International Food Research Journal 21(6): 2487-2492 (2014)
Journal homepage: *http://www.ifrj.upm.edu.my*

Isolation, identification and characterization of probiotic *Lactobacilli* spp. from Tarkhineh

*Vasiee, A. R., Tabatabaei Yazdi, F., Mortazavi, A. and Edalatian, M. R.

Department of Food Science and Technology, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Article history

Received: 30 August 2013
Received in revised form: 9 June 2014
Accepted: 13 June 2014

Keywords

Tarkhineh
Lactobacillus
Probiotic characteristics
rep-PCR

Abstract

"Tarkhineh" is a traditional fermented food produced from a mixture of spontaneously fermented butter milk and wheat flour in Iran. Nine samples of Tarkhineh were collected from different rural areas in Kermanshah province, Iran. The isolates were grouped and identified using a combination of phenotypic and genotypic methods including repetitive extragenic palindromic polymerase chain reaction (REP-PCR) fingerprinting, biochemical methods and carbohydrate profiling and then evaluation the probiotic properties of them. According to the results these 54 isolates belonged to *Lactobacillus plantarum* (19), *Lactobacillus fermentum* (17), *Lactobacillus pentosus* (9), *Lactobacillus brevis* (8) and *Lactobacillus diolivorans* (1) that profile bonding from rep-PCR showed *Lactobacillus plantarum* have a high intra-species diversity. Media of pH = 2.0–7.0 and bile salt concentrations of 0.0-0.5% were used as stress conditions. Antibacterial activity of the probiotic *Lactobacillus* was determined by means of the spot-on-lawn method. In conclusion, our results showed that 3 strains have potential probiotic value that two of them was *Lactobacillus fermentum* and one of them was *Lactobacillus plantarum.*

Introduction

Tarkhineh is a traditional Iranian fermented cereal based food product. An overview of the production of Tarkhineh is shown in Figure 1. It is widely prepared and used as a soup especially on cold days. Lactic acid bacteria "LAB" have an important role in the fermentation of Tarkhineh. LABs are widely distributed in the nature and present in fermented foods. The lactic acid fermentation which these bacteria perform has long been known and applied for making foodstuff (Hoque *et al*., 2010; Barakat *et al.*, 2011) are Generally Recognized As Safe (GRAS) and can be used well for medical and veterinary applications (Hoque *et al*., 2010). LABs are known as microorganisms that have probiotic attributes. They can produce inhibitory compounds such as lactic acid, diacetyl, acetaldehyde, hydrogen peroxide and bacteriocin. These compounds are able to inhibit the growth of harmful and pathogen microorganisms (Allameh *et al*., 2012). Probiotic microorganism, as defined in a FAO/WHO (2002) report, "are live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Sieladie *et al*., 2011). Family of LAB are Grame-positive bacteria that ferment carbohydrate into energy and lactic acid (Liu *et al*., 2009). In the processed fermented food, the number of probiotic bacteria should be 10^6-10^7 CFU/g or 10^8-10^9 CFU in 100 g or 100 ml daily food consumption in order to get the medicinal benefit (Setyawardani *et al*., 2011). Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, promote good digestion, inhibit the growth of harmful and pathogen bacteria, boost immune function and increase resistance to infection (Dhanasekaran *et al*., 2010; Sieladie *et al*., 2011). Some other benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance (Sieladie *et al.*, 2011). Two of the famous understood probiotic bacteria include *Lactobacillus* and *Bifidobacterium* genera (Iñiguez-Palomares *et al*., 2007; Kisworo, 2008). During the last fifteen years, the *Lactobacillus* genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts, fermented milks and fermented cereal based food. *Lactobacillus* comprise a large and diverse group of gram positive, non-spore forming, catalase negative, rod bacteria, non-mobility, able to produce lactic acid as the main end-product of the fermentation of carbohydrates. In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and prove safety for consumer. To survive in the gut, organisms must be tolerant to low pH and bile salts in the upper digestive tract (Sieladie *et al*., 2011). Potential probiotic usually evaluate based on some criteria such as acid and bile salts

