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Detection, identification and phylogenetic analysis of lactic acid bacteria isolated from Tarkhineh, Iranian fermented cereal product, by amplifying the 16s rRNA gene with universal primers and differentiation using rep-PCR

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Abstract

Tarkhineh is a traditional fermented cereal based food which is produced in different rural areas in Kermanshah province, Iran. The aims of this study were to identify the predominant lactic acid bacteria isolates. A total of 75 Gram-positive and catalase-negative isolates were subjected to grouping and identifying using the carbohydrates fermentation profiles and 16S rDNA sequencing, respectively. The sequencing results showed that the majority of the strains belonged to the genus of *Lactobacillus*, and were dispersed into five species: *L. plantarum* (25.33%), *L. fermentum* (22.66%), *L. pentosus* (12%), *L. brevis* (10.66%), and *L. diolivorans* (1.33%). Minor populations of the other genera, included: *Enterococcus* (*faecium* and *faecalis*) (18.66%), *Leuconostoc citreum* (5.33%) and *Pediococcus* (*acidilactici* and *pentosaceus*) (3.99%). All of the strains of *L. plantarum* and *L. brevis* were subjected to rep-PCR typing. High intra-species diversity was found among *L. plantarum* and *L. brevis* isolates. It was concluded that poly-phasic strategies, based on molecular techniques, are necessary for reliable and accurate studying of the microbial composition of fermented products.

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Introduction

Fermented products have significant functional properties introducing some health benefits to consumers owing to presence of beneficial microorganisms, which has probiotics properties, antimicrobial, antioxidant, bioactive peptides production, etc. Benefits regarding to some fermented foods including: nutrients synthesis, avoiding of cardiovascular disease, prevention of cancer, gastrointestinal disorders, allergic reactions, diabetes, among others (Tamang *et al*., 2016). Tarkhineh is a kind of fermented food product based on cereal and dairy products and used in some countries, including Turkey, Egypt, Iraq, Greece, Hungary and Finland with different names such as Tarhana, Kishk, Kushuk, Talkhuna and etc. (Ibanoglu *et al.*, 1999; Tamang *et al*., 2005). Due to its high nutritional value, it can be a good source of proteins, minerals, fatty acids and free amino acids which make it healthy for children, the elderly and medical patients. In addition, it is a good source of vitamins such as thiamine, riboflavin and B12 (Ibanoglu *et al*., 1999). Tarkhineh is produced in western provinces of Iran. It is generally prepared with wheat grout, doogh (butter milk) and turnip. The mixture is kneaded with vegetables, salt, leaven and spices and then fermented with doogh micro flora at room temperature (20◦C) for 3 days. Furthermore, dried Tarkhineh slices are consumed in different ways such as nugget or snack in local regions (Mashak *et al*., 2014). The low moisture content (3–9%) and low pH value (4.0–4.5) of the final product provide a bacteriostatic effect against pathogenic and food-spoilage microorganisms and increase the product shelf life (Ibanoglu *et al*., 1999). Fermentation occurs mainly by the naturally-occurring microorganisms of the raw materials which contain numerous micro flora, including lactic acid bacteria (LAB) (Tamang *et al*., 2016). LAB comprise a large and diverse group of gram positive, non-spore- forming bacteria, catalase negative, acid-tolerant, facultative anaerobic organisms, and a strictly fermentative rod or cocci, able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Moraes *et al*., 2013). They need some complex growth factors such as vitamins and amino acids. The use of Polymerase Chain Reaction (PCR) for rapid identification of LAB has the potential to overcome the poor sensitivity of culture methods and has been spreading over the last 20 years (Chakrabarti *et al*., 2009; Kesmen *et al*., 2012). The sequencing of the 16S rRNA gene has become the most widely used technique to study bacterial communities in fermented food products. Also, rep-PCR is considered to be a reliable tool for

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the classification and typing of very closely related species, especially in the differentiation of bacterial strains within one species (Kesmen *et al*., 2012). To the best of our knowledge, no study has been performed so far on the microbiota of Tarkhineh and their subsequent differentiation with molecular methods. Therefore, the aim of this study was to determine the lactic acid bacterial profile of Tarkhineh from different regions of Kermanshah province, Iran, using cultural methods and different molecular strategies, based on culture-dependent methods.

Materials and Methods

Sampling

Nine Tarkhineh samples were collected randomly from nine regions of Kermanshah province, Iran. Then, the samples were stored aseptically in low temperature (4◦C) to protect contamination and deterioration. Finally, the samples were transported to the laboratory for further analysis (Edalatian *et al*., 2012).

Microbiological and chemical analysis

The pH of the samples were determined at 25°C using a pH meter (WTW-Inolab Level 3 Terminal, Weilheim, Germany) (Panagou *et al*., 2008). Titratable acidity, protein, ash, fat and salt contents were determined according to AOAC (2000). For LAB isolation, 10 gr of each samples were transferred to 90 ml of 0.1% peptone water (Merck, Germany) and homogenized with a Stomacher)Seaward model, Germany). Serial dilutions up to 10^{-7} were prepared and appropriate dilutions were plated on to de Man Rogosa and Sharpe agar (MRS, Merck) in duplicate and all plates were incubated at two different temperatures, namely 30°C and 45°C for 48h under anaerobic condition (Gas-pack system). Colonies were sub-cultured three times on MRS agar to obtain single pure colonies (Sengun *et al*., 2009). For long time storage, the isolates were kept at −80°C in MRS broths containing 15% (v/v) glycerol (Edalatian *et al*., 2012). In order to determine coliform bacteria, Violet Red Bile Agar was used at 37°C for 24 h. Moreover, *Staphylococcus aureus* was isolated on Baird Parker Agar after incubation at 35°C for 48 h (Sagdic *et al*., 2014).

