ÇOMÜ Zir. Fak. Derg. (COMU J. Agric. Fac.)
2020: 8 (1): 51–59
ISSN: 2147–8384 / e-ISSN: 2564–6826
doi: 10.33202/comuagri.624715

Araştırma Makalesi/Research Article

Isolation of Lactic Acid Bacteria from Tarhana

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Geliş Tarihi: 25.09.2019 Kabul Tarihi: 25.12.2019

Abstract

Tarhana is a staple food in Turkey and produced mainly by the fermentation of wheat flour and yogurt. There are variations in its use, it can be consumed as a snack, or as a soup, and its exact ingredients vary between different geographical regions. Our study aims to isolate and identify lactic acid bacteria in tarhana samples via biochemical and molecular biology identification techniques. Three different tarhana samples were studied for bacterial Gram reactions, catalase activity, gas production, growth at 10°C and 45°C, in 6% and 16% NaCl concentrations and at pH 4,4 and pH 9,6 for the biochemical tests. For the molecular biology experiments, PCR-RFLP, sequencing and RAPD-PCR were performed to identify organisms at the species and strain level.
Keywords: Tarhana, lactic acid bacteria, PCR, identification, typing

Tarhanadan Laktik Asit Bakterilerinin İzolasyonu

Öz

Tarhana ülkemizin temel gıda maddelerinden biridir ve aslen buğday unu ve yoğurdun fermentasyonundan üretilir. Kullanım alanları farklılık gösterebilir, atıştırmalık ya da çorba olarak tüketilebilir, içeriğindeki malzemeler de üretildiği coğrafi bölgeye göre değişiklik gösterir. Çalışmamızın amacı biyokimyasal ve moleküler biyoloji tekniklerini kullanarak tarhana örneklerinden laktik asit bakterilerini izole etmek ve tanımlamalarını sağlamaktır. Üç farklı tarhana örneği kullanılmış olup, biyokimyasal testler kapsamında bakterilerin Gram reaksiyonları, katalaz aktiviteleri, gaz üretimleri, 10°C and 45°C'de, %6 ve %16 NaCl konsantrasyonlarında ve 4,4 ve 9,6 pH değerinde büyümeleri çalışılmıştır. Mikroorganizmaların tür ve suş düzeyinde tanımlanmaları amacıyla çalışılan moleküler biyoloji teknikleri arasında ise PCR-RFLP, dizileme ve RAPD-PCR bulunmaktadır.
Anahtar Kelimeler: Tarhana, laktik asit bakterileri, PCR, tanımlama, tiplendirme

Introduction

Humans have been consuming fermented foods for several thousands of years. Fermentation was primarily used in order to preserve the foods for long periods of time (Kabak and Dobson, 2011). However, nutritional value of foods can also be increased by fermentation, since it is a natural way for synthesizing various amino acids and vitamins (Simsek et al., 2017). Tarhana is a traditional Anatolian fermented food that is composed of wheat flour, yogurt, sourdough, vegetables and spices (including tomato, red pepper, onion, mint, dill, and salt) in either dried or fresh form, and is fermented at room temperature (25°C) for about 1-7 days (Bayrakçı and Bilgiçli, 2015; Simsek et al., 2017). Tarhana is a staple of Turkish cuisine as well as Balkans and Middle East, due to its high nutrient value and long shelf-life. Fermentation of tarhana is achieved by lactic acid bacteria (LAB) in a critical process to increase the nutritional values and to produce a unique savor and aroma (Celik et al., 2005; Ozdemir et al., 2007). "

The metabolites produced by LAB (lactic acid, ethyl alcohol, carbon dioxide and aromatic compounds) (Sengun et al., 2009) give tarhana its characteristic taste and flavor and increase its storage life by increasing the acidity (Simşek et al., 2017). Studies (Sengun et al., 2009; Settanni et al., 2011) showed that the bacterial population of an ordinary homemade tarhana is composed of the genera *Lactobacillus, Enterococcus* and *Pediococcus.*

LAB are generally rod- or spherical-shaped gram-positive bacteria that are catalase negative. They have been important for humans for centuries as they contribute positively to the nutritional value of food products and biological protection of nutrients, thus are important microbial agents in

human nutrition and health. LAB can be isolated from various sources including plants and animals, and used for both traditional and industrial biotechnological processes (Gezginç and Akyol, 2010).

