

Biochemical characterization and technological properties of predominant *Lactobacilli* isolated from East-Azarbaijan sourdoughs (Iran)

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Abstract

Seventeen traditional sourdough samples were collected from the Northern regions in East-Azarbaijan province of Iran. The pH and total titratable acidity (TTA) values of the sourdoughs varied from 3.71-4.07 and 15.2-26.8 ml, respectively. A total of 50 *Lactobacillus* strains were isolated from the sourdoughs and identified by biochemical methods. Of the isolates, 74% were identified as *L. plantarum* and *L. curvatus* or *L. casei* subsp. *rhamnosus*, 10% as *L. morinus* or *L. paralimentarius* and 4% as *L. sake* or *L. bavaricus* and *L. casei* subsp. *Casei*. Three isolates from the main groups were selected and identified using 16S rRNA gene sequencing. The results of gene sequencing revealed the isolates as *L. plantarum*, *L. curvatus* and *L. paralimentarius*. All three *Lactobacillus* strains produced exopolysaccharide from sucrose in MRS broth medium and also showed proteolytic activity. *Lactobacillus curvatus* had weak proteolytic activity compared to other strains. The strains showed some differences in acidification properties both in MRS broth and sourdough fermentation. All the strains had good acidifying activity in sourdough fermentation and were able to reduce the pH to less than 4 during 9-12 h incubation at 30°C.

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Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of non-sporulating Gram-positive bacteria which grow under microaerophilic to strictly anaerobic conditions (Coeuret *et al.*, 2003). These bacteria have been used as starter cultures in sourdough fermentation (Şimşek *et al.*, 2006). Sourdough is a mixture of wheat or rye flour and water for making bread. Every sourdough has a specific range of microorganisms, and so each traditional bread has its own peculiar characteristic (Ricciardi *et al.*, 2005). Yeasts and lactic acid bacteria are the key groups of fermenting organisms in sourdoughs. The lactic microflora of sourdoughs have been studied by many researchers (Gobbetti, 1998; Rocha and Malcata, 1999; Corsetti *et al.*, 2001). The main lactic acid bacteria isolated from sourdoughs belong to the genus *Lactobacillus* (Ehrmann and Vogel, 2005; Palacios *et al.*, 2006; Ferchichi *et al.*, 2007), although *Leuconoctoc* spp., *Enterococcus* spp. and *Weissella* spp. are also found (Catzeddu *et al.*, 2006; Anjum *et al.*, 2008; Zotta *et al.*, 2008). Several *Lactobacillus* species have been isolated from American, German and Italian sourdoughs but *Lactobacillus plantarum*,

Lactobacillus sanfranciscensis, *Lactobacillus pontis* and *Lactobacillus panis* are considered as the most important *Lactobacilli* in sourdoughs (Corsetti *et al.*, 1996; Catzeddu *et al.*, 2006; Arendt *et al.*, 2007). Traditional sourdoughs have been classified as type I doughs which are dominated by *Lactobacillus sanfranciscensis* and *Lactobacillus pontis* (Vogel *et al.*, 1999; Clarke and Arendt, 2005; Ricciardi *et al.*, 2005).

In Iran, sourdoughs are used in household bakeries for making traditional breads. East-Azarbaijan province is located in Northwest of Iran with mountainous climates. In most parts of East-Azarbaijan, the bakers use baker's yeast to raise the dough. Only a few household bakers in rural regions use sourdough process as a form of leavening. However, traditional breads made from these sourdoughs have a good taste, soft and elastic texture, and long shelf-life. The microorganisms involved in the sourdoughs may play an important role in quality of these traditional breads. Therefore, the aim of this study was the biochemical identification of *Lactobacilli* associated with sourdoughs of North and Northeast regions in East-Azarbaijan province for which no data are available

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in the literature. Technological properties of the predominant *Lactobacillus* strains isolated from the sourdoughs were also reported as a basis for selecting starter cultures for bread production. This is the first report on the properties of traditional sourdoughs of collected from these regions.

