Hindawi
Journal of Food Quality
Volume 2019, Article ID 4693513, 12 pages
https://doi.org/10.1155/2019/4693513

Research Article

Preliminary *In Vitro* Evaluation of the Probiotic Potential of the Bacteriocinogenic Strain *Enterococcus lactis* PMD74 Isolated from Ezine Cheese

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Received 21 January 2019; Revised 28 March 2019; Accepted 30 April 2019; Published 19 May 2019

Academic Editor: Barbara Speranza

Ezine cheese is a nonstarter and long-ripened cheese produced in the Mount of Ida region of Çanakkale, Turkey, with a protected designation of origin (PDO) status. The nonstarter fermented foods serve as sources for the isolation of novel strains. The present study aimed to report the novelty of the bacteriocinogenic *Enterococcus lactis* PMD74 strain and the *in vitro* assessment of its potential as a probiotic candidate. Additionally, the present study aimed to describe the technological and safety-related properties of the aforementioned strain. The strain exhibited high viability at pH 3.0, in the presence of pepsin, pancreatin, and bile salts (0.3% and 0.5%), and considerable survival passage through the stimulated digestion tests. The strain PMD74 exhibited substantial autoaggregative (41%) and coaggregative properties, which increased as a function of time. The highest coaggregation percentage was obtained with *Salmonella enterica* serotype Typhimurium SL1344 (23%), followed by *Staphylococcus aureus* ATCC 6538 (10.3%) and *Escherichia coli* ATCC 26922 (7.4%), respectively. The strain PMD74 was able to inhibit the growth of a number of Gram-positive bacteria, including *Listeria monocytogenes*, *Lactobacillus sake*, *Staphylococcus aureus*, and *E. faecalis*. The antimicrobial activity of the proteinaceous compound was calculated as 6400 $AU \cdot mL^{-1}$ by the critical dilution method against *E. faecalis* ATTC 29212. γ-Hemolytic PMD74 was observed to be sensitive to vancomycin, ampicillin, penicillin, gentamicin, tetracycline, chloramphenicol, and tylosin. Among the four genes tested, *E. lactis* PMD74 was observed to be positive for three virulence determinants, *ace*, *sprE*, and *gelE*, and negative for *esp*. The amino acid decarboxylase activities were detected negative for histidine, tyrosine, and ornithine. *E. lactis* PMD74 was classified as a low acidifier, which suggested its possible role as an adjunct culture. *E. lactis* PMD74 exhibited considerable survival ability (8.86 log $CFU \cdot mL^{-1}$) in the acidic condition of fermented milk for a four-week-long storage period.

1. Introduction

The bacteria belonging to genus *Enterococcus* form the third largest group of lactic acid bacteria (LAB), with the genera *Lactobacillus* and *Streptococcus* being the first and the second largest ones. The members of this group are Gram-positive, facultative anaerobic, and coccus-shaped, and were initially classified as group D streptococci. These bacteria are able to grow in the presence of 6.5% NaCl, 40% bile salts, and at pH 9.6 and are able to survive for 30 min at 60°C [1]. To date, 58 species and 2 subspecies have been identified and classified within the genus *Enterococcus* [2]. The species belonging to the genus *Enterococcus*, *Enterococcus faecalis* and *Enterococcus faecium* in particular (with counts ranging from 10^5 to 10^7 $CFU \cdot g^{-1}$ and from 10^4 to 10^5 $CFU \cdot g^{-1}$, respectively), constitute a large proportion of the autochthonous flora of mammalian gastrointestinal (GI) tract [3]. Enterococci are able to form colonies in various niches, such as soil, water, sewage, and plants, because of their exceptional ability of resistance and growth in the extraenteric environments [4]. Enterococci may contaminate raw milk or meat through intestinal or environmental contamination and may become an important part of the fermented food microflora due to their ability to

survive in harsh conditions, such as extreme pH and temperatures, and salinity [3, 4].

The species belonging to the genus *Enterococcus* may present as nonstarter LAB flora in a variety of traditional cheese prepared from both raw and pasteurized milk, such as Cheddar, Feta, Water-buffalo Mozarella, Cebreiro, Venaco, and Hispanico [1, 3]. It has been reported that the count of enterococci in the traditional cheese from Mediterranean countries may range from 10^4 to 10^6 CFU·g^{-1} in curd and from 10^5 to 10^7 CFU·g^{-1} in fully ripened cheese [3]. Enterococci contribute to ripening and the development of aroma in cheese, through the processes of proteolysis, lipolysis, and citrate breakdown [1]. Additionally, the ability of enterococci to produce antimicrobial substances such as lactic acid, hydrogen peroxide, and bacteriocins (enterocins) provides a great advantage to the food they are present in [4, 5]. It has been well documented that the use of bacteriocins as biopreservatives extends the shelf life of foods and inhibits the proliferation of harmful bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* [4, 6]. In addition to the studies emphasizing the aforementioned technological properties of enterococci, several types of research have focused on the potential probiotic properties and the health-promoting effects of enterococci [5, 7–9]. According to FAO/WHO [10], several probiotic strains (mainly from the *Lactobacillus* and *Bifidobacterium* spp.) have been investigated in terms of their ability to survive during passage through the GI tract of the consumer, as well as for their ability to conserve their beneficial properties during the production and commercialization of the product containing them.

The strains of enterococci have been used as probiotics to a considerably smaller extent. *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany) and *E. faecium* SF68 (NCIMB 10415; Cerbios-Pharma SA, Barbengo, Switzerland) have been the established enterococcal probiotics and produced in the form of pharmaceutical preparations [4, 11]. Although the technological and health-promoting properties of enterococci portray these bacteria as an interesting constituent for the development of starter or coculture in the production of fermented foods, the genus *Enterococcus*, contrary to the other LAB, is not considered "generally recognized as safe" (GRAS) [12]. The safe use of enterococci, which are also known to be opportunistic pathogens, remains controversial as these bacteria are one of the major causes of nosocomial infections [4, 13]. The presence of virulence factors, which are carried mainly by *E. faecalis* and to a lesser extent by *E. faecium*, has also been a cause for concern [14]. In this respect, the research works on the isolation of novel enterococcal strains other than *E. faecalis* as well as on their biotechnological and safety potentials hold great importance, especially in the Mediterranean nations. *E. lactis*, which was first isolated in Russian sour milk products [15], and later in South African fresh sheep milk [16], was described by Morandi et al. [17] as a novel species belonging to genus *Enterococcus*. In several studies conducted by different research groups, the biotechnological, probiotic, and safety-related properties, as well as the bacteriocin-producing potential of *E. lactis*, have been investigated [6, 8, 9, 18, 19].