Phenotypic identification of isolates can be performed according to colony morphology on agar, gram staining, catalase production, growth at different pH values (pH 4,4, pH 9,6), different salt concentrations (6% and 16% NaCl (g/mL)) and different temperatures (10°C, 45°C), and gas production. Phenotypic identification methods of bacteria, however, may not provide accurate results 100% of the time, as there can be instability of phenotypic properties under different environmental conditions (Rossello-Mora and Amann, 2001). Therefore, a combination of biochemical and molecular biology-based identification of LAB is commonly preferred. For our study, the molecular biology-based techniques included PCR-RFLP, RAPD-PCR, and DNA sequencing. PCR-RFLP and sequencing of the 16S rRNA gene were used for identification of isolates at the species level and RAPD-PCR is used to differentiate the isolates at the strain level.

Materials and Methods

Bacterial Strains and Growth Conditions

Twenty-eight bacterial strains isolated from traditional Turkish food “tarhana” were used in this study. Two out of three traditional samples were collected from Central Anatolia Region of Turkey, city of Ankara and the third sample was collected from Black Sea Region of Turkey, city of Sinop.

Phenotypic identification of the 28 isolates was performed according to colony morphology on agar, gram staining, catalase production, and growth at different pH values (pH 4,4, pH 9,6), salt concentrations (6% NaCl, 16% NaCl), and temperatures (10°C, 45°C). Gas production was detected by using Durham test tubes.

Bacterial Growth and Isolation of Strains

Tarhana samples were diluted with sterile 1x PBS (Amresco, USA) solution (pH 7,4). 1 gram of each tarhana sample was added to 10 ml of MRS and M17 broths (Merck, USA) and incubated at 37°Cfor 20 hours for further dilutions. Stock cultures of isolated strains were prepared in 20% glycerol and stored at -80°C. All the experiments were done under aseptic conditions. T

Biochemical Identification of the Strains

Gram Staining

Gram-colour staining of overnight-incubated liquid cultures was performed for each strain by using a Gram stain set (Merck, Germany) by following manufacturer’s instructions.”

Catalase Test

H_2O_2 (30%) was applied onto a loopful of isolate on a microscopic slide. Absence of bubbles indicated negative catalase activity.

Growth at 10°C and 45°C

Growth at different temperatures was determined in MRS broth and M17 broth for 48 hours at 10°C and 45°C.

Growth at pH 4,4 and pH 9,6

MRS and M17 growth media at pH 4,4 were prepared by using diluted HCl (Merck, Germany), and growth media at pH 9,6 were prepared by using 1N NaOH solution (Merck, Germany).pH values were measured with a pH meter (ThermoScientific, USA). Fresh cultures were inoculated into the prepared tubes. Cultures were incubated for 24-48 hours.

Growth at 6% NaCl and 16% NaCl

MRS and M17 broths containing 6% NaCl (g/mL) and 16% NaCl (g/mL) were prepared and isolates were incubated for 24-48 hours.

Gas Production

Gas production was tested using Durham tubes in both MRS and M17 broth containing 2% glucose (g/L).