Materials and Methods

Sourdough samples and physicochemical analysis

Seventeen sourdoughs were taken from household bakeries in Northern regions of East-Azarbaijan province, Iran. Eight samples came from Kalebar, six samples from Heris and Varzaghan, and three samples were collected from Ahar. The samples were aseptically collected and refrigerated at 4°C until analysis. Sourdough samples (10 g) were blended with 90 ml of distilled water and the pH value was determined by using pH-meter (Digital Metrohm 827, Switzerland). The acidity was titrated using 0.1N NaOH to final pH 8.5. The total titratable acidity (TTA) was expressed in ml 0.1N NaOH (Paramithiotis *et al.*, 2005; Iacumin *et al.*, 2009).

Isolation and phenotypic identification

Ten grams of each sourdough were diluted with 90 ml of sterile 0.1% Peptone water. Further dilutions were prepared with 0.85% NaCl (Ricciardi *et al.*, 2005). Selective medium de Man, Rogosa, Sharpe (MRS) agar was used for colony selection and isolation of pure cultures. The plates were incubated under anaerobic conditions at 30°C for 48-72 h. The pure isolates were checked for morphology, Gram stain and catalase reactions. Gram-positive, catalase-negative and rod shaped isolates were selected for identification. Biochemical tests were used to classify and identify the isolates. The isolates were subjected to carbohydrate fermentations (glucose; lactose; melibiose; arabinose; fructose; cellobiose; sucrose; trehalose; melezitose; rhamnose; raffinose; ribose; mannose; mannitol; xylose; galactose; salicin), different temperatures, and also different concentrations of NaCl (2, 4 and 6.5%). The growth of the isolates in MRS broth at 15°C and at 45°C was observed after incubation for 7 days and 48 h, respectively (Corsetti *et al.*, 2001; Ricciardi *et al.*, 2005).

Molecular identification

The *Lactobacillus* isolates were clustered according to biochemical tests. Three isolates from main groups were selected and identified by molecular methods. DNA extraction was carried out as described by Cardinal *et al.* (1997). The size and quality of DNA was checked by 2% agarose gel

electrophoresis in TBE buffer with a 100 bp DNA ladder. The PCR products of 16S rRNA gene of the isolates were sequenced and aligned using Chromas software and then compared with those in the NCBI database.

Technological properties of predominant Lactobacillus strains

Assessment of proteolytic activity

Proteolytic activity was assessed on MRS agar plates containing 10% skimmed milk. 10 µl of cell suspension of the strains were spotted on the surface of the plates and incubated at 30°C for 72 h. Proteolytic activity was determined by measurement of the diameter of the clear zone around the inoculated spots (mm) (Essid *et al.*, 2009).

Exopolysaccharide production

The strains were tested for exopolysaccharide (EPS) production in MRS-sucrose broth without glucose and peptone. A volume of 1.5 ml of the 24 h culture was centrifuged at 5000 × g for 10 min (4°C) and 1 ml of the supernatant was put in a glass tube and then an equal volume of ethanol was added. The positive strains formed an opaque link at the interface (Sawadogo-Lingani *et al.*, 2007).

Acid production ability

The acidification activity was measured according to the method of Zotta *et al.* (2008). An overnight MRS broth sub-culture (OD₆₅₀ = 1) was used to inoculate (5% v/v) sterile MRS broth. The pH was measured immediately after inoculation, at 9 h, and 24 h and the decrease in pH (ΔpH) was calculated for each incubation time.

Acidification properties during sourdough fermentation

The strains were tested for acidification properties during sourdough fermentation in laboratory conditions. The sourdoughs (DY 350) were prepared by mixing 200 g whole flour, tap water and the inoculums of the *Lactobacillus* strains (10⁸ cfu/g) and placed in an incubator at 30°C for 20 h. An uninoculated dough was prepared under the same conditions as a control. The pH values and TTA were determined at intervals of 1.5 h during incubation time (Robert *et al.*, 2006).

Statistical analysis

The phenotypically identified isolates of *Lactobacillus* were grouped by the clustering method. Analyses were performed using the NTSYS PC package version 2.02e (Corsetti *et al.*, 2001). All tests

were measured in triplicate and the average of data was calculated. Statistical analysis was performed using SAS software (version 9.1).