Ezine cheese is a type of nonstarter, long-ripened white cheese with a protected designation of origin (PDO) status. Ezine cheese is produced from a mixture of the pasteurized ewe (approximately 45%–55%), goat milk (at least 40%), and cow milk (at most 15%), in the Mount of Ida region of Çanakkale, Turkey [20]. It is well known that the nonstarter fermented foods serve as sources for the isolation of both potential probiotic and potential bacteriocinogenic novel strains [14]. The present study aimed to report (i) the novelty of the bacteriocinogenic *E. lactis* strain isolated from Ezine cheese (PDO), (ii) the *in vitro* assessment of the potential of this strain as a probiotic candidate, and (iii) the details of the technological and safety properties of the strain. The present study, to the best of our knowledge, is the pioneer study on the isolation of an *E. lactis* strain from dairy products in Turkey and on the evaluation of the probiotic and bacteriocinogenic potentials of the strain. The findings of the present study suggest potential applications of this strain as a novel probiotic strain or/and a biopreservative in the food industry.

2. Materials and Methods

2.1. Bacterial Strains and Growth Media. Table 1 enlists the source and the growth conditions of the indicator strains that were used in the present study. All the indicator strains were stored at −80°C in their corresponding suitable broths supplemented with 30% glycerol. The working cultures for each strain were prepared by transferring the respective frozen culture to the appropriate broth, followed by overnight incubation.

2.2. Isolation and Identification of the Potential Probiotic Strain. Ezine cheese samples were homogenized and diluted in sterile ¼ strength Ringer's solution (Merck, Germany) and were plated on kanamycin aesculin azide agar (KAA, Merck, Germany), followed by aerobic incubation at 37°C for 48 h. Subsequent to incubation, colonies with morphology typical to *Enterococcus* were selected randomly and spotted onto plates containing brain heart infusion (BHI; Merck, Germany) agar using sterile toothpicks. After an overnight incubation, 5 mL BHI soft agar (0.7%, w/v) containing 10^6 CFU·mL^{-1} count of the indicator strains (*E. faecalis* ATCC 29212 and *E. faecalis* LMG2708) was poured into the plates, followed by further incubation [21]. The colony exhibiting a clear inhibition zone (against the indicator strain) larger than 10 mm in diameter was isolated, purified using same media, and then was stored in BHI broth containing 20% (v/v) glycerol at −20°C for further investigations. Gram staining, catalase reaction, growth at different temperatures (10 and 45°C) and at pH 9.6, and growth in the presence of NaCl (6.5%), bile salt (40%), and aesculin hydrolyse (Bile Aesculin Agar; Merck, Germany) were the experiments that were performed for the preliminary identification of the strain. In order to determine the carbohydrate utilization profile of

Table 1: Indicator strains and the antimicrobial activity spectrum of *E. lactis* PMD74.

Strains	Source[a]	Growth conditions[b]	Antimicrobial activity[c]	
			Agar spot test	Well diffusion
Lactobacillus plantarum LMG2003	NLH	MRS, 37°C	+++	NZ
Lactobacillus sake NCDO2714	NLH	MRS, 37°C	+++	+++
Lactococcus lactis SIK83	NLH	M17, 30°C	NZ	NZ
Pediococcus pentosaceus FBB611	NLH	MRS, 37°C	+++	++
Pediococcus pentosaceus BH105	AUFF	MRS, 37°C	NZ	NZ
Bifidobacterium longum CHL28	NLH	MRS, 37°C	++	NZ
Bacillus cereus FM1	NLH	LB, 37°C	NZ	NZ
Listeria monocytogenes ATCC 7644	AUFF	LB, 37°C	+++	+++
Staphylococcus aureus ATCC 6538	AUFF	LB, 37°C	+++	++
Salmonella enterica serotype Typhimurium SL1344	AUFF	LB, 37°C	+++	NZ
Micrococcus luteus NCIMB8166	AUFF	LB, 37°C	NZ	NZ
Enterococcus faecalis LMG2708	NLH	BHI, 37°C	+++	++
Enterococcus faecalis ATCC 29212	AUFF	BHI, 37°C	+++	++
Escherichia coli ATCC 26922	AUFF	LB, 37°C	+++	++
Escherichia coli LMG3083 ETEC	NLH	LB, 37°C	+++	++

[a]NLH: Agricultural University of Norway; AUFF: Faculty of Science, University of Ankara, Tukey. [b]MRS: de Man Rogosa Sharpe (Merck, Germany); BHI: brain heart infusion (Merck, Germany); LB: Luria Bertani (Merck, Germany); M17 (Merck, Germany). [c]NZ: no inhibition zone (diameters of inhibition zones are +: 1–5 mm, ++: 6–10 mm, and +++: ≥11 mm).

the isolate, API 20 Strep system (BioMérieux Inc., France) was used, in accordance with the manufacturer's recommendations, and the result was analyzed using APILAB Plus version 1.2.1 (BioMěrieux Inc., France). Genetic identification of the isolate was performed using 16S rRNA sequence analysis. In this analysis, the genomic DNA of the samples was isolated according to the method described by Cancilla et al. [22], and the region between nucleotide 8 and nucleotide 928 in the 16S rDNA gene was amplified through polymerase chain reaction (PCR) using universal primers (Table 2). The PCR product thus obtained was sequenced by MedSanTek (Istanbul, Turkey) after post purification (Thermo Fischer Scientific, USA). Basic local alignment search tool (BLAST) was employed to compare the similarity of the sequences against the nucleotide database available in the National Centre for Biotechnology Information (NCBI). All the tests were performed in duplicate in order to confirm their reproducibility and *E. faecalis* ATCC 29212 was used as the control for both biochemical and genetic identification.