Genetic Identification of Strains

DNA Isolation

Genomic DNAs were prepared using Bacterial Genomic DNA Miniprep Kit (Axygen, USA). Before starting the isolation, OD600 value of 1 mL bacterial culture was measured by Genesys 10S spectrophotometer (Thermo Scientific, USA), for which the OD600 value should be between 1 and

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1.5. Briefly, 1mL of overnight grown culture was centrifuged at 12.000g for 30 second in MicroCL 17R Centrifuge (ThermoScientific, USA). This step was performed twice. The pellet was suspended in 170µL Buffer S solution containing RNase A and 20µL lysozyme. Bacterial suspension was incubated at 37°C for 30 min. The suspension was incubated on ice for 5 min after the addition of 30µL of 0.25 M EDTA (pH 8.0). 450µL Buffer G-A was added and incubated in a water bath at 65°C for 10 min. The suspension was centrifuged at 12.000g for 2 min. 400µL of Buffer G-B and 1 mL of Buffer DV (pre-chilled to 4°C) were added. The suspension was centrifuged at 12.000g for 2 min. The upper phase was discarded without disturbing the interphase. 1ml of Buffer DV was added to the remaining interphase and lower phase and then centrifuged at 12.000g for 2 min. Lower phase was transferred to a spin-filter, placed in a 2 mL microfuge tube (both provided) and centrifuged at 12.000g for 1min. 400µL of Buffer BV was added to the filtrate. Miniprep column was placed in a 2 mL microfuge tube and the binding mix was transferred to the miniprep column and centrifuged at 12.000g for 1 min. The filtrate was discarded and miniprep column was replaced back to the 2 mL microfuge tube and 500µL of Buffer W1 was added to the miniprep column and centrifuged at 12.000g for 1 min. The filtrate was discarded and 70µL of Buffer W2 was added to the replaced miniprep column and centrifuged at 12.000g for 1 min. This step was performed twice and the filtrate was discarded. The miniprep column was replaced back to the 2 mL microfuge tube and centrifuged at 12.000g for 1 min. The column was then placed into a provided clean 1.5 mL microfuge tube. 130µL pre-warmed 65°C eluent was added to the center of the membrane. Centrifugation was done at 12.000g for 1 min. Genomic DNA samples were stored at -20°C for further experiments.

Determination of DNA Concentration

The concentrations of isolated genomic DNA were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Polymerase Chain Reaction (PCR)

The polymerase chain reactions were performed on SimpliAmp Thermal Cycler (Thermo Scientific, USA) machine. A master mix was prepared for each reaction. A total of 50µL mixture was prepared by adding 50mM MgCl2, 0.62 µL of 10mM dNTP mix, 1X ammonium buffer, 300U Taq DNA polymerase (Dr. Zeydanlı, Turkey), 10mM forward primer 9699 (5'-ATCCGAGCTCAGAGTTTGATCCTGGC-3'), 10mM reverse primer 9700 (5'-TCAGGTCGACGCTACCTTGTTACGAC-3') (Oligomer, Turkey), and 100ng/µL genomic DNA. Amplification conditions were as follows; initial denaturation starts at 95°C for 2mins, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP was performed on SimpliAmp Thermal Cycler machine. Four different restriction enzymes (*Taq*I, *Lwe*I, *Xba*I, *Mbo*I) were used in this study. The reaction mixtures and conditions were done according to manufacturer's instructions (ThermoFisher Scientific, USA).

Sequencing of PCR Products

PCR products were purified by EasyPure PCR purification kit, according to manufacturer's instructions. Purified PCR products were sequenced at MedSanTek (Istanbul, Turkey). Sequences were then analyzed using MEGA X (Kumar et al., 2018) and BLAST analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were done to assign species.

Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

RAPD-PCR experiments were performed on SimpliAmp Thermal Cycler. For every reaction, 50 mM MgCl2, 10 mM primer M13 (Oligomer, Turkey), 10mM dNTP mixture, 1X ammonium buffer, 300U Taq DNA polymerase, and 300ng genomic DNA were added into a total of 50µL reaction mixture. Amplification conditions were as follows; after an initial denaturation at 94°C for 2 mins, 40 cycles of denaturation at 94°C for 1min, annealing at 42°C for 35secs and extension at 72°C for 2mins.

