar
		MRS	broth deMan Rogosa Sharpe broth
		NADH	nicotinamide adenine dinucleotide
		NBT	Nitrotetrazolium blue chloride
		NCBI	National Center for Biotechnology Information
		O ₂ ^{•-}	Superoxide Radical
		OH•	Hydroxyl Radical
		PBS	Phosphate-Buffered Saline
		PCR	Polymerase Chain Reaction
		PDA	Potato Dextrose Agar
		PDB	Potato Dextrose Broth
		ROS	Reactive Oxygen Species
		TSA	Tryptic Soy Agar
		TSB	Tryptic Soy Agar

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Introduction

Probiotics are defined as non-pathogenic viable microorganisms that have positive effects on human health and can be taken from foods or food supplements with diet (Kerry et al. 2018; Kook et al. 2019). World Health Organization defines them as living microorganisms that confer a health benefits to the host when administered adequately (Taheur et al. 2016; Jang et al. 2019).

Probiotic microorganisms have wide applications in the food, feed, dairy and fermentation industries (Plessas et al. 2017; Gao et al. 2021). They are also associated with many pharmacological approaches. For example, probiotics can inhibit the growth of pathogenic organisms, reduce the symptoms of lactose intolerance, exhibit anticarcinogenic, antimutagenic, antiobesity, antidiabetic, antiallergic, antihypertensive, anti-inflammatory, and lowering-cholesterol. They can modulate the immune system, play a role in enzyme inhibitions and improve digestion (Górska et al., 2019; Shi et al., 2019; Das et al. 2022). Furthermore, probiotic microorganisms exhibit antioxidative properties, thereby decreasing reactive oxygen species (ROS)-induced oxidative stress (Tang et al. 2017; Yang et al. 2019). For example, there are the studies showing that probiotic bacteria protect humans against ROS-induced oxidative stress-related gastrointestinal disorders, psychiatric disorders and cardiovascular diseases (Vasquez et al. 2019; Amirani et al. 2020; Fiorani et al. 2023).

Lactic acid bacteria (LAB) which are generally recognized as safe (GRAS) consist of a non-pathogenic group that may be Gram-positive, catalase-negative, non-spore forming, cocci or rod-shaped (Cholakov et al. 2017). LAB can be isolated from human faeces, fresh vegetable products, milk, cheeses, fermented foods and beverages. LAB are known as the most important group of probiotic microorganisms. There are numerous strains of probiotic LAB, such as *Lactobacillus acidophilus*, *L. brevis*, *Lactobacillus casei*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. plantarum*, *L. rhamnosus*, *L. johnsonii*, *L. bulgaricus*, *L. salyarius*, *L. reuteri*, *Bifidobacterium infantis*, *B. lactis*, *B. bifidum*, *Pediococcus acidophilus* and *Streptococcus thermophilus* (Plessas et al. 2017; Jang et al. 2019; Gomathi et al. 2014; Wang et al. 2019; Gao et al. 2021; Alameri et al. 2022). However, exploring new probiotic LAB strains is accepted as an important approach to meet the increasing demand of the market and to obtain probiotic cultures that are more active and have better probiotic properties than those available in the market.

It is well known that probiotic microorganisms must possess some potential properties to exert their beneficial effects. For example, probiotic microorganisms must be able to pass through gastrointestinal tract, survive in the

acidic conditions of the stomach, be resistant to bile salts, adhere to the intestinal mucosa, and at least temporarily colonize the colon (Belicová et al. 2013; Plessas et al. 2017). Therefore, while investigating the probiotic potential of microorganisms, the mentioned properties are tested using the in vitro methods.

As a result, it can be said that isolating a LAB and revealing its probiotic and antioxidant potential is a very beneficial approach for human health. Therefore, this study was performed to LAB from different white cheeses (also known as “Beyaz Peynir” in Turkey), and to then investigate the in vitro probiotic and anti-oxidant potential of isolated LAB.

Materials and methods

Isolation of lactic acid bacteria

The bacteria were isolated from white cheese samples (made from cow milk by traditional methods) obtained from small-scale dairies in Erzurum, Turkey. For this purpose, a small piece (about 0.1 g) of any of cheese samples (a total of 11 different cheese samples) was transferred into a tube containing sterile saline water (0.9%), the tube was vortexed and the suspension was serially diluted with saline water. After dilutions of 10^5 and 10^6 were spread on petri dishes containing MRS agar (deMan Rogosa Sharpe agar), the petri dishes were incubated anaerobically at 37 °C for 48 h. Bacterial colonies were picked and then further purified on MRS agar at 37 °C for 48 h.

Biochemical, morphological and staining characteristics were used for the preliminary identification of lactic acid bacteria (LAB), as previously described (Mokoena 2017; Amelia et al. 2020). Stock cultures of the selected lactic acid bacteria were maintained –20 °C in MRS broth with 15% (v/v) glycerol.

Investigation of haemolytic activities of isolates

The selected isolates were initially cultured in MRS broth (deMan Rogosa Sharpe broth) for 24 h at 37 °C and then streaked onto Columbia agar plates containing 5% (v/v) of sheep blood. The plates were then incubated at 37 °C for 24 h. Haemolytic reactions were recorded by the presence of a clear zone of hydrolysis around the colonies (β -haemolysis), a green zone of a partial hydrolysis around the colonies (α -haemolysis) or no reaction indicated by the absence of a zone around the colonies (γ -haemolysis). The isolates with γ -haemolysis were considered as safe.

Evaluation of resistance potential of isolates to simulated-gastrointestinal conditions

The abilities of LAB to resist the simulated-gastrointestinal conditions (low pHs, bile salt, pepsin and pancreatin) were investigated as demonstrated in the previous studies (Plessas et al. 2017; Mantzourani et al. 2019; Pradhan and Tamang 2021).

To test the tolerance of the isolated LAB to low pH, each isolate was incubated anaerobically in MRS broth at 37 °C. After 18 h incubation period, the cells were collected by centrifugation at 10.000×g for 5 min, washed twice with phosphate-buffered saline (PBS) (pH 7.2), and resuspended in 10 mL PBS buffers adjusted to different pHs (2.0, 3.0 and 4.0). After the cell suspensions were incubated at 37°C for 2 h, they were spread on MRS agar plates (Before spreading agar plates, the suspensions were serially diluted at 0 and 2 h of incubation). After the plates were incubated under anaerobic conditions at 37°C for 48 h, the counting of colonies was performed. The isolates with resistance against low pH were selected for the subsequent stages of the study.