2.3. In Vitro Evaluation of Probiotic Properties and Safety of the Strain

2.3.1. Tolerance of Strain through the Simulated Gastrointestinal Tract. The tolerance of the selected strain through the human upper GI system was assessed by using an *in vitro* model that stimulated gastric and pancreatic juices, as described previously in a report by Conway et al. [27]. Briefly, bacterial cells in their logarithmic phase were harvested through centrifugation (10,000 × g for 5 min at 4°C) and washed three times with phosphate buffered saline (PBS; pH 7.2). In order to determine tolerance to pH, the cells were resuspended in PBS with pH 2.0 and 3.0 (pH adjusted using 5 mmol·L^{-1} HCl) and the tolerance to pepsin was determined by resuspending the cells in PBS containing pepsin (3 mg·mL^{-1}; Merck) at pH 2.0 and 3.0. The resistance was evaluated in terms of viable colony counts (expressed as Log CFU·mL^{-1}) using plate count on MRS agar after incubation at 37°C for 0, 1, 2, and 3 h, the time durations reflecting the time spent by food in the stomach. Resistance to pancreatin (1 mg·mL^{-1}, pH 8.0; Merck, Germany) and resistance to 0.3% and 0.5% (w/v) bile salts (Sigma-Aldrich, Germany) were assessed in order to determine the tolerance of the *E. lactis* PMD74 strain to the intestinal juice [28].

Transit tolerance of the strain in the digestive system was determined through the sequential incubation of the bacterial suspension in solutions simulating oral cavity, gastric compartment, and intestinal compartments [29]. Briefly, bacterial cells prepared as described earlier were incubated with lysozyme solution at a final concentration of 100 ppm for 5 min in order to simulate the possible hydrolysis in the oral cavity. Subsequently, the cells were harvested and incubated with simulated gastric fluid (PBS containing 3 mg·mL^{-1} pepsin at pH 3.0) for 90 min. Finally, following the incubation at 37°C, the cells were harvested and resuspended in PBS containing 1 mg·mL^{-1} pancreatin and 0.3% or 0.5% (w/v) bile salt at pH 8.0, which simulated intestinal digestion conditions. The samples were incubated at 37°C for 150 min. The cell viability was evaluated through plate counting using the samples collected prior to and after oral, gastric, and intestinal digestion. The experiments were performed three times independently.

2.3.2. Bile Salt Hydrolysis (BSH) and Mucin Degradation Activity. The overnight bacterial culture was grown on MRS agar containing 0.5% (w/v) taurodeoxycholic acid (TDCA; Sigma-Aldrich, Germany) using the streaking method. After 48 h of incubation at 37°C, the hydrolysis effect was indicated by a colony morphology different (partial hydrolysis) from that observed in the control MRS plates [30].

The potential of the strain to degrade gastric mucin was *in vitro* evaluated following the plate procedure developed by Zhou et al. [31]. *Salmonella enterica* ssp. *Enterica* serovar Typhimurium ATCC 14028 was used as positive control. The experiments were performed three times independently.

Table 2: Primers and PCR conditions used for the 16S rRNA and virulence genes.

Target gene	Primer sets	Product size (bp)	Reference
16S rRNA	F: CCGTCAATTCCTTTGAGTTT R: AGAGTTTGATCCTGGCTCAG	928	[23]
esp	TE34: TTGCTAATGCTAGTCCACGACC TE36: GCGTCAACACTTGCATTGCCGAA	933	[24]
ace	F: GAATTGAGCAAAAGTTCAATCG R: GTCTGTCTTTTCACTTGTTTC	1008	[25]
sprE	F: TTGAGCTCCGTTCCTGCCGAAAGTCATTC R: TTGGTACCGATTGGGGAACCAGATTGACC	591	[26]
gelE	TE9: ACCCCGTATCATTGGTTT TE10: ACGCATTGCTTTTCCATC	419	[24]

2.3.3. Determination of Cell Surface Characteristics. The autoaggregation (Auto-A%) and coaggregation (Co-A%) abilities of the strain were assessed using the method described previously by Collado et al. [32] and were expressed as the percentage of the total number of bacteria present. Briefly, the stationary-phase culture was centrifuged (6000 × g, 15 min), washed, and resuspended in PBS to reach a count of 10^8 CFU·mL^{-1}, followed by incubation in aliquots at room temperature (20 ± 1°C). In order to determine the percentage of autoaggregation, absorbance was monitored at different time points (4, 18, and 24 h) in a spectrophotometer (Shimadzu, Japan) at a wavelength of 600 nm and was calculated by using the following equation:

$$\text{Auto-A}\,(\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100, \quad (1)$$

where A_t is the absorbance at 600 nm at time point t and A_0 is the absorbance at time zero.

In order to evaluate the coaggregation percentage, *E. coli* ATCC 26922, *S. enterica* serotype Typhimurium SL1344, and *S. aureus* ATCC 6538 were used as the coaggregation partners. Cell suspensions were prepared as described earlier, and equal volumes (2 mL) of the cell suspensions of presumptive probiotic and pathogenic strains were mixed and incubated at room temperature (20 ± 1°C) for 4 h without agitation. Cell suspensions of each strain were used as controls. The absorbance of the probiotic (pro) and pathogenic (pat) strains in the control and mixture (mix) tubes was measured at 600 nm. Percentage of coaggregation was calculated using the following equation:

$$\text{Co-A}\,(\%) = \left[\left(\frac{A_{\text{pro}} + A_{\text{pat}}}{2}\right) - \left(\frac{A_{\text{mix}}}{\left(A_{\text{pro}} + A_{\text{pat}}\right)/2}\right)\right] \times 100. \quad (2)$$

Standard deviations were derived from the coaggregation values of three independent experiments.