Visualization of Gels

A horizontal electrophoresis apparatus Mini-Sub Cell GT Cell (Bio-Rad, USA) was used for electrophoresis. To visualize genomic DNA on agarose gel, 1.5% agarose gel was prepared in 50 mL of 1X TAE Buffer (Dr. Zeydanlı, Turkey). Electrophoresis was performed at 50 V, 70 min for PCR and PCR-RFPL, and 80V, 80 min for RAPD-PCR. Finally, the gel was monitored at molecular imager GelDocXR+ (Bio-Rad, USA) under UV and photographed.

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Phylogenetic Analysis

Analyses of the RAPD-PCR patterns were done with GelComparII (AppliedMaths, Belgium). UPGMA analyses were performed and grouping assignments were set arbitrarily at 80% similarity level (Cebeci and Gürakan, 2008).

Results and Discussion

Biochemical Tests of Tarhana Isolates

The isolates were studied for the gram reactions, catalase activity, bacterial morphology, and growth under different temperatures and pH values (Table 1). The isolates were grown on MRS or M17 media, and isolates that are either rod or spherical, gram positive, and catalase-negative are presumed as lactic acid bacteria. All of the isolates were able to grow at 45°C, pH 9,6 and 6% NaCl.

Table 1. Testing of tarhana isolates according to pH, salt concentration, temperature and gas production

Isolate	Gram stain	Shape	Catalase activity	Temperature		pH		NaCl		Gas production
				10°C	45°C	4,4	9,6	6%	16%	
M17 H1	+	Cocci	-	-	+	+	+	+	-	-
M17 H2	+	Cocci	-	-	+	+	+	+	-	-
M17 H3	+	Cocci	-	-	+	-	+	+	-	-
M17 H4	+	Cocci	-	-	+	-	+	+	-	-
M17 H5	+	Cocci	-	-	+	-	+	+	-	-
M17 H6	+	Cocci	-	-	+	+	+	+	-	-
M17 H7	+	Cocci	-	-	+	+	+	+	-	-
M17 N1	+	Cocci	-	-	+	-	+	+	-	-
M17 N2	+	Cocci	-	-	+	-	+	+	-	-
M17 N3	+	Cocci	-	-	+	-	+	+	-	-
M17 N4	+	Cocci	-	-	+	-	+	+	-	-
M17 N5	+	Cocci	-	-	+	-	+	+	-	-
M17 N6	+	Cocci	-	-	+	-	+	+	-	-
M17 N7	+	Cocci	-	-	+	-	+	+	-	-
MRS T1	+	Cocci	-	-	+	+	+	+	-	-
MRS T2	+	Cocci	-	-	+	+	+	+	-	-
MRS T3	+	Cocci	-	-	+	+	+	+	-	-
MRS T4	+	Cocci	-	-	+	+	+	+	-	-
MRS T5	+	Cocci	-	-	+	+	+	+	-	-
MRS T6	+	Cocci	-	-	+	+	+	+	-	-
MRS T7	+	Cocci	-	-	+	+	+	+	-	-
MRS N1	+	Cocci	-	-	+	+	+	+	-	-
MRS N2	+	Cocci	-	-	+	-	+	+	-	-
MRS N3	+	Cocci	-	-	+	+	+	+	-	-
MRS N4	+	Cocci	-	-	+	+	+	+	-	-
MRS N5	+	Cocci	-	-	+	+	+	+	-	-
MRS N6	+	Cocci	-	-	+	+	+	+	-	-
MRS N7	+	Cocci	-	-	+	+	+	+	-	-

Genetic Identification of Tarhana Isolates

Presuming that the isolates are lactic acid bacteria (LAB), PCR-RFLP was performed. Unfortunately, the fingerprinting patterns of four different restriction enzymes (*Taq*I, *Mbo*I, *Lwe*I and *Xba*I) have not shown any resemblance to the patterns of bacteria in our laboratory culture collection (data not shown). Thus, in order to identify the isolates, the samples were sequenced for 16S rRNA gene. Sequencing results were used in order to do BLAST analysis and isolates were successfully assigned (Table 3.2). It can be seen in Table 3.2 that the majority of the isolates grown on MRS media belong to either *Lactobacillus plantarum* or *Pediococcus pentosaceus* species. *L. plantarum* species are commonly known for their association with fermented vegetables (Cebeci and Gürakan, 2003; Mujagic et al., 2017). The species also comprise several probiotics, including commercialized *L. plantarum* 299v strain. Thus, it was no surprise to isolate *L. plantarum* strains from tarhana. *P.*

