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RESEARCH ARTICLE OPEN ACCESS

Identification and Potential Biotechnological Characterization of Lactic Acid Bacteria Isolated from White Cheese Samples

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Abstract

In this study, the isolation of lactic acid bacteria was carried out from one hundred white cheese samples collected from different regions of Turkey. Subsequently, phenotypic and genotypic characterization of the isolates was performed. Biochemical characteristics of the isolates were determined by API 50CHL. Furthermore, the biotechnological enzyme production potential of the isolates was screened. Genomic fingerprint profiles of the test isolates were detected by using rep-PCR (BOX-PCR), which has been used successfully in the differentiation of microorganisms at the species, subspecies, and even strain levels. The results showed that a total of forty-one bacteria were isolated and seventeen of which are found to be different species. The isolates generally grew at 4-6 pH values, 0-8% NaCl and 30-40°C. Later, isolates thought to be different species were identified by 16S rRNA gene sequence analyses. According to 16S rRNA sequence results, MA56 showed a 96.41% similarity match to *Lentilactobacillus buchneri*, it is thought to be a new species. In addition, MA19, MA25, MA43, and MA47 were determined to have multi-enzyme production potential. MA43 has a plantaricin gene and it showed a high antagonistic effect on *Escherichia coli* O157:H7 ATCC 43888 and *Pseudomonas aeruginosa* ATCC 9027. Inhibition zones were measured at 19 mm and 16 mm respectively.

Keywords: Lactic Acid Bacteria, Bacteriocin, Molecular Characterization, Biotechnological Enzymes

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INTRODUCTION

Cheese is produced by coagulating the milk with the effect of a suitable proteolytic enzyme or organic acids.[1,2] The unique taste and aroma of cheeses are obtained by the addition of lactic acid bacteria to the culture. Lactic acid bacteria (LAB) are gram-positive bacteria that produce lactic acid as the main product during the fermentation of carbohydrates.[3-5] They are non-spore-forming, anaerobic or microaerophilic, and acid-tolerant organisms with a rod or coccal cellular shapes.[6,7] LAB is a group of bacteria that consists of the genus including *Streptococcus, Lactococcus, Pediococcus, Enterococcus,* and *Lactobacillus* which is commonly found in dairy and fermented foods.[8-11] Many studies are reporting the health benefits of fermented dairy products. Fermented foods typically contain microorganisms considered to be Generally Regarded As Safe (GRAS), which can produce a range of beneficial by-products/ metabolites such as antimicrobial peptides (e.g. bacteriocins), ethanol, organic acids, fatty acids, and carbon dioxide.[12-14] It is known that products resulting from LAB-induced fermentations have anti-cancer, immunomodulatory,[15] anti-gastritis,[16] antihypertensive,[17] and anti-allergenic effects.[18] In addition, Mozaffarian et al reported that consuming LAB fermented foods had positive effects on body fitness.[19,20] Other studies have shown that the consumption of fermented yogurt and dairy products might reduce the risk of developing cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).[21,22] In addition, researchers reported that fermented milk and dairy products associated with LAB have hypocholesteremic[23] and anti-cancer properties.[24]

Besides the health aspect, LABs can also be the source of new species with enzymatic activities for biotechnological properties.[25] Microbial enzymes are more preferred than other enzymes because they have high catalytic activity and efficiency. Amylases, proteases, and lipases are commonly used in biotechnological processes. LAB with amylase, lipase, xylanases, and protease activities have been reported in previous studies.[26-29] Considering these properties, interest in lactic acid bacteria is increasing day by day and there are many LAB species with biotechnological potential yet to be discovered. In this study, isolation, identification, and molecular characterization of lactic acid bacteria from cheese samples collected from different regions (Erzurum, Van, Konya, Karaman, and Kars) of Turkey. Later, biotechnologically important enzyme and bacteriocin production potentials of the isolates were determined.