Results and Discussion

Sourdough characteristics

The main characteristics of sourdoughs obtained from household bakeries are shown in Table 1. Only one bakery used baker's yeast in addition to the sourdough. The age of sourdoughs varied considerably from 8 h to 96 h. The household bakeries stored the sourdoughs at refrigerator or freezer temperature. The used sourdough for baking ranged at the level of 4-12%. The pH values of the sourdoughs ranged from 3.71 to 4.07 and TTA ranged from 15.2 to 26.8 (Table 1). Sourdough is a mixture of flour and water that is fermented by microorganisms (Ferchichi *et al.*, 2007). In sourdough, lactic acid bacteria produce different organic acids and increase the acidity. Therefore, the pH value of sourdough decreases and according to Clarke and Arendt (2005), it reaches to 3.5 - 4.3 for wheat sourdoughs. The rate of sourdough addition to bread dough depending on sourdough type varies from 5-40% (Katina, 2005). Crowley *et al.* (2002) and Chiavaro *et al.* (2008) reported that the addition of 20 g sourdough/ 100 g dough was most favorable for production of bread with the highest volume and lowest rate of staling. However, the sourdoughs analyzed in this study were traditional type I sourdoughs and their characteristics were similar to those reported for other sourdoughs.

Identification of the isolates by phenotypic tests

Fifty isolates from MRS countable plates were subjected to preliminary tests for identification. All isolates were Gram positive and catalase negative rods with the classical characteristics of *Lactobacillus* (Kandler and Weiss, 1986). In addition to the preliminary assays, sugar fermentation pattern and growth at different temperatures and different concentrations of NaCl were also used to identify the isolates. The physiological and biochemical characteristics of the presumptive *Lactobacillus* isolates are summarized in Table 2. All isolates were able to grow at 15°C and in different concentrations of NaCl. Only 15 of the 50 isolates under study could not grow at 45°C. All strains formed acid from glucose, fructose, sucrose and salicine but acid production from other sugars was variable and strain dependent. The majority of the isolates (37 isolates) were grouped in cluster A which the phenotypic properties of eighteen of them suggest their close resemblance to *Lactobacillus plantarum*. The rest of

Table 1. Main properties of the sourdoughs collected from household bakeries in Northern regions of East-Azərbayjan province, Iran

| Sample ^a | Sourdough age ^b (h) | sourdough percent added to dough | Use of baker's yeast | pH | TTA ^c |
|---------------------|--------------------------------|----------------------------------|----------------------|------|------------------|
| A1 | 48 | 10 | No | 3.82 | 16.6 |
| A2 | 24 | 10 | No | 3.99 | 15.2 |
| A3 | 48 | 10 | No | 3.94 | 18 |
| K1 | 72 | 10 | No | 3.75 | 19.2 |
| K2 | 48 | 12 | No | 3.75 | 22 |
| K3 | 8 | 10 | Yes | 4.04 | 17 |
| K4 | 96 | 10 | No | 4.07 | 17.2 |
| K5 | 24 | 10 | No | 3.91 | 19 |
| K6 | 48 | 10 | No | 3.86 | 19 |
| K7 | 48 | 10 | No | 3.87 | 19 |
| K8 | 48 | 10 | No | 3.77 | 22.8 |
| HE1 | 48 | 4 | No | 3.87 | 21.6 |
| HE2 | 24 | 5 | No | 3.73 | 17.8 |
| HE3 | 48 | 5 | No | 3.71 | 21.8 |
| V1 | 48 | 5 | No | 3.74 | 19.6 |
| V2 | 48 | 5 | No | 3.80 | 26.8 |
| V3 | 48 | 5 | No | 3.76 | 19 |

^a A, Ahar; K, Kalebar; HE, Heris; V, Varzaghan

^b Time of storage after preparation

^c ml 0.1N NaOH

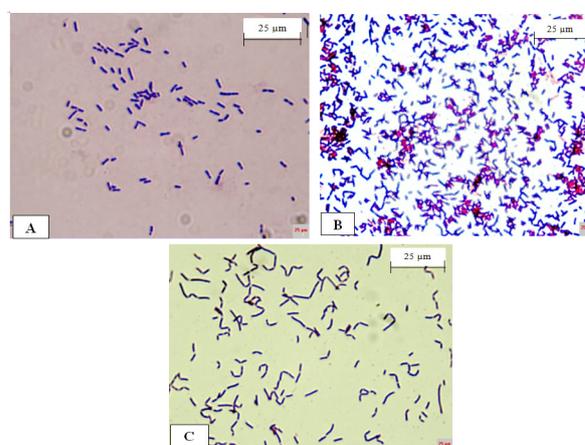


Figure 1. Microscopic shapes of predominant *Lactobacillus* strains isolated from Iranian traditional sourdoughs. A, *Lactobacillus paralimentarius*; B, *Lactobacillus plantarum*; C, *Lactobacillus curvatus*