To investigate the resistance of the isolates against pancreatin or pepsin, each isolate was initially grown in MRS broth at 37°C for 18 h and then its grown cells were collected by centrifugation (10.000×g for 5 min) as described above. The cells were washed twice with PBS and then resuspended either in 10 mL PBS solution (pH 2.0) containing pepsin (3 mg/mL), or in PBS solution pH 8.0 containing pancreatin (1 mg/mL). The cells were incubated for 3 h in the presence of pepsin or pancreatin. The cell suspensions were serially diluted after an incubation of 3 h. The dilution samples (up to 10⁻⁴) were spreaded on Petri dishes containing MRS agar, and the petri dishes were incubated anaerobically at 37 °C for 48 h before enumeration.

To investigate the tolerance of the isolates to the bile salts, each isolate was initially grown in MRS broth at 37°C for 18 h. After growing, the cells were collected by centrifugation (10.000×g for 5 min), washed twice with PBS and resuspended in 10 mL MRS broth lacking or containing bile salt. During the experiments, bile salt was added to the cultures at a final concentration of 0.3% and the cultures with 0% bile salt served as the control. The cultures were incubated anaerobically at 37 °C for 3 h. The samples taken from the cultures were serially diluted and then spreaded on Petri dishes containing MRS agar. Petri dishes were then incubated anaerobically at 37 °C for 48 h before enumeration. Survival rate (%) = [number (log CFU/mL) of survived cells in modified conditions/ initial number (logCFU/mL) of inoculated cell] x 100 (Taheur et al. 2016; Plessas et al. 2017).

Evaluation of lactase-producing potentials of isolates

To increase the secretion of β-galactosidase (lactase), the selected isolates were incubated at 37 °C on MRS broth medium containing 1% (v/v) lactose. After an incubation period of 24 h, the cultures were centrifuged and the supernatants were used for the measurement of β-galactosidase activity. For the activity assay, the reaction mixture contained 25 μL of 0.1% p-NP-β-D-galactopyranoside solution, 75 μL of 50 mM phosphate buffer (pH 7) and 25 μL of supernatant. After the reaction mixtures were incubated at 37 °C for 60 min, their absorbances were measured at 405 nm. A unit of enzyme activity was defined as 1 μmol of galactose liberated per min in 50 mM phosphate buffer (pH 7) at 37 °C. The molar extinction coefficient under these assay conditions was 13,700 M⁻¹ cm⁻¹ (Van Laere et al. 2000).

Evaluation of B12-producing potentials of isolates

The selected isolates were grown anaerobically in MRS broth medium at 37 °C. After an incubation period of 24 h, the cultures were centrifuged and the obtained cell pellets were washed three times with sterilized PBS (pH7.2). Then, pellets were inoculated into 10 mL of vitamin B12-free assay medium (B3801, Sigma-Aldrich) and were grown anaerobically at 37 °C for 24 h. At the end of growth period, the cultures were centrifuged, the collected supernatants were passed through a membrane filter (0.45 μm) and then were used for B12 analysis. Commercial vitamin B12 (≥ 98% purity, Sigma) was used as standard for the preparation of the calibration curve. The analyses were performed on a High-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a DGU-20A5 degasser, a CTO-20 A column oven, an SIL-20 A HT autosampler and a SPD-20 A Prominence UV/VIS detector. Separation was performed on a 5 μm, 4.6×250 mm C18 (Technochroma brand) column. The amount of B12 in the samples was given as μg/mL.

Evaluation of cholesterol-lowering potentials of isolates

Each isolate was inoculated in MRS broth with supplemented with 100 μg/mL water-soluble cholesterol (C4951, Sigma-Aldrich) and the cultures were incubated at 37°C. The uninoculated sterile MRS broth was used as the control. After a growth period of 24 h, the cultures and the control medium were centrifuged at 10,000 rpm for 15 min and the residual cholesterol contents of the collected supernatants were analyzed according to the OPA (o-phthalaldehyde) method

as described previously (Gilliland et al., 1985; Shobharani and Halami 2016). In brief, 0.5 mL of the supernatant was mixed with 2 mL KOH (50% wt/vol) and 3 mL absolute ethanol. The mixture was vortexed for 1 min, heated at 60 °C for 15 min and cooled to 25 °C. After adding 3 mL distilled water and 5 mL hexane, the mixture was vortexed for 1 min. After the mixture was kept at 25 °C for 15 min, 2.5 mL of the hexane layer was collected and the solvent (hexane) was removed at 60 °C using an evaporator. The final material (residue) was dissolved in 4 mL OPA reagent (0.5 mg/mL in acetic acid), the mixture was incubated at room temperature for 10 min and then 2 mL concentrated sulfuric acid was added into the mixture. The mixture was vortexed for 1 min and then left at 25 °C for 10 min. Finally, the absorbances of the mixtures were read at 550 nm using a Beckman Coulter DU730 spectrophotometer. When compared to the cholesterol content of the control medium, the decreases in the cholesterol contents of the culture supernatants were considered as the reduced amount of cholesterol. The reductions in cholesterol levels were given as percentage.

Evaluation of antimicrobial potentials of isolates

The antimicrobial activities of the selected isolates were investigated against two gram-negative (*Escherichia coli* ATCC 43,894 and *Salmonella Typhimurium* ATCC 13,883) and two gram-positive bacteria (*Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 33,019), as well as the fungal pathogen *Candida albicans* ATCC 14,053.

The agar well diffusion method was used to detect antimicrobial activities of the culture supernatants of the selected isolates against these pathogens (Tagg and McGiven 1971). For this purpose, the precultures of pathogenic test bacteria were prepared in TSB medium for 24 h, while the preculture of the yeast *C. albicans* was prepared in potato dextrose broth (PDB) for 48 h. The cultures were diluted with saline solution (0.9% [w/v]) to 0.5 Mc Farland standard at 600 nm. Then, 100 µL of the dilution samples were spreaded on the Petri dishes containing TSA or PDA using a drigalski spatula. The precultures of selected isolates were anaerobically grown in MRS broth medium at 37 °C. After an incubation period of 24 h, the cultures were centrifuged (4000 rpm at 4 °C for 30 min). The supernatants were then filtered using a 0.22 µm filter (Millipore, Billerica, MA, United States) to remove completely bacterial cells, and the obtained cell free supernatants were transferred into 6 mm diameter wells (100 µL/well) which were previously prepared on the agar medium (TSA or PDA). After an incubation of 24–48 h at 37 °C, the diameters of the zones around the wells were measured. Each test was performed in triplicate and mean zone diameters were recorded.