MATERIALS AND METHODS

Sampling and lactic acid bacteria isolation

A total of one hundred cow cheese samples taken from markets in different regions of Turkey (Erzurum, Van, Konya, Karaman, and Kars) were brought to the laboratory under aseptic conditions and kept at + 4°C until use. 225 ml of sterile physiological water (0.9% NaCl) was added to a 25g cheese sample and homogenization process was carried out and dilution series (100-10-7) were prepared.[30] The dilution aliquots were spread on MRS and M17 Agar media and incubated at 35°C for 48 hours. Pure cultures of isolates were obtained and stored at -86°C in a stock medium containing 15% glycerol.[31]

Phenotypic Characterization

To determine phenotypic characterization, test strains were grown in MRS and M17 medium at different temperatures 15°C to 50°C (with 5°C intervals) for up to 72 h. The pH ranges were analyzed in MRS and M17 at pH 3.0–11.0 (1 pH unit intervals), and tolerance of NaCl was determined using MRS and M17 supplemented with 0–12 % NaCl (at intervals of 1.0 %) for 72 h. All experiments were tested in triplicate and growth was measured at OD600 nm.[32] Gram-staining of test strains was performed according to the method by Gerhardt et al.[33] Catalase activity was performed by the production of bubbles of a drop of 3 % H_2O_2 (v/v). Oxidase reagent (Sigma) was used for testing oxidase activity.[34] Furthermore, biochemical characterization of isolates was conducted using API 50CHL test.[35]

Genotypic Characterization

Genomic DNA isolation was performed according to the WizardR Genomic DNA Purification Kit (Promega, Southampton, UK, A2360) protocol. The rep-PCR reactions were carried out in a Sensequest Thermal Cycler

(Göttingen, Germany) using BOXAIR primer (5'-CTACGGCAAGGCGACGCTGACG-3'), PCR mixtures contained: 5 µl Gitschier Buffer, 12.7 µl ddH_2O, 2.5 µl dimethyl sulfoxide, 1.25 µl bovine serum albumin, 1.25 µl dNTP, 4 µl primer, 0.3 µl Taq DNA polymerase and 3 µl template DNA. PCR Cycles were, initial denaturation at 94°C for 7 min., 36 cycles of 1 min. at 94°C, 1 min. at 45°C, 8 min. at 65°C. Final extension at 65°C for 16 min. At the end of PCR, samples were run in 1% agarose gel for 90 minutes.[36]

16S rRNA region was amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers[37]. 30 µL volume of PCR mixture containing, 13.1 µl ddH_2O, 3 µl 10X PCR buffer, 1.8 µl MgCl2, 1.2 µl DMSO, 0.6 µl dNTP, 3 µl (5 µM) forward primer (27F), 3 µl (5µM) reverse primer (1492R), 0.3 µl Taq DNA polymerase and 4 µl template DNA (70 ng). The amplified fragments were cloned into *Escherichia coli* JM101 strain with the pGEM-T Easy Cloning Vector (Promega, Southampton, UK) according to the instructions of the manufacturer. After the cloning, plasmid isolation was performed by selecting colonies that gave the positive result, and the sequence analysis was made by the Macrogen Company (Netherlands). The 16S rRNA obtained was compared with other bacterial series in GenBank and EzTaxon (http://blast.ncbi.nlm.nih and http://www.eztaxon.org), the similarity rate between them was determined and GenBank accession numbers were received.[38,39]

Preliminary enzyme assays

Lipase

To test lipase enzyme production, isolates were inoculated into a tributyrin agar medium containing 1 % tributyrin (glycerol tributyrate) and incubated at 35°C for 48 h. Isolates with a clear zone were considered positive for lipase.[40,41]

Amylase

Test strains were streaked on plates containing MRS (de Man, Rogosa and Sharpe) agar with 1% starch instead of glucose that were incubated at 35°C for 48 h. Then, Petri dishes were treated with Lugol's solution. Isolates showing clear zones were evaluated as amylase positive.[42]