Classification of LAB according to biochemical and carbohydrate fermentation tests

Following isolation, morphological tests, including Gram staining and biochemical analysis such as catalase test, growth at various temperatures (10 and 45°C), survival at various pH (4.4 and 9.6), survival at 6.5% NaCl and ability to produce CO_2 were done. Next, grouping of LAB isolates was conducted using 10 different types of carbohydrates (glucose, sucrose, galactose, fructose, lactose, maltose, sorbitol, raffinose, mannitol and melibiose) using Phenol Red Broth based (Quelab, Canada) (Fitzsimons *et al*., 1999).

Molecular identification of LAB species

Cryopreserved cultures were recovered in the MRS media and identified with a poly-phasic molecular procedure, including extraction of total DNA, partial amplification of 16S rRNA genes, sequencing and sequence comparison.

DNA extraction

Total DNA was extracted from 23 Gram-positive and catalase-negative isolates. Genomic DNA was extracted from 0.1 ml sample of overnight cultures grown in MRS broth using a Genomic DNA isolation kit, Dena Zist Asia (Mashhad, Iran).

Amplification of 16S rRNA genes

The primers used for the amplifications of 16S rRNA genes were 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) based on the conserved regions of the 16S rRNA gene. PCR was performed in 25 µL reaction volumes containing 10 mmol.L^{-1} Tris-HCl, 50 mmol.L^{-1} KCl, 1.5 mmol.L^{-1} $MgCl_2$, 0.2 mmol.L^{-1} of each dNTPs, 0.2 mmol.L^{-1} of the primers, and 5 U of Taq-polymerase. PCR amplifications were carried out in Labcycler Gradient 011-101 (Sensquest Goettingen, Germany). Amplification procedure was as follows: initial denaturation of DNA for 5 min at 95°C, 30 cycles of DNA denaturation for 1 min at 94°C, annealing for 1 min at 53°C, extension for 2 min at 72°C, and final incubation for 5 min at 72°C. Almost 5 µl of the PCR products were analyzed by 1% agarose gel electrophoresis stained with Green viewer (Fermentase, Burlington, ON, Canada) in 1X TBE buffer at 90 V for 45 min (Alegría *et al*., 2009).

Sequencing and sequence comparison

Following grouping of the isolates according to phenotypic and biochemical analysis, representative amplicons were sent to Macrogen (Seoul, South Korea) for sequencing through cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, Ca., USA) with the primer 27FYM. Averagely, 1200 bp was obtained per sequence, which was then compared with those in GenBank using the BLAST program (*http://www.ncbi.nlm.nih.*

gov/BLAST/). The strains whose sequences showed an identification percentage of at least 97% compared to other in databases, were allocated to the same species, as considered elsewhere (Stackebrandt and Gobel 1994; Palys 1997).

Phylogenetic analysis

The sequences of 16S rRNA gene were edited using the Chromas Lite 2.1 program and after deleting the regions containing ambiguous nucleotides, the sequences of the length 1200 base were aligned by CLC Main Workbench 5.5 program. The phylogenetic trees were constructed by neighbor-joining statistical method. *Escherichia coli* ECSC1 was used as an out group. In order to determine the stability of our phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis using Mega version 5 with 50% cut-off (Chao *et al*., 2007; Park *et al.*, 2010).

Typing of the isolates

All *L. plantarum* and *L. brevis* isolates were grouped by rep-PCR typing using the primer BoxA2R (5′-ACGTGGTTTGAAGAGATTTTCG-3′) according to Edalatian *et al.* (2012). PCR amplifications were performed in Labcycler Gradient 011-101 (Sensquest Goettingen, Germany). Amplification products of rep-PCR were subjected to electrophoresis in 1.5% agarose gels in 1xTBE buffer for 90 min and 75V. The bands were visualized under UV light after staining with DNA Green Viewer and photographed. Rep-PCR bands were treated as binary characters and coded accordingly (presence: 1, absence: 0). Pattern similarity was expressed via the simple matching (SM) coefficient, and patterns were clustered by the Un-weighted pair group method using arithmetic averages (UPGMA).

Statistical analysis

Statistical analysis applied was complete randomized design with factorial experiment. The data of counting lactic acid bacteria were analyzed using Minitab version 17. Graphs were plotted using Excel 2013 software.