2.3.4. Screening of Antimicrobial Activity. Agar spot test [33] and well diffusion [34] methods were used to evaluate the antimicrobial activity of the strain. Briefly, colonies from an overnight culture were seeded onto the surface of MRS agar and incubated at 37°C for 24 h. The bacterial strains used as indicators (Table 1) were inoculated (approximate count: 10^5–10^6 CFU·mL^{-1}) into BHI, MRS, M17, and LB soft agar and poured over the plates. In order to perform the well diffusion assay, the cell-free supernatant (CFS) of an overnight culture was obtained by centrifugation (10,000 × g, 15 min, 4°C). Subsequent to the pH adjustment to 6.5 using 1 mmol·L^{-1} NaOH, the CFS was sterilized using 0.45 μm pore size membrane filter (Millipore, France). The sterilized CFS (100 μL) was placed into wells that were cutout on the medium containing the indicator strains in the plates. After 24 h of incubation at the optimal growth temperature of the respective indicator strains, the plates were examined for inhibition zones. The diameters (mm) of the inhibition zones were scored as: NZ (No inhibition zone), + (a clear zone of 1–5 mm), ++ (a clear zone of 6–10 mm), and +++ (a clear zone of ≥11 mm). In order to determine the proteinous structure of the CFS, it was treated with the proteolytic enzymes proteinase-K (Thermo Fisher Scientific, USA), trypsin (Sigma-Aldrich, Germany), and catalase (Sigma-Aldrich, Germany), at a final concentration of 1 mg·mL^{-1}, against the indicator strain *E. faecalis* ATCC 29212. The bacteriocinogenic activity was measured using the critical dilution method proposed by Daba et al. [35]. Three independent experiments were performed.

2.3.5. Antibiotic Susceptibility and Hemolytic and Gelatinase Activity. The antibiotic susceptibility of the *E. lactis* PMD74 strain was determined using the disk diffusion method on Mueller–Hinton Agar (Merck) as described previously [36]. The antibiotics, which were selected according to the recommendations provided by the European Food Safety Authority (EFSA) [37], are listed in Table 3. The level of susceptibility to the tested antibiotics was expressed as resistant, intermediate, or sensitive, according to the guidelines provided by the Clinical and Laboratory Standards Institute [38] and EFSA [37].

The overnight culture was grown on the surface of Columbia blood agar plates (Oxoid) containing 5% (w/v) sheep blood using the streaking method, and the plates were then incubated at 37°C for 48 h. The plates were subsequently examined for the hemolytic reaction.

To determine the presence of gelatinase activity, the overnight culture was streaked on the surface of Todd–Hewitt agar (Merck, Germany) containing gelatin (30 g·L^{-1}; Merck, Germany) and the plates were incubated overnight at 37°C. The zones of turbidity around the colonies were examinated.

Table 3: Antibiotic susceptibility of *E. lactis* PMD74 and *S. aureus* ATCC 6538.

Group number[a]	Antibiotic	Concentration (μg/mL) (Oxoid)	Strains[b]	
			E. lactis PMD74	*S. aureus* ATCC 6538
I	Penicillin G	10 U	S	R
	Ampicillin	10	S	R
	Vancomycin	30	S	R
II	Gentamicin	10	S	R
	Kanamycin	30	R	R
	Streptomycin	10	R	R
	Tetracycline	30	S	S
	Chloramphenicol	30	S	S
	Erythromycin	15	I	R
	Clindamycin	2	R	R
	Tylosin	30	S	R
III	Rifampicin	5	S	R

[a]Group I: cell wall synthesis inhibitors; group II: protein synthesis inhibitors; group III: nucleic acid synthesis inhibitors. [b]Susceptibility is expressed as resistant (R), intermediate (I), and sensitive (S) (CLSI, 2012, EFSA 2018).

In both experiments, *S. aureus* ATCC 6538 was used as the control strain, and the tests were performed in triplicate.

2.3.6. Screening for the Presence of Virulence Genes. The PCR screening of the *E. lactis* PMD74 strain for virulence genes was conducted targeting specific virulence factors *esp* (enterococcal surface protein), *ace* (collagen-binding protein), *sprE* (serine protease), and *gelE* (gelatinase) using the primer sets listed in Table 2. *E. faecalis* ATCC 29212 and OG1RF strains were used as positive controls. Genomic DNA was isolated from the overnight cultures as described earlier. The amplification products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 mg·mL^{-1}) in 0.5X TAE buffer for 30 min at 100 V and were visualized in UV light using Kodak Gel Logic 200 Imaging System (Kodak, USA). The O'GeneRuler 100 bp DNA molecular weight ladder (Fermentas, Finland) was used as the standard for size.

2.3.7. Amino Acid Decarboxylase Detection. Modified decarboxylase base broth medium, the preparation of which has been described previously by Maijala [39], was used for the determination of the decarboxylase activity of the potential probiotic strain. The corresponding amino acids (L-histidine monohydrochloride (Sigma-Aldrich, Germany), L-tyrosine (Sigma-Aldrich, Germany), L-ornithine monohydrochloride (Sigma-Aldrich, Germany), and L-arginine monohydrochloride (Merck, Germany)) were added individually to the medium at a final concentration of 0.5% (w/v), and purple bromocresol was added as the pH indicator. The overnight culture was inoculated (2%) into the same medium with or without the corresponding amino acids. The amino acid-free medium was used as the control. After incubation at 37°C for three days, while the color was yellow in the control tubes, the conversion of the color to purple in the tubes containing amino acids was considered an amino acid decarboxylase positive reaction. The experiments were performed in triplicate independently.

2.4. Technological Properties

2.4.1. Acidifying Activity and Viability in Fermented Milk. The strain was evaluated for its acidifying activity, which is the ability to produce fermented milk, and for its viability, using the methods proposed by Morandi et al. [14] and Chiu et al. [40], respectively.

The acidifying ability was assessed in skimmed milk (Merck, Germany) by measuring using the pH meter (Sartorius PB 21, USA) during 24 h of incubation at 37°C, and the acidification rate was calculated using the following formula:

$$\Delta\text{pH} = \text{pH zero time} - \text{pH after certain time} \quad (3)$$

The values of ΔpH at 6 h and 24 h were used to evaluate the acidifying ability of the strain.

The overnight bacterial culture was inoculated (2%, v/v) into skimmed milk (4%, w/v) that had been previously sterilized and cooled to room temperature. The inoculated milk was then incubated at 37°C for 24 h to allow fermentation, followed by storage at 4°C for four weeks. During the storage period, the fermented milk was examined every week for the viability of bacteria and changes in pH.

2.4.2. Growth at Different pH Values and NaCl Concentrations. The tolerance to different pH values and NaCl concentrations was tested in the MRS broths with pH ranging from 2.0 to 10.0 and in the MRS broths containing increasing concentrations of sodium chloride (1%, 2%, 3%, 4%, 5%, and 6%), respectively. The aforementioned broths were inoculated with fresh culture and incubated at 37°C for 48 h. At 24 and 48 h of incubation, absorbance was measured at 600 nm. Cultures grown in commercial MRS broth were used as a control, and experiments were performed in triplicate independently.