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pentosaceus is also a frequently recognized member of the fermented foods and has been studied for their potential as probiotics (Jonganurakkun et al., 2008; Damodharan et al., 2015). Two isolates were identified as *Enterococcus dispar* and one isolate was identified as *E. faecium*. *Enterococcus* species are common inhabitants of the human and animal intestinal tracts and are associated with several fermented foods. M17 isolates were all assigned to one species, *E. faecium*. The food associated enterococci are thought to contribute to flavor development in cheese, and generally accepted as safe in foods (Gelsomino et al., 2002; Inoglu and Tuncer, 2013). However, although most of the members of the genus are non-pathogenic, some members of the genus are important causes of nosocomial infections, by being highly resistant to vancomycin (VRE: vancomycin resistant enterococci).

Table 2. Identification of tarhana isolates according to sequencing of the 16S rRNA gene

Isolate	Source	Species
M17-H1	Sinop	*Enterococcus faecium*
M17-H2	Sinop	*Enterococcus faecium*
M17-H3	Sinop	*Enterococcus faecium*
M17-H4	Sinop	*Enterococcus faecium*
M17-H5	Sinop	*Enterococcus faecium*
M17-H6	Sinop	*Enterococcus faecium*
M17-H7	Sinop	*Enterococcus faecium*
M17-N1	Ankara	*Enterococcus faecium*
M17-N2	Ankara	*Enterococcus faecium*
M17-N3	Ankara	*Enterococcus faecium*
M17-N4	Ankara	*Enterococcus faecium*
M17-N5	Ankara	*Enterococcus mundtii*
M17-N6	Ankara	*Enterococcus faecium*
M17-N7	Ankara	*Enterococcus faecium*
MRS-T1	Ankara	*Lactobacillus plantarum*
MRS-T2	Ankara	*Enterococcus dispar*
MRS-T3	Ankara	*Lactobacillus plantarum*
MRS-T4	Ankara	*Lactobacillus plantarum*
MRS-T5	Ankara	*Lactobacillus plantarum*
MRS-T6	Ankara	*Enterococcus dispar*
MRS-T7	Ankara	*Enterococcus faecium*
MRS-N1	Ankara	*Pediococcus pentosaceus*
MRS-N2	Ankara	*Pediococcus pentosaceus*
MRS-N3	Ankara	*Pediococcus pentosaceus*
MRS-N4	Ankara	*Pediococcus pentosaceus*
MRS-N5	Ankara	*Pediococcus pentosaceus*
MRS-N6	Ankara	*Pediococcus pentosaceus*
MRS-N7	Ankara	*Pediococcus pentosaceus*

After identification at the species level, we performed RAPD-PCR analysis of isolates according to their sampling sources. For this purpose, we used primer M13, an established primer for discriminating between lactic acid bacteria (Cebeci and Gürakan, 2011). RAPD-PCR is an easy to