Results and Discussion

Microbiological and chemical analysis

Total titratable acidity was calculated based on the lactic acid percentage. The concentrations of these organic acid increased continuously during fermentation; consequently, the pH values decreased until the end of the fermentation course. The increase in acidity followed by a decrease in pH value might have been due to the formation of organic acids resulting from the fermentation of sugars, mostly by lactic acid bacteria (Table 1). As shows in Table 1, for all tested Tarkhineh samples, fermentation time had no significant effect (Pvalue<0.01) on protein, ash, fat and salt. However, it had significant effect (Pvalue<0.01) on the pH value and acidity of Tarkhineh samples. The protein content of Tarkhineh ranged from 14.72 to14.80 g/100g. Similar results were observed about the protein content of Tarhana: 19.87-19.90 g/100g (Gabrial *et al.*, 2010) and 16.79 g/100g (Erbas *et al*., 2005). The ash content of Tarkhineh samples varied from 8.47 to 8.60. Erbas *et al.* (2005) reported that the ash content of Tarhana was 8.94 g/100g. The fat percentage of Tarkhineh was in the range of 1.41-1.50 g/100g. Fat content variations can be due to different types of dairy ingredients which are used in their formulations. The salt content of Tarkhineh samples was 4.91-5.00 g/100g with refers mainly to the variation of salt addition during the manufacture of the foods. Generally, ingredients type and their proportions, as well as processing techniques are the factors influencing the chemical compositions of Tarkhineh (Kose and Cagindi, 2002).

Table 1 shows the log (cfu/g) of lactic acid bacteria (LAB) of Tarkhineh samples during fermentation, determined on MRS agar incubated at 30◦C and 42◦C for 48-72 h. The number of LAB in Tarkhineh samples on MRS ranged between 2.31 and 10.21 log CFU/g, depending on the sample analyzed and the incubation temperature. Results indicated that the microbial counts increased by increasing the fermentation time reaching the highest level at the second day of fermentation (except the sample T6), subsequently, in the third day of fermentation the LAB counts were either stable or showed a decrease probably reflecting lack of nutrients . Daglioglu *et al.* (2002) mentioned that LAB counts of Tarhana samples increased from 10^3 to 10^5–10^6 CFU/g during the first 3 days of fermentation, decreased to 10^4 CFU/g during further fermentation. In this study, the counts of LAB were higher at 30◦C than those at 45◦C, which shows the predominance of mesophilic LAB in our samples. The increase in the counts of LAB correlated with the rapid decrease in the pH values of Tarkhineh during the first 2 days of ripening, due to the production of lactic acid by some bacteria. Obviously, various stages of fermentation had significant effect on LAB count grown on MRS agar medium (P<0.01). Furthermore, our results demonstrated that all Tarkhineh samples were free from Coliforms and *S. aureus* during the fermentation period, indicating no contamination seen from the environment or the raw materials. The ability of LAB, including *L. plantarum*, to produce

Table 1. Changes in pH, acidity, protein, ash, fat, salt and microbial counts of Tarkhineh samples during the fermentation time