Evaluation of antibiotic susceptibility of isolates

The resistance of the isolates to antibiotics was tested by disk diffusion method. For this, 2 mL of the active culture of the isolates developed in MRS broth (at 37 °C for 18 h) was taken and centrifuged at 4000 rpm for 10 min. The pellet was then washed with 1 mL of PBS and this process was repeated 2 times. The pellet was then dissolved in PBS and adjusted to the 0.5 McFarland turbidity standard. A 300 µL of the obtained cell suspension was spreaded on MRS agar medium. Then, antibiotic discs (ampicillin, chloramphenicol, amoxicillin, ciprofloxacin, ofloxacin, kanamycin and sulfamethoxazole) were placed on MRS agar medium. Petri dishes were incubated at 37 °C for 18 h. After incubation, the inhibition zone diameters (mm) formed around the antibiotic discs were measured. The results were categorized as resistant, moderate or sensitive (Taheur et al. 2016; Jang et al. 2019; Lu et al. 2021).

In vitro evaluation of antioxidant potential of isolates

The isolates were anaerobically grown in MRS broth (at 37 °C for 18 h). Then, the cultures were centrifuged (at 4000 rpm for 10 min) and the 1 mL of the obtained supernatants were used for antioxidant activity assays. During all the assays, deionized water was used as the control and vitamin C solution (1 mg/mL) was used as the positive control. Scavenging potential was calculated according to the Eq. 1.

The scavenging activities of the supernatants against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were measured according to the method suggested by Blois (1957). For this, approximately 0.9 ml of DPPH solution (2 mg/mL) was added to 0.3 mL of the sample (supernatant, MRS broth or vitamin C) and the mixture was allowed to react at room temperature in the dark for 30 min. Absorbance was measured at 517 nm. The scavenging activities (%) of the tested samples against DPPH radical were calculated according to the Eq. 1.

$$\% \text{ Scavenging} = \left(1 - \frac{A_1 - A_2}{A_0} \right) \times 100 \quad (1)$$

[A_0 is the control: absorbance of the solution containing deionized water and DPPH, A_1 : absorbance of the solution containing supernatant/MRS broth/vitamin C and DPPH, A_2 : absorbance of the solution containing other components without DPPH (instead of deionized water)].

The scavenging activities of the supernatants against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical were determined according to the method recommended by Cheng et al. (2021)

and Ji et al. (2022). For this, first, 9.9 mg potassium persulfate was mixed with 15 ml ABTS aqueous solution (5.55 mmol/L) and the solution was incubated for 15 h at 25 °C in a dark environment to develop a blue-green color. Then, this solution was diluted with PBS (100 µmol/L, pH 7.4) solution until its absorbance became 0.7 at 734 nm. Then, 1 mL of the sample (supernatant, MRS broth or vitamin C) was reacted with 2 mL of ABTS solutions for 25 min, and then the absorbance value of the mixture was measured at 734 nm. The scavenging activities (%) of the tested samples against ABTS radical were calculated according to the Eq. 1 [A_0 is the control: absorbance of the solution containing deionized water and ABTS, A_1 : absorbance of the solution containing supernatant/MRS broth/vitamin C and ABTS, A_2 : absorbance of the solution containing other components without ABTS (instead of deionized water)].

The superoxide ($O_2^{\cdot-}$) radical scavenging activities of the supernatants were determined according to the method specified by Liu et al. (1997) and Xu et al. (2009). For this, 0.1 mL of the sample (supernatant, MRS broth or vitamin C) was mixed with 1 mL of 16 mM Tris-HCl (pH 8.0) containing 0.1 mL of 78 µM nicotinamide adenine dinucleotide (NADH), 1 mL of 16 mM Tris-HCl (pH 8.0) containing 10 µM phenazine methosulfate and 16 mM Tris-HCl (pH 8.0) containing 50 µM nitrotriazolium blue chloride (NBT). After the solution was incubated for 5 min at 25 °C, the absorbance of the solution was measured at 560 nm. The $O_2^{\cdot-}$ radical scavenging potential was calculated according to the Eq. 1 [A_0 is the control: absorbance of the solution containing deionized water (in stead of the sample) and other components, A_1 : absorbance of the solution containing the sample and other components, A_2 : absorbance of the solution containing other components without NBT solution (instead of deionized water)].

The hydroxyl radical (OH^{\cdot}) scavenging activity of the supernatants was determined according to the method specified by Qiao et al. (2009). For this, 0.5 mL of the sample (supernatant, MRS broth or vitamin C) was mixed with 0.5 mL salicylic acid solution (9 mmol/L), 0.5 ml $FeSO_4$ solution (9 mmol/L) and 0.5 ml H_2O_2 solution (9 mmol/L). The prepared final solution was incubated at 37 °C for 40 min. At the end of this period, the absorbance of the mixture was measured at 510 nm. The OH^{\cdot} scavenging activity (%) was calculated according to the Eq. 1 [A_0 is the control: absorbance of the solution containing deionized water (in stead of the sample) and other components, A_1 : absorbance of the solution containing the sample and other components, A_2 : absorbance of the solution containing other components without H_2O_2 solution (instead of deionized water)].

Molecular identification of the most potent isolate

The identification of the most potent isolate was carried out according to the nucleotide sequences analysis of the 16 S rRNA gene. In brief, the total genomic DNA of the most isolate was extracted from the purity-controlled culture using Promega wizardR genomic DNA purification kit (A2360). PCR amplification of 16 S rRNA was performed using the following oligonucleotide primers: UNI16S-F (5'-ATTCTAGAGTTTGATCATGGCTCA) and UNI16S-R (5'-ATG GTACCGTGTGACGGGCGGTGTGTA). The cloning of the PCR product into the pGEM-T vector system was performed according to the manufacturer's instructions (Promega, Southampton, UK). The recombinant plasmids were isolated using Wizard® Plus SV Minipreps (Promega). The clone was sequenced (Macrogen) and the sequence was compared with the complementary sequences within GenBank and EzTaxon (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> and <http://www.eztaxon.org>). After the similarity rate was determined, GenBank accession number for the most potent isolate was received. The phylogenetic tree was constructed by Neighborjoining method using MEGA 7.0 Software Package.