Protease

To test whether isolates can produce protease enzymes, the strain was inoculated into MRS agar containing 1% Skimmed Milk Powder and incubated at 35°C for 48 h. Isolates with a clear zone were considered positive for protease.[40]

Xylanase

Isolates were inoculated into medium containing xylan (10 g/L), NaNO3 (1.2 g/L), KH_2PO_4 (13 g/L), K_2HPO_4 (6 g/L), $CaCl_2$ (0.05 g/L), $MgSO_4$ (0.01 g/L), $ZnSO_4$ (0.001 g/L) and agar (15 g/L) incubated at 35°C for 48 hours. After incubation, the plates were stained with 0.1% congo red for 20 min and then washed with 1M NaCl. Isolates with orange-colored zones were evaluated for xylanase positive.[29]

Bacteriocin gene detection

After the characterization of test strains, bacteriocin production characteristics of isolates were investigated. For this purpose, PCR analysis was performed using primers specific to each bacteriocin gene.[43,44] The 16S rRNA PCR program given above was performed except for changing the annealing temperatures of bacteriocin primers.

Detection of Antibacterial Activity

For the detection of antibacterial activity, a disc diffusion assay was used. Pathogenic bacteria were spread on the surfaces of Mueller Hinton agar media. Overnight cultures, on MRS medium, of the strains to be tested were centrifuged and cell-free supernatant was loaded on discs placed in the middle of the petri dish. The plates were incubated at 37°C for 24h. The antagonistic effects of the test strains were determined by measuring the zone of inhibition diameters.[45] The target test strains used in this study were *Escherichia coli* O157:H7 (ATCC 43888), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 810), *Pseudomonas aeruginosa* (ATCC 9027), *Streptococcus pyogenes* (ATCC 12344), *Klebsiella pneumoniae* (ATCC 13883), *Listeria monocytogenes* (ATCC 7644), *Staphylococcus epidermidis* (ATCC 12228), *Shigella dysenteriae* (ATCC 13313) and *Staphylococcus aureus* (ATCC 6538).

RESULTS AND DISCUSSION

Isolation of LAB

In this study, a total of 41 bacterial isolates were isolated from cheese samples taken from provinces (Erzurum, Kars, Karaman, Konya, and Van). Since seventeen of the isolated bacteria belonged to different species (according to rep-PCR results), stock cultures of these strains were prepared and the study was continued with these isolates.

Phenotypic Characterization

According to conventional analysis, all isolates were gram-positive and oxidase negative and showed cocci or bacilli cell morphology. In general isolates grow at 4-6 pH values, 0-8% NaCl and 30-40°C. Similarly, Ni et al determined that isolated lactic acid bacteria can usually grow at 35-45°C at pH 3 and 6.5% NaCl.[46] Interestingly, it has been found that the MA7 strain can thrive in a wide range of pH and salt concentrations such as pH:2-11 and 0-10% NaCl. So, MA7 can be suitable for many biotechnological processes. Detailed phenotypic characteristics and API test results are given in Table 1.

Genotypic Characterization

Previous studies have reported that rep-PCR is an easy method that can be used to classify bacteria. Mohammed et al used BOX-PCR analysis to characterize lactic acid bacteria isolated from traditional milk samples.[47] We also performed genomic fingerprint analysis of isolates using BOX-PCR in this study. While 12 polymorphic bands were observed in some of the test strains, it was observed that there was 1 band in some isolates (Figure 1). It was observed that the BOX-PCR was not sufficient to classify all LAB.