Tarkhineh samples	Fermentation time (days)	pH	Acidity	Log cfu/g		Coliform	*S. aureus*	Protein	Ash	Fat	Salt
				30 °C	45 °C			(g/100g)			
T1	0	4.47±0.03[a]	3.72±0.03[a]	3.71±0.02[a]	2.38±0.09[a]	ND	ND	14.74±0.25[a]	8.52±0.31[a]	1.44±0.20[a]	4.99±0.29[a]
	1	4.27±0.07[c]	5.02±0.04[d]	7.69±0.07[c]	5.60±0.06[d]	ND	ND	14.74±0.34[a]	8.51±0.33[a]	1.45±0.16[a]	4.98±0.51[a]
	2	4.06±0.03[e]	7.03±0.02[e]	9.17±0.10[e]	6.47±0.03[e]	ND	ND	14.72±0.24[a]	8.52±0.30[a]	1.45±0.05[a]	4.98±0.46[a]
	3	4.00±0.05[e]	7.16±0.02[f]	7.58±0.09[b]	6.17±0.10[c]	ND	ND	14.73±0.23[a]	8.49±0.25[a]	1.45±0.24[a]	4.99±0.42[a]
T2	0	4.29±0.05[a]	3.27±0.04[a]	2.75±0.07[a]	2.44±0.14[a]	ND	ND	14.79±0.29[a]	8.50±0.16[a]	1.47±0.41[a]	4.95±0.63[a]
	1	4.10±0.07[c]	4.57±0.02[c]	6.66±0.04[c]	6.64±0.07[c]	ND	ND	14.79±0.28[a]	8.52±0.24[a]	1.46±0.46[a]	4.95±0.56[a]
	2	3.79±0.06[e]	7.34±0.06[e]	7.42±0.06[b]	7.86±0.12[e]	ND	ND	14.80±0.31[a]	8.50±0.26[a]	1.47±0.60[a]	4.94±0.50[a]
	3	3.75±0.02[e]	7.43±0.04[e]	6.43±0.08[c]	7.35±0.11[e]	ND	ND	14.80±0.28[a]	8.50±0.15[a]	1.46±0.46[a]	4.94±0.36[a]
T3	0	4.56±0.07[a]	2.21±0.09[a]	3.33±0.12[a]	2.68±0.16[a]	ND	ND	14.77±0.25[a]	8.48±0.15[a]	1.41±0.26[a]	4.98±0.50[a]
	1	4.21±0.02[c]	3.48±0.04[b]	6.56±0.19[c]	6.24±0.13[c]	ND	ND	14.77±0.30[a]	8.49±0.21[a]	1.43±0.30[a]	4.97±0.46[a]
	2	3.92±0.03[e]	6.53±0.04[e]	7.46±0.12[c]	6.87±0.10[c]	ND	ND	14.78±0.33[a]	8.47±0.24[a]	1.41±0.25[a]	4.97±0.55[a]
	3	3.82±0.02[e]	6.63±0.02[e]	6.59±0.06[c]	6.41±0.09[c]	ND	ND	14.76±0.25[a]	8.48±0.15[a]	1.42±0.31[a]	4.98±0.30[a]
T4	0	4.63±0.02[a]	3.08±0.04[a]	2.87±0.09[a]	2.99±0.14[a]	ND	ND	14.74±0.20[a]	8.50±0.42[a]	1.49±0.38[a]	4.92±0.60[a]
	1	4.43±0.02[c]	4.41±0.05[c]	6.26±0.07[c]	5.26±0.14[c]	ND	ND	14.74±0.21[a]	8.50±0.35[a]	1.48±0.30[a]	4.93±0.51[a]
	2	4.16±0.03[e]	6.95±0.01[e]	7.83±0.19[c]	7.25±0.20[e]	ND	ND	14.75±0.24[a]	8.52±0.46[a]	1.49±0.36[a]	4.93±0.35[a]
	3	4.13±0.04[e]	7.12±0.03[e]	6.56±0.14[c]	6.73±0.19[c]	ND	ND	14.74±0.21[a]	8.52±0.53[a]	1.47±0.16[a]	4.92±0.35[a]
T5	0	4.70±0.06[a]	3.47±0.03[a]	4.22±0.10[a]	3.47±0.10[a]	ND	ND	14.78±0.30[a]	8.55±0.63[a]	1.42±0.33[a]	4.96±0.48[a]
	1	4.40±0.02[c]	4.66±0.05[c]	7.61±.015[c]	7.43±0.12[c]	ND	ND	14.79±0.32[a]	8.55±0.42[a]	1.42±0.24[a]	4.95±0.56[a]
	2	4.15±0.04[e]	6.75±0.03[e]	10.26±0.01[e]	7.86±0.09[e]	ND	ND	14.77±0.31[a]	8.55±0.61[a]	1.43±0.22[a]	4.96±0.40[a]
	3	4.14±0.02[e]	6.92±0.04[e]	7.31±0.12[c]	6.22±0.11[c]	ND	ND	14.79±0.31[a]	8.57±0.55[a]	1.43±0.24[a]	4.97±0.24[a]
T6	0	4.43±0.04[a]	3.66±0.02[a]	3.68±0.09[a]	3.16±0.08[a]	ND	ND	14.74±0.29[a]	8.56±0.33[a]	1.46±0.18[a]	4.92±0.55[a]
	1	4.17±0.02[c]	4.70±0.01[c]	7.66±0.08[c]	6.75±0.09[c]	ND	ND	14.74±0.27[a]	8.56±0.40[a]	1.46±0.19[a]	4.91±0.47[a]
	2	4.07±0.02[e]	6.82±0.05[e]	8.64±0.11[c]	4.28±0.10[c]	ND	ND	14.75±0.31[a]	8.55±0.35[a]	1.45±0.35[a]	4.92±0.55[a]
	3	3.98±.04[e]	7.03±0.02[e]	6.43±0.01[c]	3.70±0.19[c]	ND	ND	14.74±0.25[a]	8.57±0.41[a]	1.44±0.43[a]	4.91±0.40[a]
T7	0	4.97±0.07[a]	2.13±0.04[a]	4.25±0.06[a]	3.52±0.14[a]	ND	ND	14.77±0.32[a]	8.60±0.28[a]	1.42±0.20[a]	4.97±0.63[a]
	1	4.62±0.04[c]	3.83±0.04[c]	7.62±0.05[c]	7.40±0.07[c]	ND	ND	14.77±0.31[a]	8.58±0.28[a]	1.41±0.23[a]	4.96±0.62[a]
	2	4.34±0.03[e]	5.94±0.02[e]	8.41±0.10[c]	8.15±0.09[c]	ND	ND	14.78±0.32[a]	8.58±0.32[a]	1.41±0.36[a]	4.96±0.35[a]
	3	4.30±0.02[e]	6.12±0.03[e]	7.87±0.17[c]	7.78±0.12[c]	ND	ND	14.77±0.30[a]	8.58±0.24[a]	1.42±0.42[a]	4.96±0.40[a]
T8	0	4.26±0.03[a]	3.82±0.02[a]	3.15±0.05[a]	2.41±0.12[a]	ND	ND	14.73±0.21[a]	8.53±0.15[a]	1.50±0.41[a]	5.00±0.53[a]
	1	4.10±0.02[c]	5.84±0.06[c]	6.41±0.09[c]	5.23±0.08[c]	ND	ND	14.72±0.22[a]	8.51±0.16[a]	1.50±0.35[a]	4.99±0.41[a]
	2	3.96±0.03[e]	7.17±0.02[e]	7.61±0.04[c]	5.52±0.12[c]	ND	ND	14.72±0.24[a]	8.53±0.21[a]	1.49±0.36[a]	4.99±0.25[a]
	3	3.91±0.04[e]	7.99±0.02[e]	6.87±0.09[c]	5.12±0.01[c]	ND	ND	14.73±0.23[a]	8.52±0.17[a]	1.49±0.32[a]	5.00±0.26[a]
T9	0	4.56±0.05[a]	2.12±0.02[a]	3.97±0.09[a]	2.62±0.12[a]	ND	ND	14.80±0.32[a]	8.55±0.49[a]	1.47±0.43[a]	4.92±0.38[a]
	1	4.35±0.04[c]	3.08±0.04[c]	7.33±0.09[c]	6.69±0.16[c]	ND	ND	14.78±0.31[a]	8.57±0.47[a]	1.48±0.36[a]	4.93±0.40[a]
	2	4.24±0.01[c]	5.43±0.04[e]	9.64±0.07[c]	7.11±0.09[c]	ND	ND	14.79±0.31[a]	8.55±0.46[a]	1.48±0.43[a]	4.93±0.26[a]
	3	4.16±0.07[c]	5.67±0.02[e]	6.52±0.14[c]	6.67±0.17[c]	ND	ND	14.78±0.29[a]	8.54±0.27[a]	1.47±0.46[a]	4.94±0.50[a]