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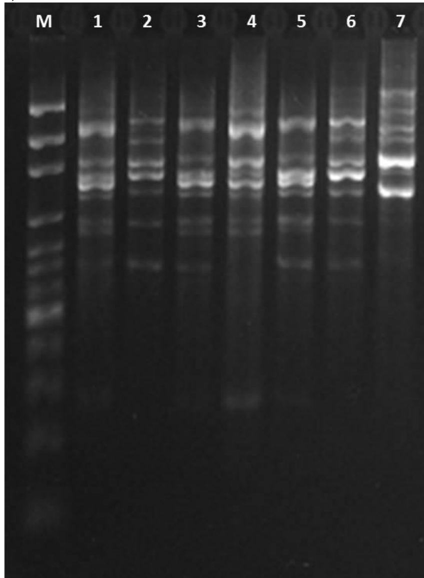
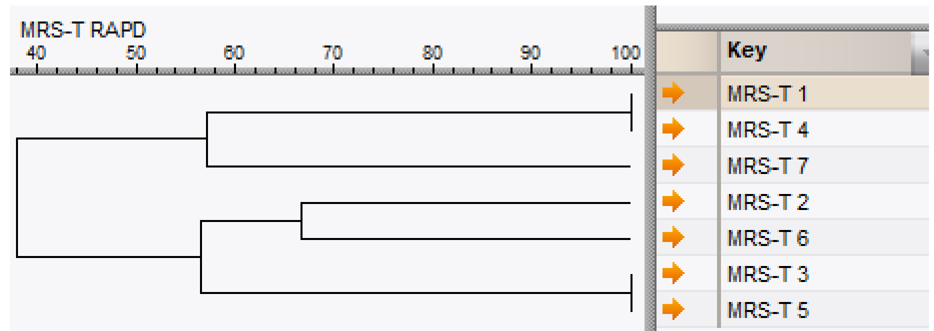
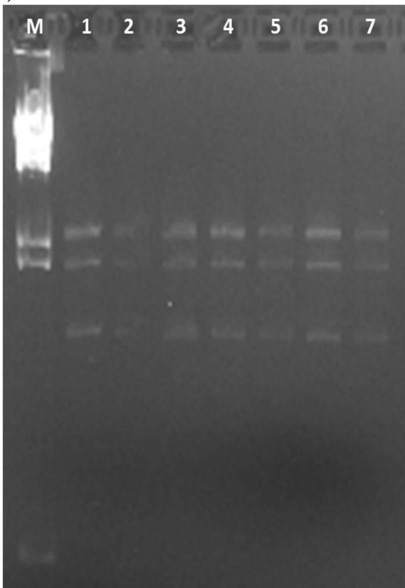
perform analysis, but strict conditions are required since reproducibility among laboratories is low and contamination issues may be encountered. For this purpose, stringent RAPD-PCR protocols were followed to make sure no such problems were encountered. Doing triplicate RAPD-PCR experiments ensured reproducibility of the method. M17 isolates have two common bands around 1200 and 3000bp, while for the MRS isolates a common band at around 2000bp is visible. When the number of bands produced by the primer is compared, it depended on the sample, MRS-T samples provided distinguishing fingerprinting patterns, while MRS-N samples provided no different patterns. Analyses of the RAPD-PCR patterns were used to construct phylogenetic trees (Figure 1). According to the dendrograms, primer M13 was successful in discriminating most of the tarhana isolates. MRS-T isolates formed 5 groups at the 80% similarity level, with several common bands, and the lowest similarity level for them was around 40%. MRS-T isolates also formed the highest number of bands with primer M13. On the other hand, MRS-N isolates were not differentiated at all, and all of the isolates were pooled into the same group at 100% similarity level. M17-N isolates formed 3 groups at the 80% similarity level and the lowest similarity level for them were around 30%. M17-H isolates formed only 2 groups at the 80% similarity level, and the lowest similarity level was 50%.

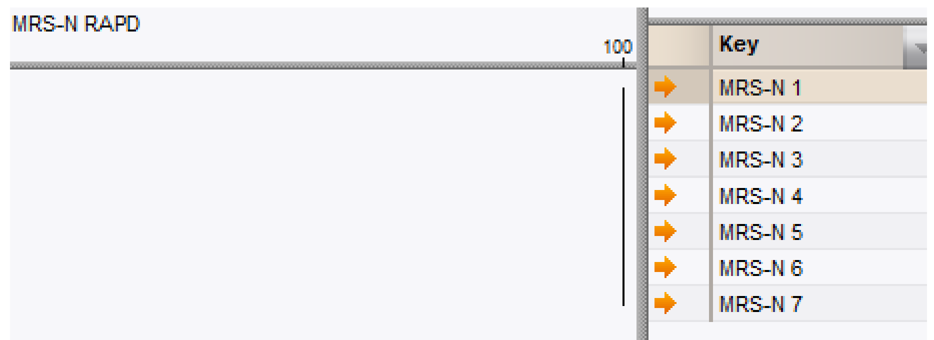
a)





b)