*Corresponding author.
Email: *ali.vasiee@gmail.com*

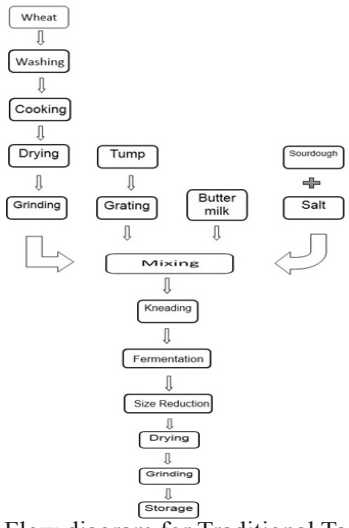

Figure 1. Flow diagram for Traditional Tarkhineh production

tolerance, antibiotic susceptibility, antibacterial test and adhesion properties as antagonistic effect against pathogens (Hoque *et al.*, 2010). Traditionally, LABs have been classified based on phenotypic properties including physiological tests and sugar fermentation patterns. Molecular techniques have been used as an effective and accurate method to identify and characterize the dominant flora in complex bacterial communities such as fermented food for the last 20 years (Kesmen *et al*., 2012). In particular, PCR-based genomic fingerprinting techniques are believed to have the highest potential for more rapid, reliable and repeatable detection, identification and classification of LAB. PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the characteristics like, high discriminatory power, low cost, suitable for a high-throughput of strains, and considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Geversa *et al*., 2001). This technique assigns molecular fingerprints based on the amplification of repetitive sequences specific to the organism and commonly found in non-coding regions of bacterial genomes (Behringer *et al.*, 2011). In this study we used three different formulations of Tarkhineh. Including local butter milk, local high-fat yogurt and local low-fat yogurt. The main objective of our study was isolation and identification of *Lactobacillus* spp. from Tarkhineh, evaluation probiotic characteristics and typing them.

Materials and Methods

Sample collection

Tarkhineh samples were collected from Kermanshah province, Iran. Then samples of Tarkhineh were stored aseptically in low temperature (4°C) refrigerator to protect contamination and deterioration. Finally, samples were transported to the laboratory for further analysis.

Isolation of lactic acid bacteria

Ten g sample was transferred to 90 ml 0.1% peptone water (Merck, Germany) and homogenized with a Stomacher (Seaward model, Germany). Then 0.1 ml of homogenized samples was spread on MRS agar (Merck) and all plates were incubated at two different temperatures, 30°C and 45°C for 48 h in anaerobic condition to remove unwanted bacteria (Sengun *et al*., 2009). Colonies sub-cultured three times on new MRS agar to obtain single pure colonies (Allameh, 2012). For long time storage, isolates were kept at (−20°C) in MRS broths containing 15% (v/v) glycerol.

Identification of Lactobacillus

At the first step after isolation, morphological characteristic tests were done which included Gram staining and physiological characteristics of LAB tests such as catalase, growth at various temperatures (10, 37, and 45°C), survival at various pHs (4.4 and 9.6), survival at 6.5% NaCl and ability to produce CO_2. The identification of *Lactobacillus* spp. isolates was conducted using 10 different types of carbohydrates and the media used for this reactions was Phenol Red Broth (based) (Quelab, Canada).

pH tolerance

Fifty four strains of lactobacilli were subjected to primary screening for acid tolerance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 minutes at 37°C. Streak method on MRS agar plates was performed to determine for survival isolates, and the growth was observed after 24-48 h after anaerobic incubation at 37°C. Isolates grown on agar were considered to be acid resistant strains. These strains were cultivated in MRS broth under anaerobic condition at 37°C. Cultures (10^7–10^8 cfu/ml) were inoculated in 10 ml of 0.05 M sodium phosphate buffer adjusted to pH 2.0, 3.0, and 7.0 with 1 N HCl. Samples were incubated at 37°C for 2 h. Cells were serially adjusted to 10-fold dilution by phosphate buffer pH 7.0. For determination of viable cells the dilution was plated on MRS agar after 48 h of incubation. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration. Experiments was performed in triplicate (Klayraung *et al*., 2008).

Bile salt tolerance

Survival of isolates in the presence of bile salts was determined by adjusting the early log phase

MRS broth culture to pH 6 with 1 N NaOH and adding suspension of bile salts to obtain different concentration (0.0, 0.1, 0.2, 0.3, 0.4, and 0.5%). Viable bacteria were determined after 24 and 48 h incubation at 37°C (Barakat *et al*., 2001).