16S rRNA sequence analysis is used as a powerful tool in determining the prokaryotic diversity in almost every environment.[48,49] So, we performed molecular identification of lactic acid bacteria isolated from cheese samples based on 16S rRNA sequencing. Except for MA56, all isolates were found to be 99% similar to related standard type strains. MA56 has a 96.41% similarity match with *Lentilactobacillus buchneri* DSM 20057. According to general acceptance, the 16S rRNA gene sequence similarity ratio below 97% is a new species indicator of the isolate.[49] In recent years, it has been reported that this rate is 98.2% - 99% and that the whole genome sequence and

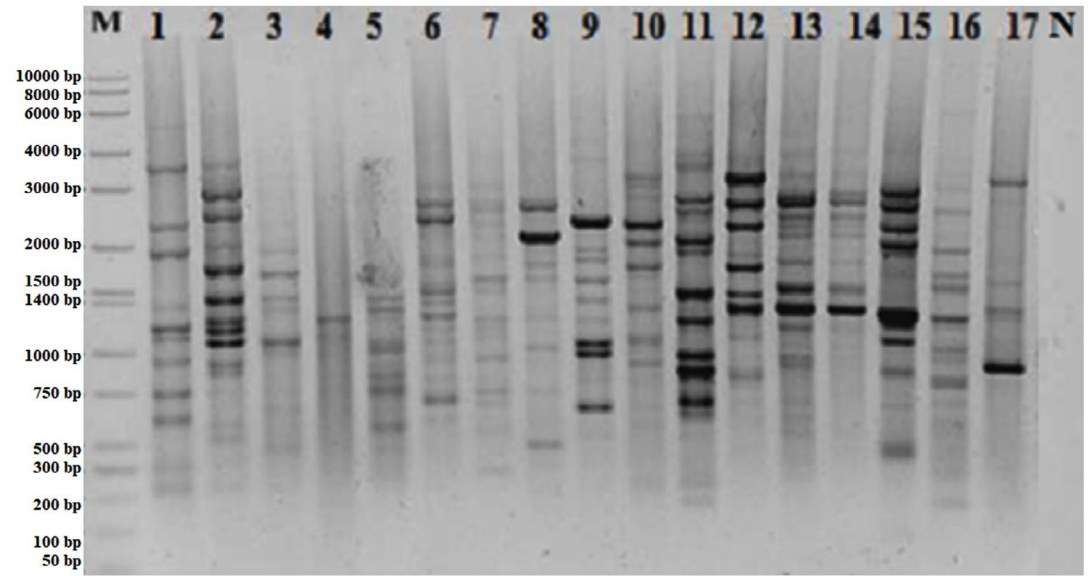

Figure 1. BOX-PCR profiles of isolates (M:Marker, 1: MA4, 2: MA7, 3: MA10, 4: MA12, 5: MA19, 6: MA25, 7: MA27, 8: MA28, 9: MA31, 10: MA33, 11: MA34, 12: MA35, 13: MA39, 14: MA43, 15: MA47, 16: MA55, 17:MA56, N: Negative Control)

Table 1. Phenotypic characterization of isolates 1: MA4, 2: MA7, 3: MA10, 4: MA12, 5: MA19, 6: MA25, 7: MA 27, 8: MA28, 9: MA31, 10: MA33, 11: MA34, 12: MA35, 13: MA39, 14: MA43, 15: MA47, 16: MA55, 17: MA56