2.5. Statistical Analysis. All statistical analyses were performed using the SPSS program (version 17, SPSS Inc., Chicago, IL, USA). Means and standard deviations were calculated, and significant differences were determined using ANOVA at the probability level of $P < 0.05$. The mean differences were compared using Tukey's test.

3. Results

3.1. Isolation and Identification of E. lactis PMD74 Strain. A total of 114 colonies exhibiting the typical morphology of *Enterococcus* on KAA agar were transferred onto BHI Agar. It was determined that eighty-four colonies exhibited inhibition zones larger than 10 mm against the indicator strains (results not shown). Among these colonies, PMD74 was selected for further experiments as it exhibited the highest antimicrobial activity. The characteristic properties (Gram-positive, catalase-negative and aesculin-positive, coccus-shaped isolate that was able to

grow effectively at 10–45°C, pH 9.6, and in the presence of both NaCl and bile salts) of the strain were consistent with the general characteristics of genus *Enterococcus* [17]. According to the results of the API 20 Strep test, the isolate was observed to generate a positive reaction for Voges–Proskauer, pyrrolidonyl arylamidase, and leucine aminopeptidase, while it was negative for α-galactosidase, β-glucuronidase, and hippurate. Additionally, the strain possessed the ability to produce acid from arabinose, lactose, L-mannitol, ribose, and trehalose, while acid production from glycogen, inulin, raffinose, and sorbitol was observed. The strain possessed weak acid production ability from starch. The finding that the PMD74 strain did not produce gas from sucrose, while it produced gas from salicin, also supported the proposition that PMD74 was a different strain than *E. hirae* [17].

According to the complete 16S rRNA gene sequence analysis, which was performed in order to ensure molecular identification, the isolate was designated as an *Enterococcus lactis* strain. *E. lactis* PMD74 was deposited in the NCBI GenBank database under the accession number MK318965–66.

3.2. In Vitro Evaluation of Probiotic Properties

3.2.1. Survival under Conditions Simulating Human GI Tract. In the present study, survival of *E. lactis* PMD74 was evaluated in both gastric and intestinal juices. Resistance to pH 2 and pepsin (at pH 2) was observed only with the lower counts (<2 log CFU·mL^{-1}) in the initial time points of the treatment (data not shown). However, the viable counts in PBS with pepsin and in PBS without pepsin, both adjusted to pH 3, remained almost unaltered, after three hours of treatment. The results demonstrated that *E. lactis* PMD74 was able to survive at all the concentrations of the bile salts tested and exhibited an exponential growth during the 4 h of incubation after inoculation. No significant differences were observed between the control and treatments with different concentrations of bile salts. However, treatment with pancreatin (pH 8) resulted in a 2 log loss in the cell viability compared to control (Table 4).

3.2.2. Bile Salt Hydrolysis. PMD74 was observed to exhibit partial bile salt hydrolase activity, which was recorded as differentiated colony morphology on the TDCA-MRS agar in comparison to the control MRS agar plates (Table 5). No significant difference was observed among the independent experiments ($P > 0.05$).

3.2.3. Determination of Cell Surface Characteristics. The strain PMD74 exhibited considerable ($P > 0.05$) autoaggregation characteristic, which increased as a function of time and was at its highest (41%) at the time point of 24 h. The strain also demonstrated great coaggregative properties with all the tested foodborne pathogens after 4 h of incubation at 20°C and exhibited the highest coaggregation percentage of 23% with *S. enterica* serotype Typhimurium SL1344, followed by 10.3% with *S. aureus* ATCC 6538 and 7.4% with *E. coli* ATCC 26922 (Table 5).

Table 4: Survival of *E. lactis* PMD74 strain in the simulated gastric and intestinal juices and digestion system (log CFU·mL^{-1}).

	Time (min)	*E. lactis* PMD74
Simulated gastric juice control		
pH 3	60	8.63 ± 0.17^a
	120	8.64 ± 0.08^a
	180	8.73 ± 0.07^a
Pepsin (3 mg·mL^{-1}, pH 3)	60	8.73 ± 0.08^a
	120	8.34 ± 0.09^a
	180	8.53 ± 0.03^a
	60	7.80 ± 0.21^a
	120	7.50 ± 0.11^a
	180	7.50 ± 0.05^a
Simulated intestinal juice control		
Bile salt (0.3%)	60	8.64 ± 0.08^a
	240	8.50 ± 0.06^a
Bile salt (0.5%)	60	8.50 ± 0.19^a
	240	8.30 ± 0.02^a
Pancreatin (1 mg·mL^{-1}, pH 8.0)	60	8.56 ± 0.11^a
	240	7.11 ± 0.01^a
	60	6.60 ± 0.16^a
	240	6.47 ± 0.08^a
Simulated digestion system control		
	0	8.90 ± 0.05^a
	240	8.87 ± 0.07^a
Oral cavity	5	9.04 ± 0.03^a
Gastric digestion	90	7.70 ± 0.12^a
Intestinal digestion	150	6.85 ± 0.08^a

± indicates standard deviation of three replicates. The values with the same letter (a) are not statistically significant ($P > 0.05$).

Table 5: Probiotic and technological properties of *E. lactis* PMD74.

	E. lactis PMD74
BSH activity	+
Mucin degradation activity	−
Gelatinase activity	−
Autoaggregation (%)	
T_4	11 ± 0.98
T_{20}	33 ± 1.00
T_{24}	41 ± 1.00
Coaggregation (%)*	
Salm. enterica serotype Typhimurium SL1344	$23\% \pm 2.00$
S. aureus ATCC 6538	$10.3\% \pm 1.70$
E. coli ATCC 26922	$7.4\% \pm 0.59$
Hemolytic activity	γ-Hemolytic
Amino acid decarboxylase detection	
Histidine decarboxylase	−
Tyrosine decarboxylase	−
Ornithine decarboxylase	−
Arginine decarboxylase	+
Acidification activity	
ΔpH_6	0.8 ± 0.09
ΔpH_{24}	1.38 ± 0.09

± indicates standard deviation of three replicates. *The result is expressed as the percentage (%) of coaggregated bacteria with the tested foodborne pathogens after 4 h incubation at 20°C.