Antibacterial activity

The antibacterial activity of isolates was determined by agar spot-on-lawn method. The indicator bacteria used in this study were *Escherichia coli* TISTR 780, *Listeria innocua* ATCC 33090 and *Staphylococcus aureus* TISTR 029. One µl of each overnight culture of selected *Lactobacillus* was spotted on MRS plates and incubated under anaerobic conditions for 48 h. A portion of 0.25 ml of 1:10 dilution of an overnight culture of the indicator bacteria was inoculated in 9 ml of Brain Heart Infusion (BHI) (Merck, Darmstadt, Germany) soft agar. The medium poured over the MRS plate on which the *Lactobacillus* was grown. The plates were incubated aerobically at 37°C for 24 h. The antibacterial activity was related to the inhibition zone which calculated as the difference between the total of inhibition zone and the diameter of growth spot of selected strains (Klayraung *et al.*, 2008).

DNA extraction

DNA extraction of isolates was performed according to procedure of Denazist extraction DNA kit, Iran.

Typing of isolates

All *Lb. plantarum* isolates were grouped by rep-PCR typing using 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 from rep-PCR were subjected to electrophoresis in 1.5% agarose gels in 1x TBE buffer for at 90 min and 75V (Tamang *et al*., 2005). Bands were visualized under UV light after staining with DNA Green Viewer and photographed. 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) (Edalatian *et al.*, 2012).

Results and Discussion

Identification and typing of LAB species

All 54 strains isolated from Tarkhineh were identified as *Lactobacillus* based on their morphology, Gram staining, non-motility and catalase reaction.

Table 1. Species and numbers of majority cultured microorganisms identified through different formulation of Tarkhineh using molecular methods and sequencing

Species	Formulation F1	F2	F3	Media	Total
Lactobacillus plantarum	7	9	3	MRS	19
Lactobacillus fermentum	2	14	1	MRS	17
Lactobacillus pentosus	2	7	-	MRS	9
Lactobacillus brevis	4	4	-	MRS	8
Lactobacillus diolivorans	-	-	1	MRS	1

Formulation: F1: Tarkhineh with butter milk, F2: Tarkhineh with High-fat yogurt, F3: Tarkhineh with Low-fat yogurt

Table 2. Morphological, physiological and biochemical characteristics of isolated *Lactobacillus*

Biochemical characteristics	*Lactobacillus plantarum*	*Lactobacillus brevis*	*Lactobacillus fermentum*	*Lactobacillus pentosus*	*Lactobacillus diolivorans*
Gram reaction	+	+	+	+	+
Catalase test	_	_	_	_	_
Growth at:15/45°C	+/_	+/_	_/+	_/+	+/_
CO_2 production	_	+	+	+	_
Growth at pH=4.4	+	+	+	+	_
Growth at pH:9.6	+	+	+		_
6.5% NaCl	+	_	_	+	_

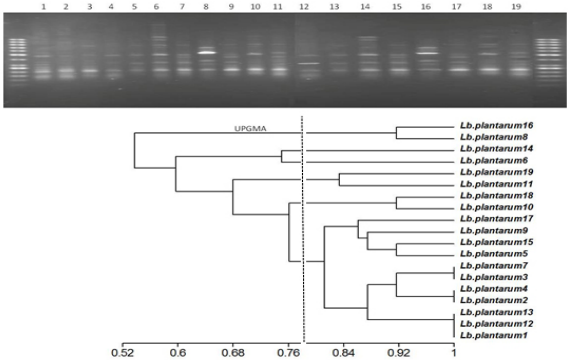

Figure 2. (A): REP-PCR typing profiles obtained with primer BoxA2R among the 19 *Lb. plantarum* strains or isolates from Tarkhineh. (B): dendogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient.