T1: Samp l, T2: Sample 2, T3: Sample 3, T4: Sample 4, T5: Sample 5, T6: Sample 6, T7: Sample 7, T8: Sample, T9: Sample 9
ND = Not Detected

oxygen peroxide may hasten the elimination of Coliforms and *S. aureus* (Arizcun *et al*., 1997). As a result, the absence of gram-negative and food-borne pathogens, low pH and the presence of large numbers of LAB make Tarkhineh as a healthy food.

Classification and grouping of LAB using physiological and biochemical tests

In a total of 185 colonies isolated from Tarkhineh, 75 presumptive LAB isolates strains were found to be gram-positive, catalase negative, non-motile, cocci or rod-shaped. The results of the physiological and biochemical tests are presented in Table 2. The majority of the isolates were heterofermentative (64.00%) and bacilli (72.00%). Out of 75 strains of LAB isolated from Tarkhineh samples, 10 strains were homofermentative rods, and 3 isolates were tetrad-forming cocci. All cocci forming tetrads were presumptively identified as pediococci. In addition, the isolates that were cocci and heterofermentative, belonged to *Leuconostoc* and those of the isolates that were cocci and could grow at 10 and 45°C, were identified as *Enterococcus* (Tamang *et al*., 2005). A total of 75 isolates, purified from MRS were examined using biochemical tests and carbohydrate fermentation profiles. According to biochemical tests, all these 75 isolates were identified up to genus level and classified under 4 separate genus including *Lactobacillus, Enterococcus, Pediococcus* and *Leuconostoc* (Table 2). After observing the carbohydrate fermentation profile (10 carbohydrates), these 75 isolates were classified under 10 distinct groups (Table 2).

Identification and typing of LAB using molecular culture-dependent methods

Based upon phenotypic results, representative isolates from each group were selected for DNA extraction and subsequent PCR of the 16S rRNA gene. A great number of similarities (97–100%) between the isolates were obtained during the present study and the sequences deposited in Gen-bank were found. The results obtained with sequencing exhibited that the majority of the strains belonged to the genus *Lactobacillus*, and were divided into five species: *L. plantarum* (25.33%), *L. fermentum* (22.66%), *L.*

Table 2. Physiological and biochemical characteristics of lab isolated from Tarkhineh

Group number	1	2	3	4	5	6	7	8	9	10
Number of strains	19	17	9	8	1	2	1	4	7	7
Growth at 10 °C	+	–	+	+	+	–	–	+	+	+
Growth at 45 °C	–	+	–	–	–	+	+	–	+	+
Growth at pH=4.4	+	–	+	+	+	+	+	–	+	+
Growth at pH=9.6	(8/11)	(15/2)	(7/2)	(5/3)	–	–	–	–	+	+
Growth at 6.5% NaCl	(17/2)	–	–	(4/4)	–	+	+	(1/3)	+	+
CO_2 from glucose	(12/7)	+	(6/3)	+	+	–	–	+	–	–
Glucose	+	+	(8/1)	+	–	+	+	+	+	+
Sucrose	+	+	+	+	–	+	–	+	–	+
Galactose	+	+	+	+	+	+	+	–	+	+
Fructose	+	+	+	+	+	+	+	(3/1)	+	+
Lactose	+	+	+	+	–	–	–	–	+	+
Maltose	+	+	+	+	+	+	+	+	–	(6/1)
Sorbitol	+	+	+	+	–	–	–	–	–	–
Raffinose	+	+	+	–	–	–	–	–	–	–
Mannitol	+	(11/6)	+	–	+	–	–	+	(5/2)	+
Mellibiose	(14/4)	+	(4/5)	+	+	–	+	–	–	–

+, all strains positive; –, all strains negative; (../..), number of positive/negative strains