Results and discussion

Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) used as probiotic agents can be isolated from different foods such as milk, cheese, yogurt, milk and sausage (Wang et al. 2019; Miranda et al. 2021; Tarique et al. 2022). In the current study, 11 different white cheeses (made from cow milk) were used as the isolation source of LAB and a total of 58 indigenous bacterial isolates could be obtained. According to the pre-selection criteria (Gram-positive strain, rod or coccus shaped cells, non-spore forming, catalase negative, the growth ability in MRS broth, acid production etc.) (Mokoena 2017; Amelia et al. 2020), 50 out of 58 isolates were determined to be strongly LAB and the following experiments were performed by using 50 isolates.

Investigation of haemolytic activities of isolates

It is widely documented that a clear hydrolysis zone (β -haemolysis) or a green partial hydrolysis zone (α -haemolysis) around the microbial colonies on blood agar is an indicator of microbial pathogenesis. Whereas, the absence of a zone (γ -haemolysis) around the colonies usually indicate a non-pathogenic property of a microorganism. Therefore, it has been suggested that probiotic

microorganisms do not show haemolytic activity on blood agar (Olajugbagbe et al. 2020; Jawan et al. 2021).

The present experiments demonstrated that none of 50 isolates caused β -haemolysis on blood agar. It was determined that 43 of the 50 selected isolates did not form a hydrolytic zone (γ -hemolysis), an indication of safety of probiotic microorganisms. The rest 7 isolates were determined to cause a partial hydrolysis with green zone (α -haemolysis). It was assumed that these 7 isolates might be belonged to *Enterococci*. This is because, it is known that *Enterococci*, a large genus of LAB, can be isolated from food samples (cheese, milk etc.) and include both pathogenic and commensal microorganisms, and that some strains of the genus show α -haemolytic activity (Hanchi et al. 2018; Terzić-Vidojević et al. 2021). Based on these results, the next phases of the study were performed on 43 LAB which showed γ - hemolytic activity and were considered safe.

Investigation of resistance profiles of isolates to low pH

It is known that the gastrointestinal environment has an acidic pH. Therefore, it is desired that the bacteria to be used as probiotics should have high resistance to low pH (Mantzourani et al. 2019; Soares et al. 2019). Therefore, in this stage of the study, the resistance potential of 43 LAB to low pH was investigated.

The preliminary screening experiments demonstrated that the viability rates of 29 LAB at pH 2 and 3 were rather low (the survival rates were under 10%) (these results are not shown). Namely, the resistance potential of 29 isolates to low pH was found to be very low. The remaining 14 isolates were found to have higher tolerance (survival ratio over 10%) to all the pH levels tested including pH 2 (Table 1).

For example, the viabilities of 14 LAB at pH 2 ranged from 14.4 to 45%. In particular, it was determined that 9 isolates (ED8, ED9, ED13, ED20, ED25, ED36, ED39, ED47 and ED51) showed more resistance (survival ratio above 30%) to low pH (viability rates at pH 2 were 37.9, 41.6, 34.7, 39.5, 39.2, 37.8, 45.0, 44.0 and 40.0%, respectively). Therefore, the following stages of the study were performed on 9 isolates.

The cultures were pre-incubated anaerobically at 37 °C for 2 h at different pHs (2.0, 3.0, and 4.0). The samples which were taken from the cultures at the beginning (0 h) and 2th h of incubation were serially diluted and spreaded on MRS agar. Petri dishes were re-incubated anaerobically at 37 °C for 48 h before enumeration. t_0 , viable count (log CFU/mL) of strain at 0 h. t_2 , viable count (log CFU/mL) of strain at 2 h. The experiments were performed in triplicate.

Investigation of resistance potential of isolates to pepsin, pancreatin and bile salts

Pepsin, pancreatin and bile salts are known to help the digestion and/or absorption of nutritional compounds in human gastro-intestinal tract. Pepsin is secreted in stomach in the form of inactive pepsinogen and then is converted to the active enzyme by autocatalysis at pHs below 5. The active pepsin plays a significant role in protein digestion (Santos-Hernández et al. 2018). Pancreatin is produced by the pancreas and is composed of several digestive enzymes such amylase, lipase and protease. This enzyme mixture is delivered to the stomach and small intestine for the hydrolysis of complex nutrients (Whitcomb and Lowe 2007; McClements et al. 2008). Bile salts are synthesized by the liver and then secreted into bile, stored temporarily in the gallbladder, passed from the gallbladder into the duodenum and

Table 1 The resistance of the isolates to low pH

Isolates	pH 2		Survival (%)	pH 3		Survival (%)	pH 4		Survival (%)
	Viable count (log CFU/mL)			Viable count (log CFU/mL)			Viable count (log CFU/mL)		
	t_0	t_2		t_0	t_2		t_0	t_2	
ED3	8.9	1.4	15.7	9.1	1.7	18.6	9.6	2.9	30.2
ED4	8.3	1.2	14.4	8.5	1.4	16.4	9.3	4.7	27.9
ED8	7.9	3.0	37.9	8.1	3.2	39.5	8.3	4.9	59
ED9	7.2	3.0	41.6	7.5	3.3	44	7.9	5.1	64.5
ED11	7.3	1.3	17.8	7.6	1.5	19.7	7.7	2.9	37.6
ED13	7.2	2.5	34.7	7.5	2.8	37.3	7.8	4.5	57.7
ED18	8.7	1.9	21.6	8.8	2.1	23.8	8.9	4.7	52.8
ED20	8.1	3.2	39.5	8.7	3.6	41.4	9.6	5.9	61.4
ED25	7.9	3.1	39.2	8.5	3.7	43.5	9.6	6.2	64.5
ED36	8.2	3.1	37.8	8.6	3.5	40.6	9.1	5.6	61.5
ED39	7.1	3.2	45	7.7	3.6	46.7	8.6	5.8	67.4
ED43	8.9	1.7	19.1	9.3	2.1	22.6	9.5	4.1	43.1
ED47	7.5	3.3	44	7.7	3.4	44.1	8.0	5.1	66.2
ED51	8.0	3.2	40	8.2	3.6	43.9	8.5	5.3	62.3