In total, 54 isolates from Tarkhineh were identified from the counting plates of MRS-agar (Table. 1). These 54 isolates were classified into 5 groups based on biochemical tests and carbohydrate fermentations. Table 2 showed the biochemical tests of *Lactobacillus* spp. in Tarkhineh. Among 54 *Lactobacillus* strains which were isolated from Tarkhineh, 19, 17, 9, 8 and 1 isolates belonged to *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus pentosus*, *Lactobacillus brevis* and *Lactobacillus diolivorans*, respectively. The *Lb. plantarum* and *Lb. diolivorans* strains were not able to produce CO_2 from glucose, indicating they were homofermentative LAB. This LAB group was only able to ferment glucose to lactic acid. Whereas, *Lb. fermentum*, *Lb. brevis* and *Lb. pentosus* were in the group of hetero-fermentative. LAB which they were able to ferment glucose to lactic acid, ethanol/acetic acid, and CO_2. *Lb. plantarum*, *Lb. brevis* and *Lb. diolivorans* strains were able to grow at 10°C, but no growth at 45°C. While, *Lb. fermentum* and *Lb. pentosus* were able to grow at 45°C, but none of the colonies grew at 10°C (Kam *et al.*, 2012).

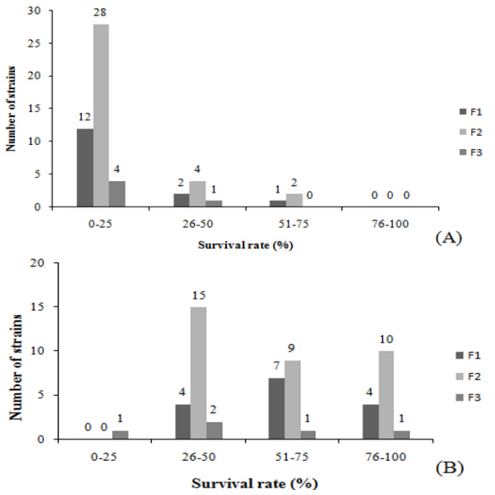

Figure 3. Survival rates of isolated strains from Tarkhineh with butter milk (F1), Tarkhineh with High-fat yogurt (F2) and Tarkhineh with Low-fat yogurt, in incubation media having pH 2.0 (A) and pH 3.0 (B).

As shown in Table 2, the results indicated *Lb. plantarum* was the predominant species in Tarkhineh followed by *Lb. fermentum*, *Lb. plantarum* isolates were all subjected to rep-PCR typing to evaluate intra-species diversity. Figure 2 shows the profiles obtained with the 19 *Lb. plantarum* isolates from Tarkhineh, plus the similarity dendrogram for the different typing patterns clustered by the UPGMA method and using the Simple Matching coefficient. Given the reproducibility of the assay isolates sharing a percentage of similarity of >78% were considered to be the same strain. Six different profiles were considered to represent different strains for *Lb. plantarum*. A high intra-species diversity was found among the *Lb. plantarum* isolates using rep-PCR, which suggests a high subsequent phenotypic diversity.

Effect of pH

It was reported that acids such as the hydrochloric acid found also in human stomach, interrupt the biomolecules of cells such as proteins, vitamins, fatty acids and DNA (Hassanzadazar *et al*., 2012). Low pH environments can inhibit the metabolism and reduce the growth and viability of *Lactobacillus*. The effect of pH range from 2.0 to 7.0 on the survival rate of the 54 selected strains was studied. Good probiotic strain should withstand at least pH 3.0 (Ferna´ndez *et al.*, 2002). The number of strains that survive and their survival rate were represented in Figure 3. It was found that the majority of strains could survive approximately less than 50% in pH 2.0. Strain no. B20 showed the highest resistance or tolerance in this regard. This strain could survive in pH 2.0 at the survival rate of 60.2%. Barakat *et al.* (2011) reported

Table 3. Antimicrobial activity of the selected *Lactobacillus* strains

Strains	Radius of inhibition zone (mm) of indicator strains		
	S. aureus TISTR 029	*E. coli TISTR*	*L. innocua ATCC 33090*
A54	6.20±0.45	4.76±0.65	5.00±0.78
B2	5.76±1.12	4.86±0.69	6.73±0.68
B20	7.26±0.65	6.10±1.34	6.36±0.74