3.2.4. Evaluation of Antimicrobial Activity. The antimicrobial activity of the strain *E. lactis* PMD74 against fifteen Gram (+) and Gram (−) indicator strains was screened by using the agar spot assay and well diffusion method. In the agar spot assay, a broad antimicrobial activity spectrum, as presented in Table 1, was determined against certain bacteria including the Gram (−) bacteria ($P < 0.05$). Neutralized CFS of *E. lactis* PMD74 exhibited antimicrobial activities against closely related as well as against unrelated Gram (+) bacteria. It was noteworthy that the highest activity of the neutralized CFS was detected against *L. monocytogenes* ATCC 7644. The antimicrobial activity against *Lb. plantarum* LMG2003, *B. longum* CHL28, and *S. enterica* serotype Typhimurium SL1344 observed in the agar spot assay could not be determined with the well diffusion assay. The treatment of neutralized CFS of *E. lactis* PMD74 against *E. faecalis* ATCC 29212 with proteinase-K and trypsin resulted in a complete loss of the antimicrobial activity. However, no alteration in the antimicrobial activity was observed with catalase treatment (data not shown). The antimicrobial activity of this proteinaceous substance was estimated to be 6400 AU/mL.

3.3. Safety Assessment

3.3.1. Antibiotic Susceptibility and Hemolytic and Mucin Degradation Activity. The results of the antibiotic susceptibility test for *E. lactis* PMD74 are summarized in Table 3. *E. lactis* PMD74 exhibited susceptibility to all cell wall synthesis inhibitors tested in the present study. The strain was also observed to be susceptible to the protein synthesis inhibitors, such as gentamicin, tetracycline, chloramphenicol, and tylosin as well as to the nucleic acid synthesis inhibitors. It exhibited intermediate sensitivity to erythromycin, while resistance to the antibiotics such as kanamycin, streptomycin, and clindamycin.

The strain *E. lactis* PMD74 did not show mucin degradation activity and exhibited γ-hemolysis after 48 h of incubation on blood agar plates (Table 5).

3.3.2. Screening for the Presence of Virulence Genes. In order to evaluate the potential pathogenicity of the *E. lactis* PMD74 strain, the presence of genes encoding the enterococcal surface protein (*esp*), adhesion of collagen (*ace*), serine protease (*sprE*), and gelatinase (*gelE*) was tested using the PCR method (Figure 1). Among the four genes tested for, *E. lactis* PMD74 was observed to be positive for three virulence determinants, *ace*, *sprE*, and *gelE*, and negative for *esp*.

3.3.3. Amino Acid Decarboxylase Detection. Arginine decarboxylase activity was detected positive for the strain *E. lactis* PMD74 and negative for the amino acids histidine, tyrosine, and ornithine (Table 5). No significant difference was observed among the independent experiments ($P > 0.05$).

3.4. Technological Properties. The values of ΔpH for *E. lactis* PMD74 at 6 h (ΔpH_6) and 24 h (ΔpH_{24}), which highlighted the acidifying behavior of the strain, are listed in Table 5.

No decrease in the viable counts of the strain *E. lactis* PMD74 was observed during the four-week-long storage at 4°C. Although the viable cell counts remained almost consistent (ranging between 8.54 and 8.86 Log CFU·mL^{-1}), a gradual decrease in the pH was observed after fermentation, which reached the value of 4.77 at the end of the fourth week (Figure 2). The results for tolerance to different pH values and NaCl concentrations are presented in Figure 3. At pH below 5.0, growth was much slower in the MRS medium compared to the control strain, and it decreased inversely with the increasing concentration of salt.

4. Discussion

The presence of enterococci during the ripening period of the cheese develops the flavor and the specific physicochemical properties of the different varieties of cheese as a result of the proteolytic and lipolytic activities of these bacteria [3]. Previous studies have reported the common presence of enterococcal strains, such as *E. faecium*, *E. faecalis*, *E. durans*, *E. gallinarum*, and *E. italicus*, in Turkish white cheese as a result of contamination from several sources, including poor hygienic conditions [41, 42]. Ezine cheese, Turkish white cheese, contains large biodiversity of spontaneous LAB microbiota and constitutes a source of the isolation for novel strains. To the best of our knowledge, this study is the pioneer in reporting the isolation and identification of *E. lactis* from dairy products in Turkey.

The properties, which are recommended by FAO/WHO [10], of the selection for a strain as a desirable probiotic microorganism are as follows: survival in the GI tract, bile tolerance, bile salt deconjugation, aggregation, and the ability to produce antimicrobial agents against the potential pathogenic bacteria. The primary requirement of a bacterium to be regarded as a probiotic is to survive in high acidic environment in the stomach and in high concentration of bile components in the proximal intestine of the host. The range of pH in the stomach is between 2.5 and 3.5, except during prolonged fasting when it is 1.5 or after a meal when it is 4.5 [43, 44]. The resistance of *E. lactis* PMD74 to simulated gastric juice, bile salts, and pancreatin is parallel to the resistances of *E. italicus*, *E. faecium*, *E. durans*, and *E. lactis* strains in the studies of Fortina et al. [45], Ahmadova et al. [5], Pieniz et al. [46], Morandi et al. [14], and Braïek et al. [9], respectively.

The BSH activity, which has been correlated with cholesterol-lowering effect, is an essential criterion for selection of a strain as probiotic [9]. The positive BSH activity exhibited by *E. lactis* PMD74 and the substantial tolerance to bile salt cause this strain to be a probiotic candidate [8, 9].