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
pH	4-6	2-11	4-6	2-8	3-6	4-6	4-7	4-5	4-6	4-6	4-6	4-6	5-7	4-6	3-6	4-6	2-8
NaCl	0-9	0-10	0-9	0-8	4-6	0-6	0-8	0-11	0-8	0-6	0-8	0-8	0-8	0-5	0-8	0-10	0-8
Temperature	30-40	30-40	15-45	30-40	30-40	30-40	15-45	15-45	15-45	30-40	30-40	15-45	30-40	30-40	30-40	30-40	30-40
Morphology	Bacil	Bacil	Bacil	Bacil	Coc	Coc	Coc	Bacil	Coc	Coc	Bacil	Coc	Coc	Bacil	Coc	Coc	Bacil
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-
L-Arabinose	+	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	+
Ribose	-	+	+	-	-	-	+	+	d	-	+	+	-	+	-	-	+
Xylose	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+
Adonitol	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+
D-Glucose	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+
D-Fructose	-	+	+	-	-	-	+	+	-	+	+	+	+	+	-	+	+
D-Mannose	-	+	+	-	-	-	+	-	-	-	+	+	d	+	-	+	-
Rhamnose	-	-	+	-	-	-	-	-	-	-	-	d	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Mannitol	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-
Sorbitol	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Met-D-Gluc.	-	-	+	-	-	-	-	d	-	-	-	-	-	-	-	-	+
NAG	-	+	+	-	-	-	+	d	d	+	+	+	-	+	-	+	+
Amygdaline	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-
Arbutine	-	+	+	-	-	-	+	-	-	-	+	-	-	+	-	-	-
Esculine	d	+	-	+	-	-	+	-	-	-	-	+	-	+	-	+	-
Salicine	-	+	+	-	-	-	+	-	-	-	+	d	d	+	-	d	-
Cellobiose	-	+	+	-	-	-	+	-	-	-	+	+	-	+	-	+	-
Maltose	-	d	+	-	-	-	+	+	+	+	+	-	+	+	-	-	+
Lactose	-	+	+	-	-	-	+	+	+	+	-	+	+	+	-	+	+
Melibiose	-	-	-	-	+	+	-	+	-	-	-	-	-	+	+	-	+
Saccharose	-	d	+	-	-	-	-	-	+	d	-	-	+	+	-	-	-
Trehalose	-	+	+	-	-	-	+	-	+	+	+	+	+	+	-	d	+
Melezitose	-	+	+	-	-	-	-	-	-	-	+	-	d	+	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Gentiobiose	-	-	+	-	-	-	+	-	-	-	+	+	-	+	-	+	-
D-Turanose	-	+	-	-	-	-	-	-	d	d	+	-	d	-	-	-	-
D-Tagatose	-	+	-	-	-	-	-	-	-	-	+	d	-	-	-	+	-
D-Arabitol	-	-	+	-	-	-	-	-	-	-	-	-	-	d	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+:Positive -: Negative d: Delayed.

DNA: DNA hybridization is required in addition to the 16S rRNA gene sequence.[50] According to this information, MA56 might be a novel species belonging to the genus *Lentilactobacillus*. It is thought that MA56 will be added to the literature as a novel species as a result of the whole genome sequence analysis in future studies. Detailed sequence results of the isolates and related species are given in Table 2. Also, the 16S rRNA gene-based phylogenetic tree is shown in Figure 2.

Table 2. Related species and similarity rates of isolates according to 16S rRNA sequence analysis

Isolate Code	Product size (bp)	Related species	Similarity rate (%)	Genbank No.
MA 4	1535	*Lentilactobacillus kefiri*	99	KY425772
MA 7	813	*Lacticaseibacillus casei*	99	KY425775
MA 10	1528	*Lactiplantibacillus paraplantarum*	99	KY425790
MA 12	1515	*Lysinibacillus sinduriensis*	99	KY425788
MA 19	1484	*Micrococcus yunnanensis*	99	KY425784
MA 25	1486	*Microbacterium paraoxydans*	99	KY425786
MA 27	1387	*Enterococcus faecium*	99	KY425810
MA 28	1527	*Levilactobacillus brevis*	99	KY425773
MA 31	1512	*Staphylococcus haemolyticus*	99	KY425785
MA 33	885	*Staphylococcus hominis*	99	KY425791
MA 34	1531	*Lacticaseibacillus paracasei* subsp. *paracesi*	99	KY425778
MA 35	1538	*Pediococcus lolii*	99	KY425782
MA 39	1488	*Rothia dentocariosa*	99	KY425811
MA 43	1528	*Lactiplantibacillus plantarum* subsp. *plantarum*	99	KY425796
MA 47	1462	*Micrococcus aloeverae*	99	KY425780
MA 55	1539	*Pediococcus parvulus*	99	KY425789
MA 56	1491	*Lentilactobacillus buchneri*	96	KY425792