Table 2 The resistance of the isolates to pepsin and pancreatin

Isolates	Control		Pepsin		Pancreatin	
	(log CFU/mL)		(log CFU/mL)	Survival (%)	(log CFU/mL)	Survival (%)
ED8	7.4		1.4	18.9	3.2	43.2
ED9	7.0		1.5	21.4	2.7	38.5
ED13	6.9		1.9	27.5	3.6	52.1
ED20	7.4		1.9	25.6	3.3	44.5
ED25	7.2		2.1	29.1	4.3	59.7
ED36	7.1		1.9	26.7	4.9	69
ED39	6.9		1.5	21.7	3.3	47.8
ED47	7.3		1.5	20.5	3.9	53.4
ED51	7.3		1.4	19.1	3.2	44.4

Table 3 The resistance of the isolates to bile salts

Isolates	Control (Bile salts-free)		0.3% Bile salts	
	(log CFU/mL)	Survival (%)	(log CFU/mL)	Survival (%)
ED13	7.1	100	3.1	43.6
ED20	7.3	100	4.2	57.5
ED25	7.4	100	4.9	66.2
ED36	7.3	100	3.6	49.3

absorbed throughout the small intestine. They play essential roles in digestion and absorption of fats (de Buy Wenniger et al. 2013). As mentioned above, pepsin, pancreatin and bile salts play very beneficial roles in the gastro-intestinal system. However, these substances are known to have toxic effects on probiotic microorganisms. Therefore, researchers have suggested that probiotic microorganisms show high resistance against the inhibitory effects of pepsin, pancreatin and bile salts (Plessas et al. 2017; Mantzourani et al. 2019).

As seen from Table 2, all of nine isolates had lower tolerance to pepsin compared to pancreatin. In particular, five isolates (ED8, ED9, ED39, ED27 and ED39) were found to have rather low pepsin tolerance (survival rates in the presence of pepsin ranged from 18.9 to 21.7%). The results also revealed that although five isolates have high survival rates at pH 2 in pepsin-free medium (Table 1), they exhibited low survival rates at pH 2 when incubated in the medium containing pepsin (Table 2). The remaining four isolates (ED13, ED20, ED25 and ED36) were found to have a good survival potential at low pH of 2.0 in the presence of pepsin. The survival rates of ED13, ED20, ED25 and ED36 in the presence of pepsin (at pH 2) were 27.5, 25.6, 29.1 and 26.7%, and the survival rates in the presence of pancreatin were 52.1, 44.5, 59.7 and 69%, respectively. Considering these results, only four isolates (ED13, ED20, ED25 and ED36) which were capable of resisting to both pepsin and pancreatin were selected for the following stages of the study. When the resistance potential of four isolates to bile salts was tested, it was seen that ED25 had the highest potential (the viability rates in the presence of 0.3% bile salts were 43.6, 57.5, 66.2 and 49.3 for ED13, ED20, ED25 and ED36, respectively) (Table 3).

The cultures were pre-incubated anaerobically at 37 °C in the presence of pepsin (at pH 2) or pancreatin (at pH 8) for 3 h). Control group was not subjected to pepsin or pancreatin treatment. After an incubation period of 3 h, the samples which were taken from the cultures were serially diluted and spreaded on Petri dishes containin MRS agar. Petri dishes were then re-incubated anaerobically at 37 °C for 48 h before enumeration. The experiments were performed in triplicate.

The cultures were incubated anaerobically at 37 °C in the presence of 0% (control) or 0.3% bile salts. After an incubation period of 3 h, the samples which were taken from the cultures were serially diluted and spreaded on Petri dishes containing MRS agar. Petri dishes were then re-incubated anaerobically at 37 °C for 48 h before enumeration. The survival ratio for control was accepted as 100%. The experiments were performed in triplicate.

Investigation of cholesterol-lowering, lactase and B12-producing properties of isolates

Lactose intolerance is a digestive disorder causing a negative effect on human health. In healthy people, a digestive enzyme called lactase which is produced by small intestine is responsible for breaking lactose down into sugars called glucose and galactose. Lactose intolerance occurs when small intestine can not produce enough of lactase to digest lactose. Therefore, lactose-intolerant people experience discomforting symptoms such as pain, diarrhea, gas, and bloating after consuming lactose-containing products (Swagerty et al., 2002; Misselwitz et al. 2013; Bayless et al. 2017). It is estimated that 75% of individuals worldwide experience

hypolactasia, or some decrease of lactase activity (Fieker et al. 2011). Today, the lactase enzyme, also known as β -Galactosidase, is used as a supplement in the preparation of lactose-free foods for the nutrition of lactose-intolerant individuals (Saqib et al. 2017; Shafi and Husain 2022). Furthermore, lactase-containing tablets can be directly used for the reduction of lactose intolerance-related clinical symptoms (Medow et al., 1990; Montalto et al., 2005). On the other hand, the studies have shown that LAB, which are used directly as probiotics, also ameliorate lactose intolerance by producing lactase (Vonk et al. 2012; Gingold-Belfer et al. 2020). Therefore, the lactase producing-abilities of four bacterial isolates were tested in the present study. The experiments displayed that all of four isolates were able to produce lactase; however, ED20 and ED25 isolates, especially ED25, were found to be more potent (Table 4).