Both autoaggregation and coaggregation abilities are considered advantageous properties for the probiotics [7]. In detail, the autoaggregation capacity provides the probiotic strains with the ability to adhere to and colonize the intestinal mucosa. Furthermore, the ability to coaggregate with pathogens is considered important for the host defense mechanism of colonization of the pathogenic bacteria, due to a reduction in the cell-cell distances between the probiotic and the pathogens, which increases the efficiency of the

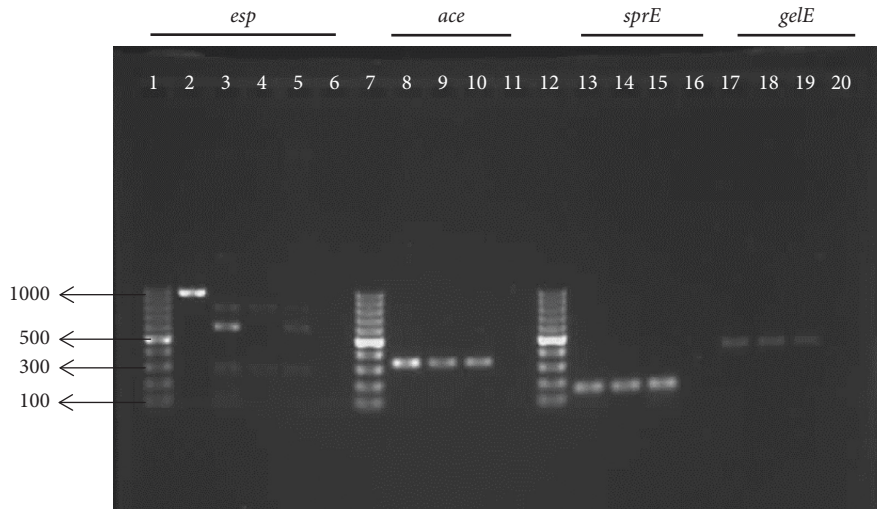

Figure 1: PCR screening for the presence of virulence genes in *E. lactis* PMD 74. Lanes 4, 8, 13, and 17: amplification products of corresponding genes obtained for OG1RF. Lanes 5, 9, 14, and 18: amplification products of corresponding genes obtained for *E. faecalis* ATCC 29212. Lanes 3, 10, 15, and 19: amplification products of corresponding genes obtained for *E. lactis* PMD 74. Lanes 1, 7, and 12: DNA ladder (100 bp, Fermentas).

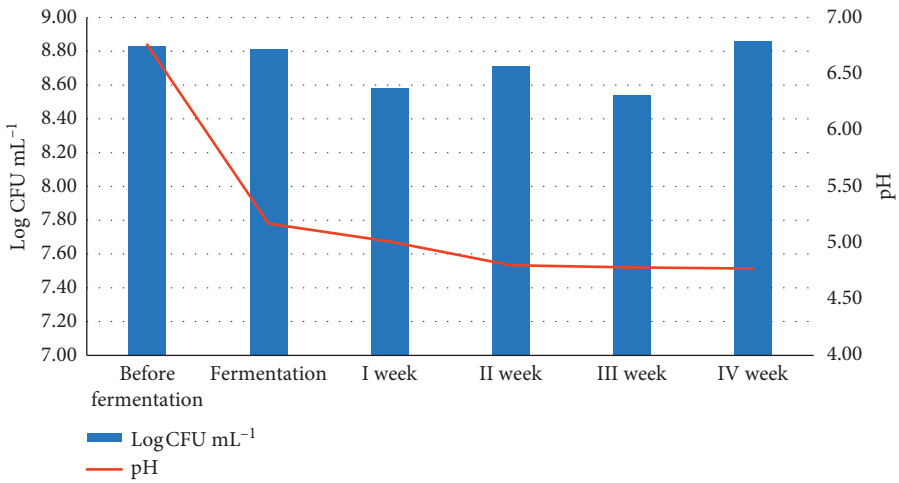

Figure 2: pH and viability in fermented skim milk during storage.

antimicrobial metabolites (organic acids, bacteriocins, etc.) [47]. Autoaggregation rates for different species belonging to genus *Enterococcus* have been reported previously by Ahmadova et al. [5] and Fortina et al. [45]. The autoaggregation rates obtained in the present study for *E. lactis* PMD74 were observed to be higher than those reported for *E. lactis* strains in two previous studies conducted by Braïek et al. [8, 9], in which the rates were in the range of 16%–31% and 20%–36%, respectively. The results of the coaggregation tests were in line with the results reported by Ahmadova et al. [5] and Braïek et al. [8, 9].

Bacteriocin production is another expected property of the probiotic bacteria as it enhances the resistance of them against the resident intestinal flora [48]. In this context, it was observed that *E. lactis* PMD74 inhibited the growth of closely related Gram (+) bacteria as well as the pathogen strains such as *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 6538 at varying levels. Similar spectra of inhibitory activities by different enterococcal strains, including *E. lactis*, have been reported by previous studies as well [3, 5, 8, 9, 42]. The high antilisterial effect observed for the tested strain PMD74 was remarkable and in line with the studies reported previously by Ahmadova et al. [5]. However, observed activity against *L. monocytogenes* in this study was higher than that reported in the studies by Braïek et al. [8, 9]. Interestingly, antimicrobial activity against the tested *E. coli* strains was detected in the present study [8, 9, 42, 49]. However, it has been reported in a few studies that the activity against Gram-negative bacteria is rare [5]. Proteinase-K and trypsin treatments of the neutralized CFS resulted in the loss of antimicrobial activity and showed the bacteriocin-producing potential of *E. lactis* PMD74.

The determinations of antibiotic susceptibility and hemolytic activity are considered as safety aspect for the selection of probiotic strains [10]. The strain PMD74 was observed to be susceptible to the clinically most relevant

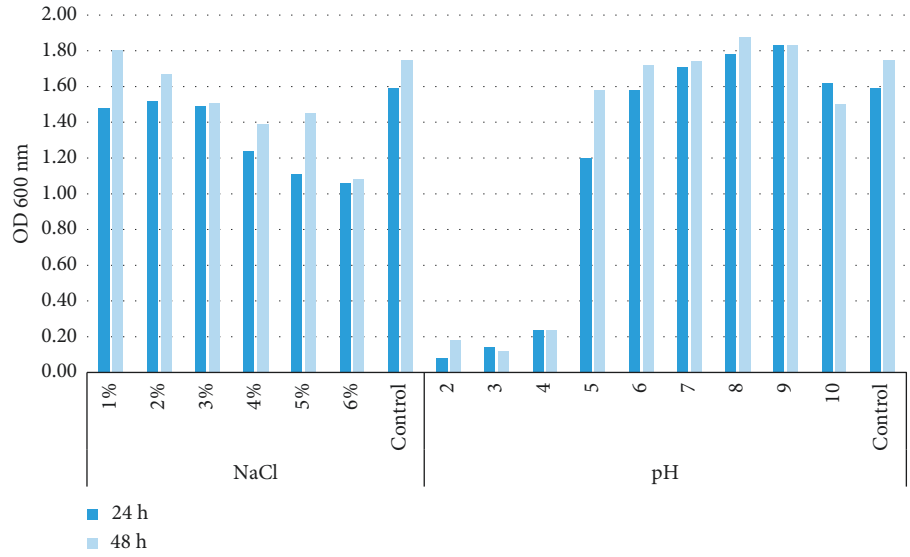

Figure 3: Tolerance to different pH values and NaCl.