pentosus (12%), *L. brevis* (10.66%), and *L. diolivorans* (1.33%). It has been reported in many investigations that lactobacilli have been predominantly present at the end of the ripening period (Sengun *et al.*, 2009; Edalatian *et al.*, 2012; Tamang *et al*., 2005). These species are more resistant to harsh conditions like higher salt concentration, anaerobic condition and reduced availability of nutrients (Marino *et al*., 2003). Minor populations of other genera, included: *Enterococcus* (*faecium* and *feacalis*) (18.66%), *Leuconostoc citreum* (5.33%) and *Pediococcus* (*acidilactici* and *pentosaceus*) (3.99%). The results of sequencing are shown in Table 3. Isolate series A was obtained from Tarkhineh immediately after production. In conclusion *En. faecium, L. plantarum* and *Le. citreum* were detected in the raw materials. Sengun *et al.* (2009) reported that the genus *Enterococci,* mainly *En. faecium,* was the most frequent isolated LAB from wheat grains. Isolates series B and C include *L. plantarum, L. fermentum, L. brevis* and *P. acidilactici* obtained on the first day. After 2 days (series D, E and F), the largest diversity of bacteria covering seven species, *L. plantarum, L. fermentum, L. pentosus, L. brevis, Le. citreum, P. pentosaceus* and *En. faecalis* was obtained and *L. plantarum* was the most abundant one. Four species, including *L. plantarum, L. fermentum, L. diolivorans* and *En. Faecium* were LAB on the sixth day (series G and H). Isolates series J include *L. fermentum* and *En. faecalis* obtained on the tenth day (seven days after the end of fermentation). *Lactobacillus* and *Leuconostoc* spp. are generally found in plants. Firstly, heterofermentative strains such as *Leuconostoc* strains began the fermentation with gas production. The absence of *Leuconostoc* in the other sampling times except for one case, may be because of acid production during fermentation and low resistance of this genus to low pH of the environment and prefer an initial medium pH of 6.5 (Schleifer 2009). The diversity of LAB in Tarkhineh depends primarily on their initial levels in raw materials, especially local doogh. In the second place, it is influenced by the ingredient compositions and fermentation conditions. Natural fermented products in different regions have their own specific properties. The micro floras were greatly affected by the local specific climatic conditions (Kurmann, 1984). Tamang *et al.* (2005) reported the presence of *L. plantarum, L. brevis* and *pediococcus* in the products from regions with a cold climate. In our study, 65.33% of the isolates could grow at 10˚C and the predominant micro flora was *L. plantarum* which is in agreement with the cold weather of Kermanshah province. *L. plantarum* has a long history of natural presence and safe use in various food products. Following *L. plantarum,* the predominant LAB was *L. fermentum* which exhibited the phenotypic properties typical of obligatory heterofermentative, gram-positive, catalase-negative rods, which occurred singly or in pairs and produced gas and DL-lactate from glucose metabolism. Similar to our results, *L. fermentum* was isolated from other fermented products such as Algerian arid zone raw goat milk, Maasai (a traditional fermented milk in Kenya), Nigerian fermented foods and the traditional fermented milk, Dahi, in Bangladesh. Also, *L. fermentum* has been traditionally and widely found in fermented cereal food (Olasupo *et al*., 2001; Guessas and Kihal 2004; Mathara *et al.*, 2004; Harun-ur-Rashid *et al*., 2007). Ten different species of LAB were observed among which *Lactobacillus* spp. were the most frequently detected ones in Tarkhineh samples. Some LAB species are beneficial bacteria in that they favorably alter the intestinal microbiota balance, promote good digestion, inhibit the growth of harmful and pathogenic bacteria, boost the immune

Table 3. Results of molecular analyses (16S rRNA PCR and sequencing)

Isolate Code	Day of sampling	Name of Bacteria	%ID in NCBI	Accession Number
A3	0	*Lactobacillus plantarum strain G1*	100	KC965107.1
A12	0	*Lactobacillus plantarum strain KLDS 1.0725*	100	EU626010.1
A25	0	*Enterococcus faecium* strain BAB-1371	98	KF535138.1
A33	0	*Leuconostoc citreum KM20*	100	NR_074694.1
B7	1	*Lactobacillus plantarum strain KLDS 1.0725*	100	EU626010.1
B23	1	*Lactobacillus brevis strain FSHS1*	100	KF307784.1
C10	1	*Lactobacillus fermentum strain FQ002*	100	KF418815.1
C17	1	*Pediococcus acidilactici* DSM 20284 strain DSM 20284	98	NR_042057.1
D2	2	*Lactobacillus pentosus strain 124-2*	100	NR_029133.1
E14	2	*Lactobacillus plantarum strain IMAU32489*	100	KF149163.1
E33	2	*Lactobacillus plantarum strain IMAU32489*	100	KF149163.1
E35	2	*Pediococcus pentosaceus ATCC 25745 strain ATCC 25745*	98	NR_075052.1
E48	2	*Lactobacillus brevis strain FSHS1*	100	KF307784.1
E52	2	*Lactobacillus fermentum strain KLDS 1.0613*	99	EU419592.1
F6	2	*Lactobacillus fermentum strain FQ002*	100	KF418815.1
F19	2	*Leuconostoc citreum strain IMAU62126*	99	KF149766.1
F36	2	*Enterococcus faecalis V583 strain V583*	100	*NR_074637.1*
G11	6	*Lactobacillus fermentum* strain FQ002	100	KF418815.1
G27	6	*Lactobacillus plantarum* strain KLDS 1.0725	98	EU626010.1
H12	6	*Lactobacillus diolivorans* strain NM130-2	99	HM218517.1
H14	6	*Enterococcus faecium* strain FS019	100	KC568549.1
J4	10	*Lactobacillus fermentum IFO 3956*	100	NR_075033.1
J16	10	*Enterococcus faecalis V583*	99	NR_074637.1