Vitamin B12, also known as cobalamin, is a water-soluble vitamin. Vitamin B₁₂ is an essential human nutrient and its deficiency causes anemia, neuropathy, hyperhomocysteinaemia in humans. Since humans cannot synthesize vitamin B12, they obtain it mainly from animal origin-foods such as milk, meat and eggs. The risk of B12 deficiency is higher in vegetarians with low consumption of animal-derived foods and in elderly populations with certain gastric dysfunctions (Madhu et al. 2010; Li et al. 2017). Since LAB have the capacity to synthesize B12, the fermented foods prepared with these bacteria can provide B12 to humans. Moreover, when LAB are used directly as probiotics, they can synthesize B12 in the gastrointestinal tract (LeBlanc et al. 2011; Melini et al. 2019). Therefore, it is recommended that the bacteria to be used as probiotics should have also the capacity to produce B12 (Li et al. 2017; Chugh and Kamal-Eldin 2020). The data summarized in Table 4 display that although all of four isolates were able to produce B12, the maximum B12 production was achieved using ED20 (19 $\mu\text{g}/\text{mL}$), followed by ED25 (12 $\mu\text{g}/\text{mL}$). In brief, it was determined that ED20 and ED25 isolates did not cause blood hemolysis which is a indicator of pathogenicity and could produce higher levels of lactase and B12 compared to the other two isolates.

Cholesterol acts as a basic building block in the formation of membranes. It also participates in the synthesis of steroid hormones and vitamin D, as well as bile acids that are involved in the emulsification of fats, their ingestion and absorption. However, an elevated level of blood cholesterol

is considered as one of major risk factors for coronary heart diseases (Kumar et al. 2012; Shobharani and Halami 2016). Today, there are some medicinal drugs that are used for lowering the high cholesterol levels in human; however, they are expensive and are known to cause side effects such as muscular pain and liver toxicity (Albano et al. 2018; Park et al. 2018). In the recent studies, it has been demonstrated that LAB reduce cholesterol levels and thus can be used for the treatment of high cholesterolemia (Tarrach et al. 2021; Frappier et al. 2022). The results of the present study revealed that all of four isolates had the potential to decrease cholesterol content in MRS broth. However, the maximum cholesterol removal (79.3%) was achieved in the culture of the isolate ED25 (Table 4). Moreover, the cholesterol-removing ability of ED25 was found to be quite promising when compared with the probiotic microorganisms tested in previous studies (Iranmanesh et al. 2014; Castorena-Alba et al. 2018; Kathede et al., 2020).

Investigation of antimicrobial potential of isolates

Probiotic microorganisms can produce antimicrobial compounds such as lactic acid, bacteriocin-like molecules and hydrogen peroxide. They are also capable of stimulating immune system and modulating intestinal microbiota. Furthermore, probiotic microorganisms prevent the adhesion of pathogens by competing for the binding sites on the intestinal epithelial cells. Due to these potential properties, probiotics can protect human body against pathogenic microorganisms in the gastrointestinal tract (Karimi et al. 2018; Monteagudo-Mera et al. 2019).

When the antimicrobial activities of the culture supernatants of the four isolates were investigated, it was found that ED13 had an antibacterial activity against only *S. aureus*, ED20 against two pathogenic bacteria (*E. coli* and *S. aureus*), and ED36 against three pathogenic bacteria (*E. coli*, *S. Typhimurium* and *S. aureus*). None of three isolates showed antifungal activity against the yeast *C. albicans* (Table 5). On the contrary, it was determined that the culture supernatant of the isolate ED25 had strong antimicrobial activity against all of the four pathogenic bacteria. Even, it was seen that ED25-induced zone diameters were larger than other isolates-induced zone diameters. Furthermore, the culture supernatant of ED25 was ascertained to have antimicrobial activity against the yeast *C. albicans*. Zone diameters of

Table 4 Lactase and B12-producing and cholesterol-lowering potential of the isolates

Isolates	Lactase (U/mL)	B12 ($\mu\text{g}/\text{mL}$)	Cholesterol removal (%)
ED13	10.9	7	23.2
ED20	13.8	19	64.7
ED25	14.7	12	79.3
ED36	13.1	6	41.6

Table 5 Antimicrobial effectiveness of the culture supernatants of isolates

Isolates	Zone diameter (mm)				
	<i>E. coli</i>	<i>S. aureus</i>	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>C. albicans</i>
ED13	NH	10	NH	NH	NH
ED20	10	11	NH	NH	NH
ED25	14	16	14	11	10
ED36	10	13	11	NH	NH

Table 6 Susceptibility of the isolates to different antibiotics

Isolates	Antibiotics						
	K	SMX	C	CIP	AMP	AML	OFX
ED13	R	R	S	R	S	S	MS
ED20	R	R	S	R	S	S	MS
ED25	R	R	S	MS	S	S	MS
ED36	R	R	S	MS	S	S	MS

The antibiotic susceptibility of the isolates were determined according to agar disc diffusion method. C, chloramphenicol; K, Kanamycin; SMX, sulfamethoxazole; CIP, ciprofloxacin; AMP, ampicillin; AML, amoxicillin; OFX, ofloxacin; R, resistant; MS, moderate sensitive and S, sensitive

ED25 against *S. aureus*, *E. coli*, *S. Typhimurium*, *L. monocytogenes* and *C. albicans* were measured as 16, 14, 14, 11 and 10 mm, respectively. In short, it was found that the isolate with the highest antimicrobial activity against pathogenic microorganisms was ED25, followed by ED36. As reported in the previous studies (Lin et al., 2019; Ibrahim et al. 2021), the antimicrobial effectiveness of ED25 could be attributed to the peptidic or proteinaceous bacteriocins, organic acids and other small molecules which it produced.

The antimicrobial effectiveness of the isolates were determined according to agar well diffusion method. After an incubation of 24 h at 37 °C, the diameter of the zones around the wells was measured. NH; no hydrolysis.

Investigation of antibiotic susceptibility of isolates

The experiments revealed that the susceptibilities of the selected four isolates (ED13, ED20, ED25 and ED36) to the antibiotics were similar to each other. Significant difference for antibiotic sensitivity was recorded only for ciprofloxacin (Table 6). All of four isolates were sensitive to amoxicillin, chloramphenicol and ampicillin but resistant to sulfamethoxazole and kanamycin. The susceptibility of four isolates to the ofloxacin was determined as moderate sensitive. Two isolates (ED13 and ED20) was resistant to the ciprofloxacin, while the sensitivity of the other two isolates (ED25 and ED36) to the ciprofloxacin was moderate. Overall, the current study indicated that five antibiotics could prevent completely or partially the growth of the isolate ED25. Low antibiotic resistance indicates that ED25 have the potential to be used as a probiotic. This is because that antibiotic resistance is not a desirable property for probiotics, and it is recommended that probiotics are sensitive to

at least two antibiotics of clinical importance to due safety problems (Sanders et al. 2010; Choudhary et al. 2019).