*mean ± standard deviation

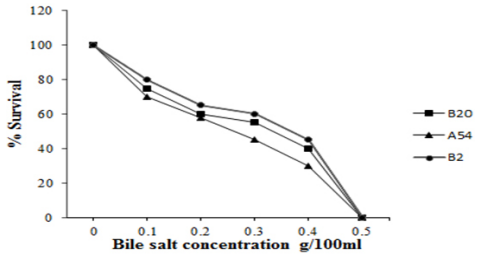

Figure 4. Effect of bile salt concentration on survival of strain B2, B20 and A54 on MRS agar incubated at 37°C under anaerobic conditions.

that high tolerance to low pH may due to the reason that reduce the lethal effect of low pH on the cell wall like production polysaccharides. When the pH was raised to 3.0, more than 40% of isolates exhibited a survival rate higher than 75%. When the pH reached up to 7.0, all isolates could survive 100%. One of the most important standard for selection of LAB as probiotic is potential viability at low pH (Allameh *et al*., 2012). As Sahadeva *et al.* (2011) and Boke *et al*. (2010) Confirmed the viability of all strains was significantly reduced at pH 2 compared with pH 3 and pH 7. Among 54 strains, only 3 of them showed significant resistance to the acid in the extremely high concentration which were selected for further studies.

Effect of bile concentration

To evaluate the potential of using LAB as effective probiotics, it is generally necessary to evaluate their ability to resist the effects of bile acid. Oxgall is a natural dried bovine bile component containing both conjugated and unconjugated bile salts (Barakat *et al*., 2011). All 3 isolates demonstrated good capacity to resist bile salts but B2 showed better resistance (Figure 4). The gradual decrease of viable cells was observed when the concentration of bile salt was increased up to 1.0% (Klayraung *et al.*, 2008; Sahadeva *et al*., 2012). The protective effect of food matrix, may prevent the bacteria from bile exposure and hence, giving rise to the increased bile resistance (Begley *et al*., 2005). Another important factor is the bile salt hydrolase (BSH) activity that help for the bile salt resistance (Sahadeva *et al*., 2011). Mourad *et al.* (2006) showed the survivability of *Lb. plantarum* strains in the conditions of high bile salt concentration and low pH values. Hutari *et al.* (2011) said that *Lb. fermentum* is the most tolerant isolate to bile salts 0.5

and 1% because its lag time is the shortest.

Detection of antibacterial activity

Lactic acid bacteria are well known producer of antimicrobial compounds especially bacteriocins which have high antimicrobial activity (Aween *et al.*, 2012). *Lactobacillus* are known for their production of various antimicrobial compounds. The production of these compounds by intestinal microflora is one of the most important mechanisms responsible for the antagonistic phenomenon and therefore it is essential to examine this property in isolates that candidates for probiotic (Bilkova *et al.*, 2011). The good probiotics should present their antimicrobial actions particularly to the pathogens in the GI system (Klayruang *et al*., 2008). In this study, *Staphylococcus aureus*, *Listeria innocua* and *Escherichia coli* were used as the test bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. Three potentially probiotic *Lactobacillus* isolates were subjected to antibacterial activity assay. The results are shown in table 3. The isolate no. B20 showed the most antibacterial potency to *S. aureus* and *E. coli* whereas the isolate no. B2 demonstrated the highest potency to *L. innocua.* The production of organic acid and hydrogen peroxide by Lactobacilli was reported to inhibit both gram positive and gram negative bacteria, whereas bacteriocin affects only the growth of gram positive bacteria (Klayruang *et al.*, 2008).

Conclusion

The present study concluded that *Lactobacillus* spp. was predominant in microflora of Tarkhineh. Although the original Ingredient of this food is butter milk, also we have used low and high-fat yogurt to investigate microbial populations and probiotic characteristics and compare with the original sample that made with butter milk. Tarkhineh where local butter milk and high-fat yogurt was used, in terms of population, diversity and probiotic characteristics was richer than low-fat yogurt. According to the results of acid test, bile salt tolerance and antibacterial activity we can conclude these 3 isolates, including *Lb. fermentum* (2 isolate) and *Lb. plantarum* (1 isolate), have good Characteristics to be considered as probiotic bacteria. However, other tests such as antibiotic resistance and adhesion properties better to be done.

Acknowledgments

The authors thank Professor Mohammd Reza Nassiri, Department of Animal Science, Shahnaz Afsharian, Director of microbiology lab, and Nafiseh Daavati, PhD student of food microbiology, for their sincere assistance for this study.

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