Table 3. Screening of Industrial Enzyme Profiles of Isolates

Isolate Code	Related species	Amylase	Lipase	Protease	Xylanase
MA4	*Lentilactobacillus kefiri*	-	-	++	-
MA7	*Lacticaseibacillus casei*	-	-	++	-
MA10	*Lactiplantibacillus paraplantarum*	-	-	-	-
MA12	*Lysinibacillus sinduriensis*	-	-	++	-
MA19	*Micrococcus yunnanensis*	++	-	++	-
MA25	*Microbacterium paraoxydans*	+	+	+	-
MA28	*Levilactobacillus brevis*	-	-	++	-
MA31	*Staphylococcus haemolyticus*	-	-	-	-
MA33	*Staphylococcus hominis*	-	-	++	-
MA34	*Lactiplantibacillus paracasei* subsp. *paracesi*	-	-	-	-
MA35	*Pediococcus lolii*	+	-	-	-
MA39	*Rothia dentocariosa*	-	-	-	-
MA43	*Lactiplantibacillus plantarum* subsp. *plantarum*	+	+	++	-
MA47	*Micrococcus aloeverae*	-	-	++	+
MA55	*Pediococcus parvulus*	+	-	-	-
MA56	*Lentilactobacillus buchneri*	-	-	++	-

-: Negative, +: Positive, ++: Strong positive.

Determination of Biotechnological Enzyme Production Characteristics of Isolates

The ability of isolates to produce amylase, lipase, protease and xylanase enzymes, which are biotechnologically important, was determined. As a result of the analysis, one strain has xylanase and lipase activity, five strains with amylase activity, and ten strains with protease activity were observed (Table 3). Also, Petri images of some isolates are given in Figure 3. Matthews et al investigated the enzyme production potential of lactic acid bacteria and determined that especially *Lactobacillus* and *Pediococcus* species are important producers of lipase, cellulase, and xylanase enzymes. In another study, Konkit and Kim examined that *Lactoccocus chungangensis* produces amylase, proteinase, and lipase enzymes.[26] These enzymes are very important for industrial processes. For example, lipases are used for transesterification acidolysis, xylanase is used for the enzymatic breakdown of agricultural wastes for the production of alcohol fuels, enzymatic treatment of animal feed to release free pentose sugars, manufacturing of dissolving pulps yielding cellulose for rayon