In vitro investigation of antioxidant potential of isolates

Reactive oxygen species (ROS) are produced by endogenous sources (plasma membrane, mitochondria, chloroplast, endoplasmic reticulum, peroxisomes etc.) and exogenous factors (pollutants, radiation, smoking, drugs, heavy metals etc.) (Bouayed and Bohn 2010; Curieses Andrés et al., 2023). When present at low or moderate levels, ROS act as signal transducers and play vital roles in various cellular process. However, excess-accumulation of ROS oxidizes the nucleic acids, membranes, proteins, and lipids, thereby causing cell and tissue damages and eventually oxidative stress-mediated diseases (Lee et al., 2021; Nakamura and Takada 2021). Therefore, maintaining ROS levels in the equilibrium is crucial for healthy cell functioning.

In living organisms including humans, this equilibrium is mainly sustained by endogenous antioxidant system consisting of enzymatic (superoxide dismutase, catalase, glutathione peroxidase etc.) and non-enzymatic antioxidants (albumin, ceruloplasmin, metallothioneins etc.) (Mirończuk-Chodakowska et al. 2018). However, exogenous antioxidants also help the endogenous antioxidant system to eliminate excess ROS in biological systems (Bouayed and Bohn 2010; Hussain and Kayani 2020). Human obtain exogenous antioxidants mainly from plants through diet; however, other organisms such as algae, bacteria, and fungi are also known as the potential producers of antioxidative compounds (Mirończuk-Chodakowska et al. 2018; Chandra et al. 2020; Ślusarczyk et al. 2021). There are numerous studies showing that probiotic bacteria have

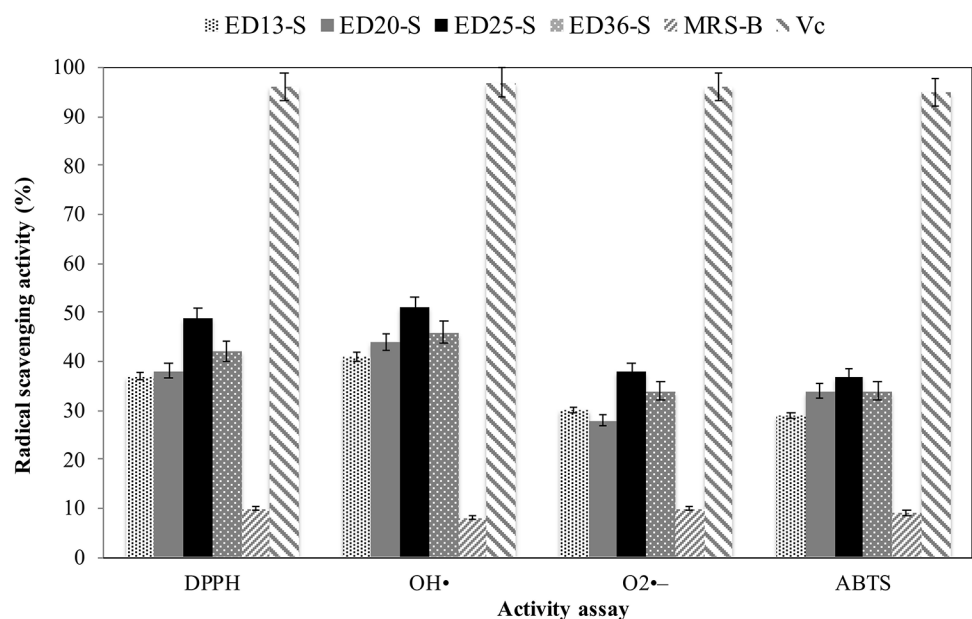
antioxidant potential (Li et al. 2012; Tang et al. 2017; Yang et al. 2019). For example, the studies have revealed that due to the antioxidant potential, probiotic bacteria can exhibit a protective role against oxidative stress-related neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) and gastrointestinal disorders (inflammatory bowel disease, colitis, and cancer) (Beltrán-Velasco et al. 2024; Li et al. 2024; Ma et al. 2024; Philip Mani et al. 2024; Valvaikar et al. 2024). For instance, it has been reported that *Lactiplantibacillus plantarum* AS21 and *Clostridium butyricum* can reduce the pathological effects of colitis in mice by improving the integrity of the intestinal mucosal barrier and suppressing inflammation and oxidative stress (Li et al. 2024). Therefore, the present study also focused on testing the antioxidant potential of the four isolates. For this purpose, the culture supernatants of four isolates were evaluated for the in vitro antioxidant activities and the results were compared with cell-free MRS broth and vitamin C (positive control). The experiments revealed that the cell-free culture supernatants of four isolates had a promising antioxidant potential to scavenge DPPH, ABTS, superoxide ($O_2^{\cdot-}$) and hydroxyl (OH^{\cdot}) radicals when compared to the vitamin C. Among four isolates, the isolate ED25 was found to be more promising in terms of radical scavenging potential. The scavenging rates of ED25 towards DPPH, ABTS, $O_2^{\cdot-}$ and OH^{\cdot} radicals were determined as 49, 37, 38 and 51%, respectively. On the contrary, no significant radical scavenging activity was measured for MRS broth when compared to vitamin C. The scavenging rates of MRS broth on DPPH, ABTS, $O_2^{\cdot-}$ and OH^{\cdot} radicals were 10, 9, 8 and 10%, respectively (Fig. 1). These results implied that the antioxidative potential of the culture supernatants was mainly due to the bacteria-derived natural compounds

rather than the nutritional components of MRS broth. For example, the polysaccharides and bacteriocins produced by the isolates may be responsible for antioxidant activities of the culture supernatants. This hypothesis can be supported by the results of the previous studies showing that LAB-derived polysaccharides and bacteriocins exhibit antioxidant potential (Mahdhi et al. 2017; Krishnamoorthi et al. 2022). Due to the strong antioxidant potential, ED25 may be used as a probiotic agent against oxidative stress-related diseases including neurodegenerative diseases and gastrointestinal disorders. However, the further in vivo studies are need to prove this hypothesis.