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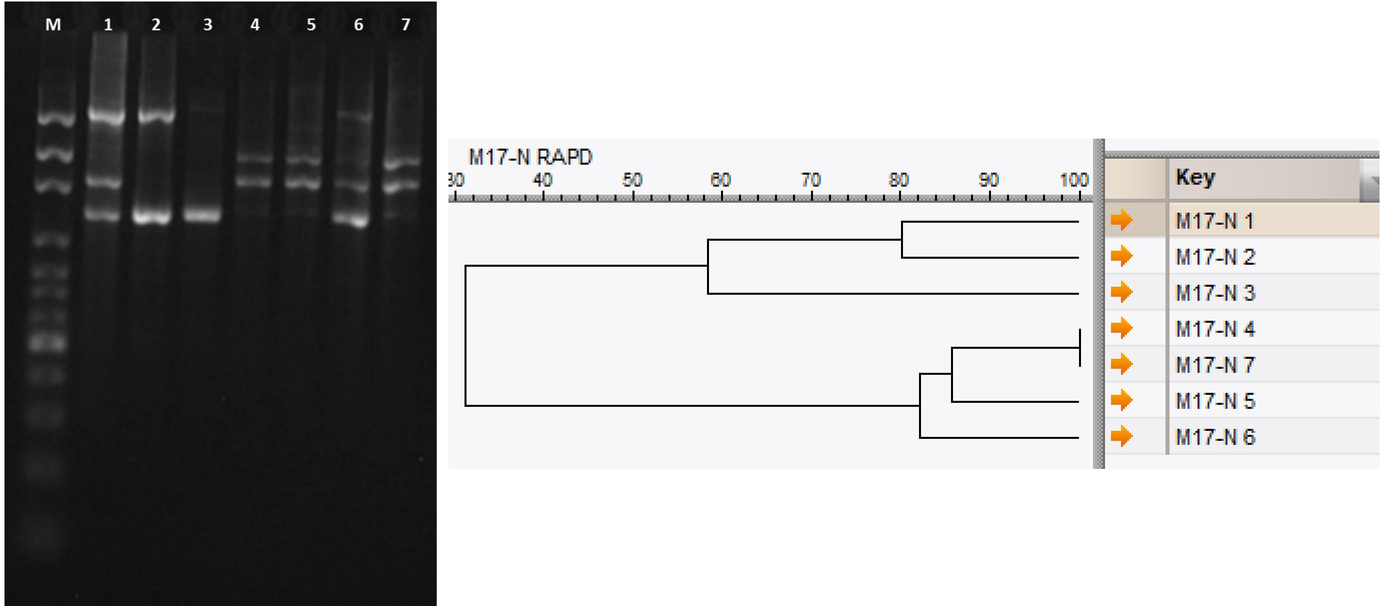
c)



d)