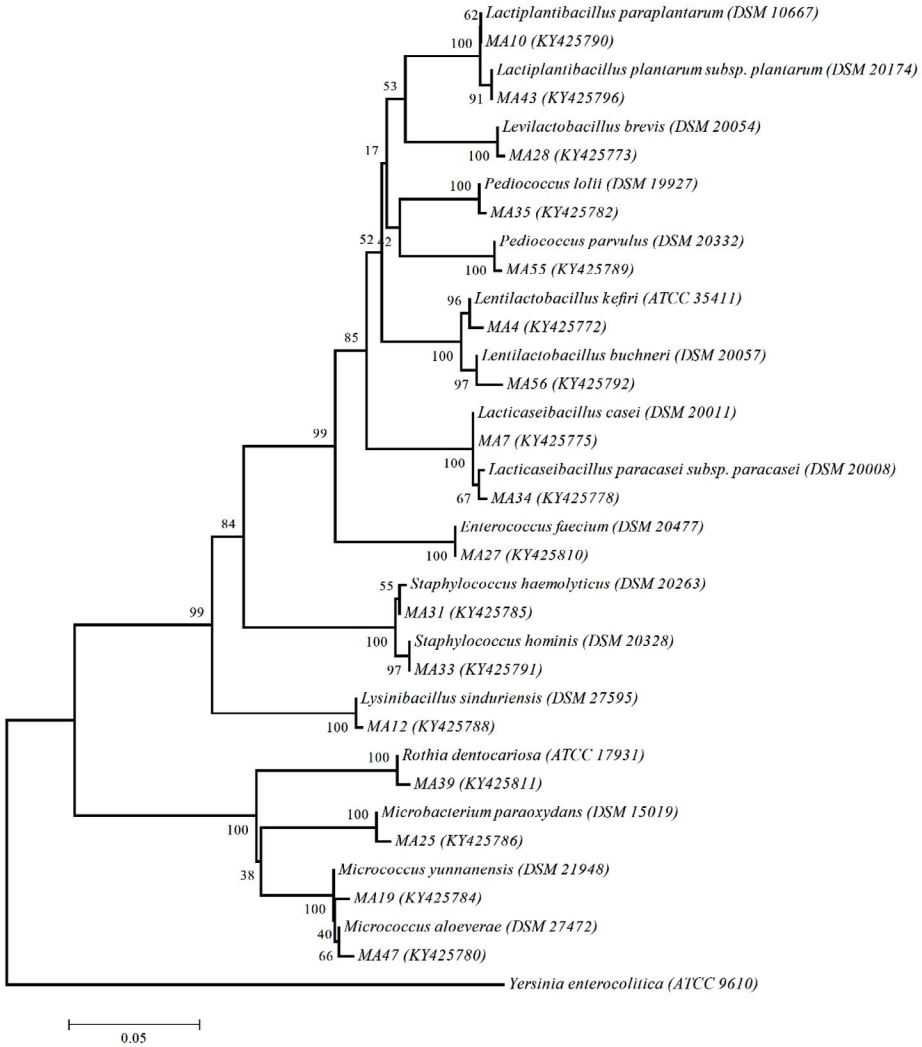

Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of test strains and related type species. *Yersinia enterocolitica* ATCC 9610 was used as an out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parentheses. The scale bar represented 0.5% divergence

production, and bio-bleaching of wood pulps, proteases used for the detergent industry, and amylases used for food, fermentation and pharmaceutical industries.

There are many studies in the literature in which enzymes obtained from lactic acid bacteria are used in biotechnological processes. For example, The xylanase enzyme purified from *Pedioccus acidilactici* was applied in clarification of fruit juices.[29] In another study, it was reported that the protease enzyme obtained from *Lactobacillus plantarum* had an antimicrobial effect on pathogenic microorganisms.[51] Therefore, in this study, isolates coded MA19, MA25, MA43, and MA47 were determined to have multi-enzyme production potential (Table 3). These isolates are attractive for biotechnological processes because they have more than one enzyme activity.

Determination of Bacteriocin Production Potential

The presence of bacteriocin genes in the strains was determined using bacteriocin-specific primers with PCR. As a result of the PCR analysis, it was determined that only the MA43 strain has a plantaricin gene region. The gel image of plantaricin belonging to MA43 and *Lactobacillus plantarum* ATCC 8014 are shown in Figure 4.

After it was determined that MA43 has a bacteriocin gene, its effect on pathogenic bacteria was investigated. It has been determined that MA43 has the highest antibacterial effect against *Escherichia coli* O157:H7 ATCC 43888

Table 4. The result of the disc diffusion test. The diameter of the inhibition zone of MA43

Pathogens	MA43
Serratia marcescens ATCC 810	5 ± 0.4
Shigella dysenteriae ATCC 13313	12 ± 1.3
Klebsiella pneumoniae ATCC 13883	12 ± 0.8
Streptococcus pyogenes ATCC 12344	10 ± 0.4
Staphylococcus epidermidis ATCC 12228	9 ± 0.3
Staphylococcus aureus ATCC 6538	12 ± 0.8
Pseudomonas aeruginosa ATCC 9027	16 ± 1.1
Salmonella typhimurium ATCC 14028	7 ± 0.6
Listeria monocytogenes ATCC 7644	9 ± 0.7
Escherichia coli O157:H7 ATCC 43888	19 ± 0.5

The measures of the inhibition zone are expressed in mm.