Identification of the isolate ED25

As seen from the results summarized in Tables 1, 2, 3, 4, 5 and 6; Fig. 1, the isolate ED25 was superior to the other isolates in terms of not causing hemolysis, resisting to the simulated gastrointestinal conditions (low pH, pepsin, pancreatin and bile salts), producing lactase, reducing cholesterol levels, inhibiting pathogenic microorganisms, possessing low antibiotic resistance, and exhibiting high antioxidative property. These results implied that when compared to the other isolates, the isolate ED25 had a higher potency to be used as a probiotic agent. The identification of the isolate ED25 was performed according to the nucleotide sequences analysis of the 16 S rRNA gene. For this purpose, the genomic DNA of ED25 was extracted and PCR amplification of the 16 S rRNA gene was carried out. The PCR product was cloned into pGEM-T Easy Vector and then sequenced at Macro-gen. As seen from Fig. 2, the sequence of ED25 (GenBank accession number: OP036674.1) had a similarity of 99.73% to *Lactobacillus paracasei* subsp. *paracasei* (reclassified to

Fig. 1 In vitro antioxidant activities of isolates. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-Diphenyl-1-picrylhydrazyl; OH^{\cdot} , hydroxyl radical; $O_2^{\cdot-}$, superoxide radical and Vc, vitamin C



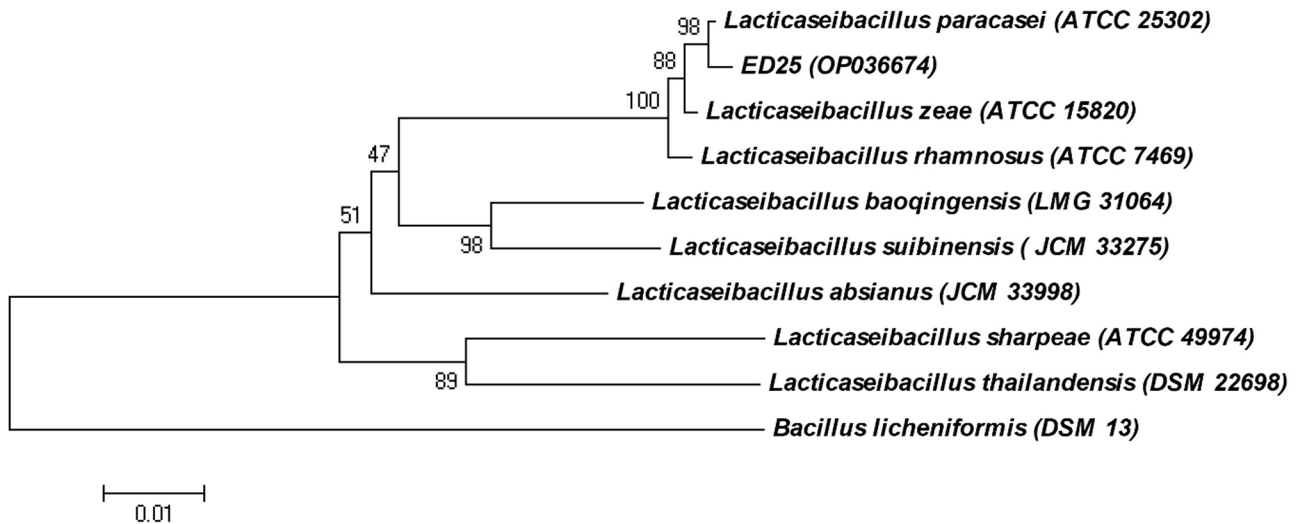


Fig. 2 Neighbour joining phylogenetic tree based on 16 S rRNA gene sequence data of the isolate ED25. Bootstrap values based on 1000 replications are listed as percentages at branching points. The acces-

sion numbers are given in parenthesis. Only bootstrap values > 50% are shown at nodes. The scale bar represented 1% divergence

Lacticaseibacillus paracasei) when compared to the data of GenBank and EzTaxon.

Lactobacillus paracasei is present in healthy individuals' intestinal microbiota (Bretto et al. 2022). Some strains of the bacterium can be also isolated from foods such as cheese and fermented milk, and the isolated strains can be used as probiotic for humans (Stefanovic et al. 2018; Mangia et al. 2019). Earlier studies have demonstrated that *L. paracasei* strains are resistant to gastrointestinal conditions, have no haemolytic activity and toxicity, and are capable of producing B12 and lowering cholesterol levels (Qureshi et al. 2020; Tarrah et al. 2021; Torres-Miranda et al. 2022). Moreover, previous studies have displayed that the strains of this species have antimicrobial activity against pathogens (*S. aureus*, *E. coli*, *S. Typhimurium*, *L. monocytogenes* and *C. albicans*) (Verdenelli et al. 2009; Bendali et al., 2014; Jam et al. 2020). Similarly, the present experiments revealed that the isolate ED25 which was identified as *L. paracasei* possessed the properties sought in a probiotic microorganism.

Conclusions

The present study revealed that *L. paracasei* subsp. *paracasei* ED25 (*Lacticaseibacillus paracasei*) isolated from Turkish white cheese (made from cow's milk) is a non-haemolytic strain (γ -hemolysis), which has a low antibiotic resistance, resists the simulated-gastrointestinal conditions (low pH, pepsin, pancreatin and bile salt tolerance), produces B12 vitamin and lactase, removes cholesterol, inhibits pathogens and exhibits in vitro antioxidant activities.

Due to these potential properties, the isolate may be used for the preparation of probiotic formulations. For example, it may be considered as a probiotic agent against gastrointestinal infections due to its antimicrobial potential, lactose intolerance due to its lactase-producing ability, high cholesterol-linked cardiovascular diseases due to its cholesterol-lowering ability, and oxidative stress-induced neurodegenerative diseases and gastrointestinal disorders due to its antioxidant properties. However, further in vivo studies are needed to fully say that ED25 can be used as a probiotic in the field of health.

Authors contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Elanur Dasedemir, Nazli Pinar Arslan, Serkan Ortucu, Gurkan Aykutoglu, Hakan Ozkan, Ahmet Adiguzel and Mesut Taskin. The first draft of the manuscript was written by Mesut Taskin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data are included in this manuscript.

Declarations

Ethics approval This study does not contain any experiments with human participants or animals.

Conflict of interest The author declares that they have no conflict of interest.

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