system function and increase the resistance to infection called probiotic. Today probiotics are using as bio-ingredients in many functional fermented products (Dhanasekaran *et al*., 2008; Sieladie *et al.*, 2011, Tamang *et al*., 2016). *Lactobacillus* genera is one of the famous understood probiotic bacteria. Some popular commercial probiotic cultures which are using in global markets include this genus (Iniguez-Palomares *et al*., 2007; Kisworo *et al.*, 2008). The high presence of this genus in Tarkhineh makes it healthy. Vasiee *et al.* (2014) studied the probiotic potential of *Lactobacillus* isolated from Tarkhineh. The authors declared some isolates belonging to *L. plantarum* and *L. fermentum* showed good probiotic properties. That is probably why people use it as a healthy food. In the fermentation of food products, *L. paracasei* and *L. rhamnosus* are usually isolated from dairy products whereas *L. plantarum* is found in fermented foods of plant origin (Molin 2001). In the case of Tarkhineh, we expected the predominant presence of *L. plantarum* because of the incorporation of wheat grout and herbal additives into the formulation. These results confirmed the selectivity and suitability of MRS medium for the genus *Lactobacillus* because of the negligible growth of other species in this medium. Lopez-Diaz *et al.* (2000) accomplished similar results that the genus *Lactobacillus* was predominant in MRS. On the other hand, the presence of some cocci-shaped LAB in this medium can be explained by the low selectivity of this medium that allows the growth of other LAB genera (Caridi, 2003). In our study, we isolated *Leuconostoc* species with a lower number (four out of 75 strains). *Leuconostoc* strains are important for flavor development in fermented products, although *Leuconostoc* exhibits a weak competitive ability during fermentation and has complex nutritional requirements (Mataragas *et al*., 2004). *P. acidilactici* and *P. pentosaceus* are difficult to differentiate using biochemical and physiological tests, suggesting a high degree of relatedness. These two species also show a higher relatedness to each other than to other Pediococci (Holzapfel *et al.*, 2005). At the phenotypic level, the inability of *P. acidilactici* to ferment maltose and its ability to grow at 50°C differentiates it from *P. pentosaceus*. Some strains of *P. acidilactici* produce the bacteriocin pediocin PA1/AcH (Marugg *et al.*, 1992). In our study, we isolated *Enterococcus,* too. The presence of *Enterococci* in fermented foods has been long considered as an inadequate sanitary condition during processing and production. But today, it has been demonstrated that the presence of *Enterococcus* strains in many food products is not always related to direct fecal contamination, therefore enterococci species might be considered as normal parts of the food microbiota (Zamfir, 2006). However, because of their proteolytic and esterolytic functions, as well as their production of diacetyl, they can develop flavor and aroma (Jurkovic *et al*., 2006). In order to improve the understanding of the lactic acid bacteria ecology present in Tarkhineh samples, we determined the distribution of the bacterial population using the results of culture-dependent methods. As can be seen in Figure 1, the population distribution is well observed in all samples. *L. plantarum* and *L. brevis* isolates were all subjected to rep-PCR typing

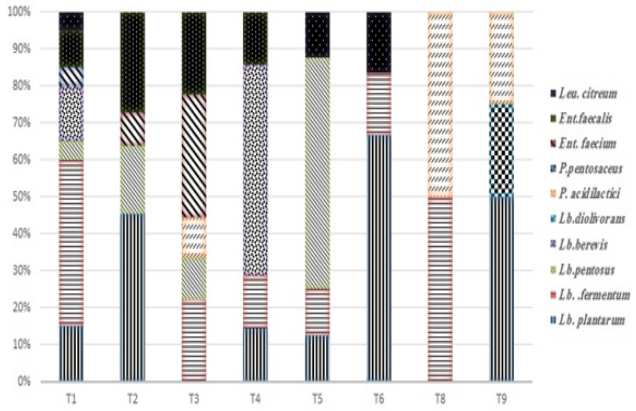

Figure 1. Distrubition of lactic acid bacteria present in Tarkineh samples, identified with culture-dependent methods.

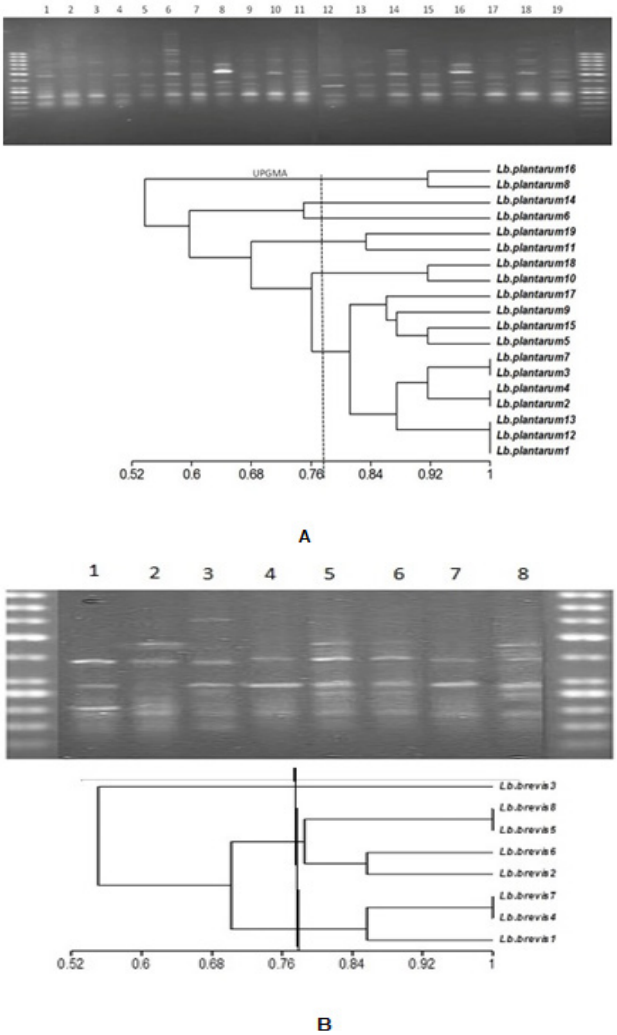

Figure 2. Typing REP-PCR profiles obtained with primer BoxA2R among the 19 *Lb. plantarum* (A) and 8 *Lb. brevis* (B) isolates from Tarkhineh. Below, dendogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient .Mmolecular weight marker GeneRuler TM (Fermentas, St. Leon-Rot, Germany). The broken line denotes the arbitrary percentage of similarity (77%) used to consider isolates as different strain; this percentage of similarity was lower than the assay reproducibility (78%).