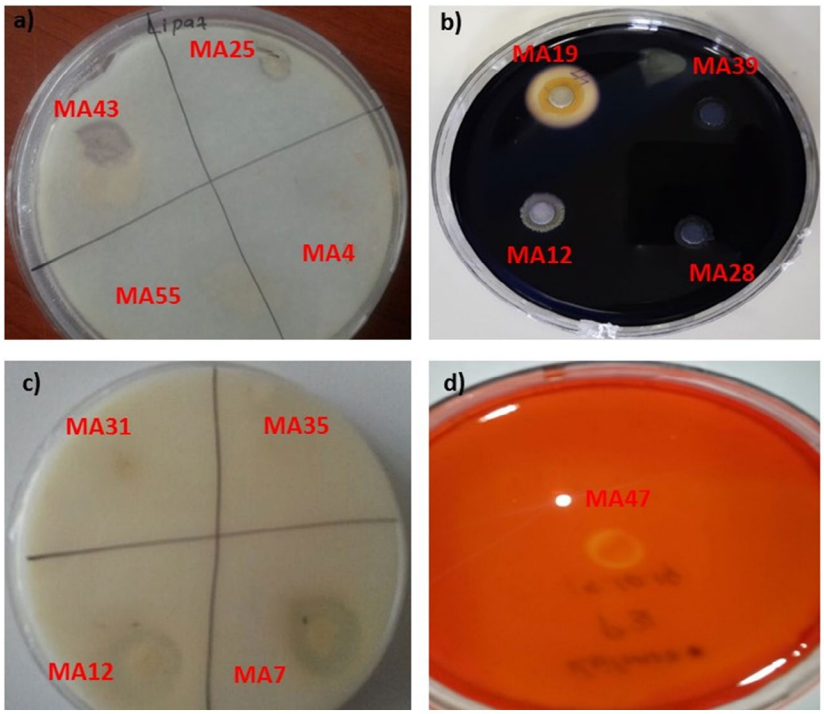

Figure 3. Screening biotechnological enzymes profiles of some isolates a) Petri image of lipase b) Petri image of amylase c) Petri image of protease d) Petri image of xylanase

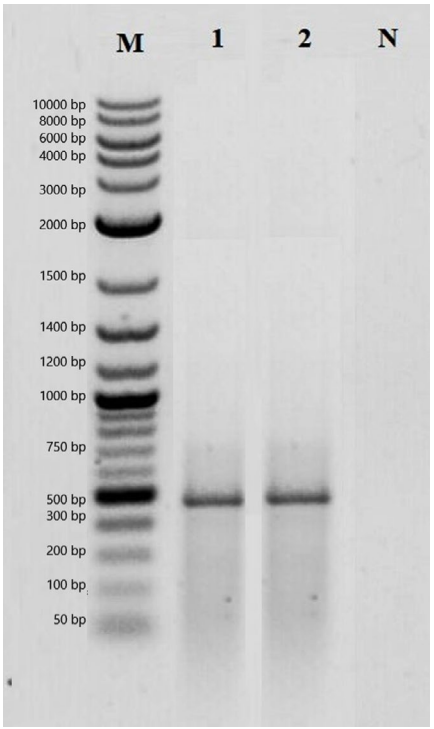

Figure 4. PCR analysis result of plantaricin genes. M: Marker, 1: *Lactobacillus plantarum* ATCC 8014, 2: MA43, 3: Negative control

and *Pseudomonas aeruginosa* ATCC 9027 (Table 4). Previous studies have also reported that *Lactobacillus plantarum* has an antimicrobial effect against various pathogens.[52,53] In this respect, MA43 has an antagonistic effect against foodborne pathogens showing that it can be used as a food preservative.

CONCLUSION

In this study, it was determined that the white cheese samples have a very wide range of microflora. 16S rRNA sequence similarity to the closest species of the MA56 was determined as 96.41%. This isolate is more likely to be a novel species of lactic acid bacteria. MA43 not only has amylase, lipase, and protease activity but also produces bacteriocin making it unique for biotechnological processes. In addition, this study will be a pioneer for future studies to be conducted on MA56 and MA43.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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