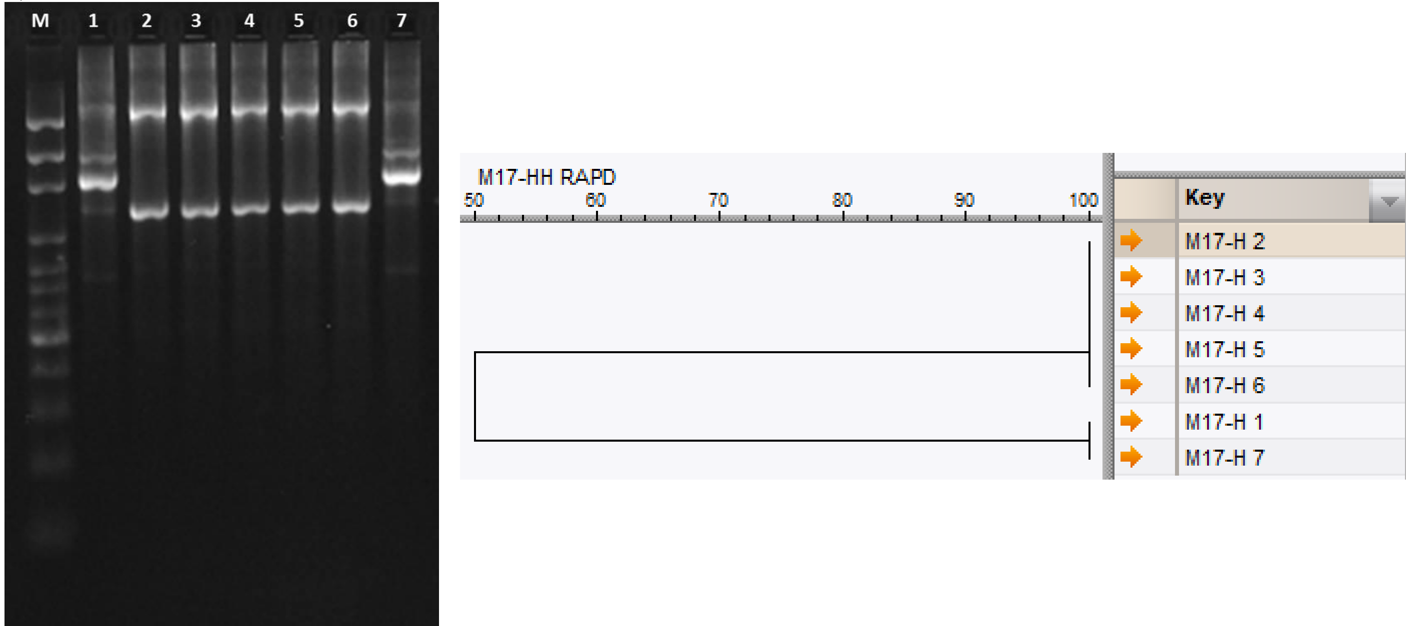


Figure 1. RAPD-PCR analyses of tarhana isolates and their corresponding dendrograms. a) MRS-T isolates. M: Phage Lambda / HindIII digest, 1: MRS-T1, 2: MRS-T2, 3: MRS-T3, 4: MRS-T4, 5: MRS-T5, 6: MRS-T6, 7: MRS-T7. b) MRS-N isolates. M: Axygen 100bp ladder plus, 1: MRS-N1, 2: MRS-N2, 3: MRS-N3, 4: MRS-N4, 5: MRS-N5, 6: MRS-N6, 7: MRS-N7. c) M17-N isolates. M: Axygen 100bp ladder plus, 1: M17-N1, 2: M17-N2, 3: M17-N3, 4: M17-N4, 5: M17-N5, 6: M17-N6, 7: M17-N7. d) M17-H isolates. M: Axygen 100bp ladder plus, 1: M17-H1, 2: M17-H2, 3: M17-H3, 4: M17-H4, 5: M17-H5, 6: M17-H6, 7: M17-H7.

Conclusion

Twenty-eight LAB isolates were successfully isolated from three different tarhana samples. The morphological and biochemical analyses were performed, followed by growth at different pH values and temperatures. These analyses were then followed by genetic analyses. Sequencing of the 16S rRNA gene resulted in identification at the species level. Dominant species were detected to be *E. faecium, P. pentosaceus* and *L. plantarum*. The differentiation of the isolates at the strain level was

obtained using RAPD-PCR. RAPD-PCR was able to discriminate most of the isolates into different groups, except for one group (MRS-N). The fingerprinting patterns were similar among the isolates from the same source, consistent with the literature. In conclusion, conventional microbiological identification of isolates as lactic acid bacteria was confirmed by molecular biology methods, and sequencing of the 16S rRNA gene together with RAPD-PCR were found to be very successful in identification and typing of lactic acid bacteria from tarhana samples.

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