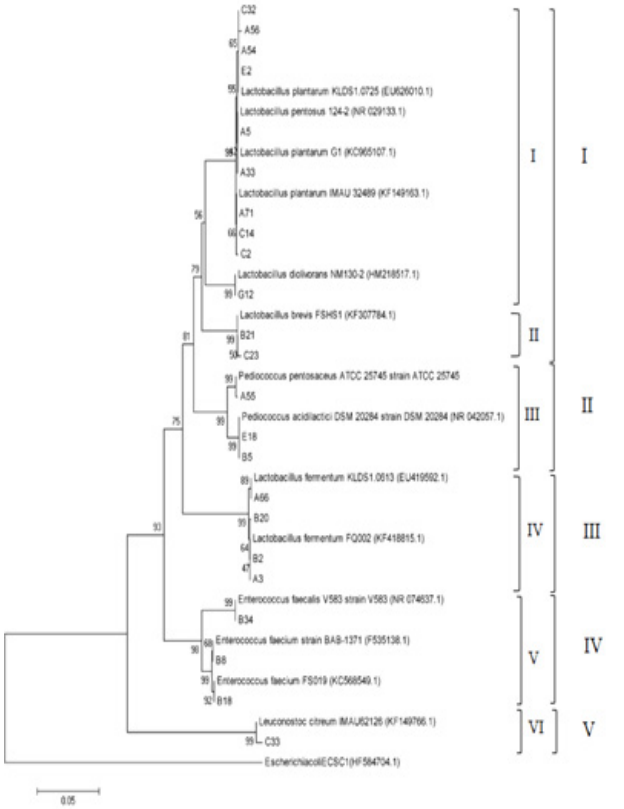

Figure 3. Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of strains isolated from Tarkhineh. The tree was constructed by the neighbor-joining statistical method, and *Escherichia coli* was used as the out group. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given.

to evaluate the intra-species diversity. Figure 2 shows the profiles obtained with the 19 *L. plantarum* isolates and 8 *L. brevis* from Tarkhineh, besides the similarity dendrogram for the different typing patterns clustered by the UPGMA method using the Simple Matching Coefficient. Given the reproducibility of the assay, the isolates sharing a similarity percentage of >78% were considered to be the same strain. Six different profiles were considered to represent different strains of *L. plantarum* and three different profiles were regarded to represent different strains of *L. brevis*. A high intra-species diversity was found among the *L. plantarum* and brevis isolates using rep-PCR, which suggests a high subsequent phenotypic diversity. In cultural detection approaches, biochemical tests and carbohydrate profiles were used for classification of the LAB isolates of Tarkhineh samples. As a result of these tests, 10 different groups of bacteria were observed. 23 isolates were randomly selected from each group and after DNA extraction, amplification of 16S rRNA gene was done. The isolates and related reference strains were used to construct the

phylogenetic tree (Figure 3), which is mainly composed of five clusters and six sub-clusters including four genera. Cluster I was *Lactobacilli* group which was composed of two sub-clusters: sub-cluster i *(L. plantarum)* and sub-cluster ii *(L. brevis)*. Cluster II (sub-cluster III) was *Pediococcus* group. Cluster III (sub-cluster IV) was *L. fermentum*. Cluster IV (sub-cluster V) was the group of *Enterococcus*. Cluster V (sub-cluster VI) was the group of *Leuconostoc*. In recent twenty years, the sequencing of gene coding for the 16S ribosomal RNA (rRNA) gene has become the most valuable tool for the identification of lactic acid bacteria (Ben Amor *et al*., 2007). On the other hand, BoxA2R-based rep-PCR is also a powerful PCR-based strain differentiation technique which has been successfully employed to differentiate between LAB species. Today, it is broadly accepted that the identification of lactobacilli to species or strain level based on physiological and biochemical measures is not reliable, very ambiguous and complicated. Even the 16S rRNA sequences analysis, which is considered to be rapid and reliable, might yield confusing results because of the large data base of sequence in NCBI data bank. Therefore, the use of poly-phasic strategies is necessary to identify the micro biota of fermented products (Edalatian *et al.*, 2012).

Conclusion

In this study, 75 LAB strains isolated from Tarkhineh were identified by 16S rRNA gene sequencing. According to our results, ten different LAB strains were determined among which *L. plantarum* was the most abundant one. Studying the biodiversity of traditional fermented food products is important as increasing the information on the natural microbiota of such products can help to prevent the loss of their diversity. Tarkhineh is produced in west of Iran whose typical features depend on local and regional traditions and on the indigenous microbiota present or selected by the environment or technology used. Moreover, this study provides raw data and a strain resource in the following culture-independent study on the microbial structure during the fermentation and evaluates the technological and probiotic properties of the isolates.

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