antibiotics for curing infections. On the other hand, the observed kanamycin resistance reflects the natural resistance of the *Enterococcus* spp. Similar results have been reported previously for *E. faecium* strains [50] and *E. lactis* strains [8, 9, 14, 19]. As a safety concern, the sensitivity of the novel strains against the commonly used antibiotics is desirable [3]. Although antibiotic-resistant enterococci may be isolated from foods, only a few are resistant to clinically important antibiotics such as ampicillin, penicillin, gentamicin, and vancomycin [1, 51]. The result of the hemolytic activity assessment was in line with the results reported by Pieniz et al. [46] on various species belonging to genus *Enterococcus* (*E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. hirae*) isolated from different sources.

The virulence potential of enterococci requires proper characterization of the wild strains in order to verify their adequacy to be used as probiotics. In the present study, it was determined that *E. lactis* PMD74 did not possess genes coding for *esp*, which is one of the adhesion-associated proteins, although it did possess the *ace* gene. The results of the present study were consistent with the results reported by Morandi et al. [14] and Braïek et al. [8, 9] for *E. lactis*. The *E. lactis* PMD74 strain was observed to be positive for another pathogenicity factor coded by the *gelE* gene, which is associated with gelatinase activity. This result was in accordance with the previous observations for both *E. durans* and *E. faecalis* [7, 42], and in contrast with the observations reported for *E. lactis* by Morandi et al. [14]. On the basis of results of the studies conducted by Carlos et al. [52], Nueno-Palop and Narbad [7] suggested that the identification of the presence of virulence genes using PCR does not imply that the gene is functional. The aforementioned studies indicated that the expression of the virulence genes is dependent on the strain and the environmental factors. However, consistent with our results, Eaton and Gasson [24] demonstrated that even when a virulence gene such as *gelE* is present, it may be silent in the phenotype. On the other side, the expression of the collagen adhesion protein coded by *ace* may confer the ability to adhere to and colonize the eukaryotic cells. The presence of this gene was also revealed in the probiotic strain *E. faecalis* Symbioflor 1, and the gene was interpreted as a probiotic factor that promotes colonization of and persistence in the gastrointestinal tract, rather than a true virulence determinate [53].

Although amino acid decarboxylations are important reactions for sensory changes in fermented foods, from a food safety standpoint, both of these reactions may also lead to the formation of biogenic amines [54]. The production of biogenic amines has traditionally been considered a species-level rather than a strain-level characteristic. The three species of the genus *Enterococcus* (*E. faecium*, *E. faecalis*, and *E. durans*), which are commonly isolated from dairy products, have been identified and reported as tyramine or putrescine producers [3]. The presence of these biogenic amines is considered an obstacle for the possible use of *Enterococcus* as a starter and/or protective culture in fermented foods with long ripening phase, especially the ones in which strong proteolysis occurs [55]. Nonetheless, this ability must not be disregarded when the enterococcal strains are being proposed as probiotics. The fact remains that a quantitative evaluation of biogenic amines rather than a qualitative one has to be used as a criterion for the selection of enterococci. However, the results of the present study indicated that *E. lactis* PMD74 was safe to be used as a probiotic in terms of biogenic amine production.

When the technological properties of *E. lactis* PMD74 were evaluated, remarkable results were achieved. According to Psoni et al. [56], *E. lac*tis PMD74 was classified as a low acidifier as it caused a decrease in the pH lower than 1.5 pH units. Generally, enterococci are classified as poor milk acidifiers. The poor acidifying capacity of the dairy-originated *E. lactis* and *E. faecium* strains have also been reported by Morandi et al. [18] and Favaro et al. [50]. This finding of the present study suggested the possible

application of *E. lactis* PMD74 as an adjunct culture rather than a starter culture. It has been stated in the legal requirements that the number of viable cells should be higher than 6 log CFU·mL^{-1} in order to exhibit health benefits in probiotic products [10]. *E. lactis* PMD74 exhibited considerable survival ability (8.86 Log CFU·mL^{-1}) for the complete storage period of four weeks when compared with the results reported by Yerlikaya et al. [57]. In order to evaluate the suitability of the candidate probiotic strain for the technological process, its physiological traits were studied. Since the strain was isolated from cheese and exhibited the general characteristics of the genus *Enterococcus*, the revelation of tolerance to both pH and NaCl was not surprising. The detected technological properties of the isolate suggested that this isolate could be suitable for application in the food industry.

5. Conclusion

In conclusion, the present study revealed that Ezine cheese (PDO), which is a nonstarter, long-ripened Turkish white cheese, serves as a source for the isolation of novel enterococcal strains. To the best of our knowledge, this is the first study on the isolation of *E. lactis* in Turkey. The results such as high resistance of the strain to the GI tract simulated conditions, other physiological characteristics, and notable antimicrobial activity to both close relative and foodborne pathogenic bacteria render *E. lactis* PMD74 a probiotic candidate. Given the fact that probiotic microorganisms should remain alive and effective during consumption and administration, the *E. lactis* PMD 74 strain exhibited notable survival ability in fermented milk during the four-week-long storage. Therefore, it is proposed that this novel strain *E. lactis* PMD74 might have a probiotic potential. For recommendation as candidate probiotic strain, further investigations including *in vitro* and *in vivo* studies must be conducted in order to elucidate the safety of this strain and its potential application in dairy fermented foods.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The author declares that there are no conflicts of interest.

Acknowledgments

The author expresses her gratitude to Yurtsev Uymaz, Mustafa Akçelik, Nefise Akçelik, and Pınar Şanlıbaba for their kind help.

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