them classified as *L. casei* subsp. *rhamnosus* or *L. curvatus*, as suggested by their sugar fermentation patterns. Five isolates in cluster B that were rhamnose and xylose negative but fermented ribose and sucrose seemed to be *L. murinus* or *L. paralimentarius*. The only isolate in cluster D seemed to be *L. sake* or *L. bavaricus* or *L. animalis*. The isolate in cluster G was identified as *L. casei* subsp. *Pseudopantarum* and *L. casei* subsp. *casei* or *L. maltaromicus* according to the species descriptions (Kandler and Weiss, 1986). The isolates in clusters C, E, F, and H were phenotypically close to each other but their identification was not possible because they did not cluster with reference strains. Gobbetti *et al.* (1994) reported that 21% of the isolates obtained from wheat sourdoughs of the Umbria region (Central Italy) belonged to *L. plantarum*. A higher percentage of facultatively heterofermentative species (88%) was isolated from Altamura bread sourdoughs (Apulia, Southern Italy), in which *L. plantarum*, *L. paracasei* and *L.*

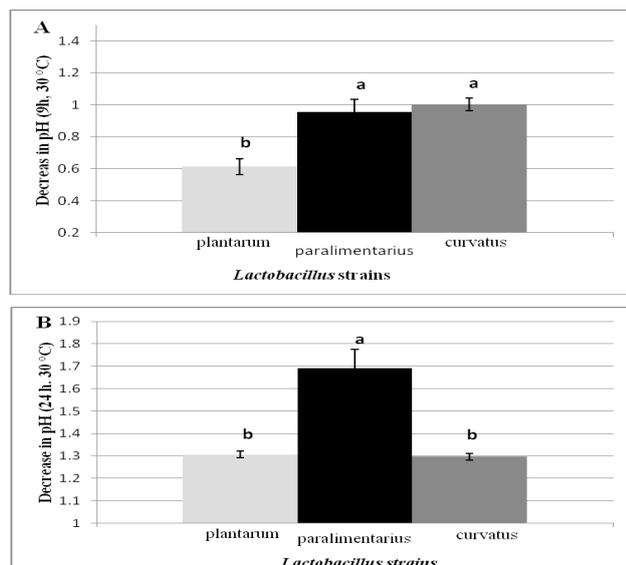


Figure 2. Acid production ability of the *Lactobacillus* strains after 9 h (A) and 24 h (B) incubation at 30°C in MRS broth. Columns marked by same letter are not different at $P < 0.05$

casei were the main isolates (Ricciardi *et al.*, 2005). Corsetti *et al.* (2001) isolated from durum wheat sourdoughs of the Apulia region, *L. sanfranciscensis* (30%), *L. alimentarius* (20%), *L. brevis* (14%), *L. plantarum* (7%) and smaller percentages of *L. fermentum*, *L. acidophilus* and *L. delbrueckii* subsp. *Delbrueckii*. In another study by Reale *et al.* (2005), *L. plantarum* also represented 7.1% of the isolates in wheat sourdoughs of the Molise region (Southern Italy). However, *L. plantarum* is the species which usually associates with sourdoughs and our results also have a good agreement with the results of others. According to phenotypic tests, only 4% of the isolates identified as *L. sake* or *L. bavaricus* and *L. casei* which corresponded to those often isolated from sourdoughs (Salim ur *et al.*, 2006; Şimşek *et al.*, 2006; Ferchichi *et al.*, 2007).

Technological properties of predominant *Lactobacillus* strains

Proteolytic activity and EPS production

As mentioned previously, fifty *Lactobacillus* strains were isolated on MRS agar from the 17 samples of traditional sourdough and grouped based on biochemical tests. Three isolates from the main groups were selected and identified by molecular method. The partial sequence analysis of 16S rRNA gene revealed three isolates as *L. paralimentarius*, *L. plantarum* and *L. curvatus* which their microscopic shapes are shown in Figure 1. The *Lactobacillus* strains were tested for their proteolytic activity, EPS production and acidification properties. Three strains of *Lactobacillus* were able to produce EPS

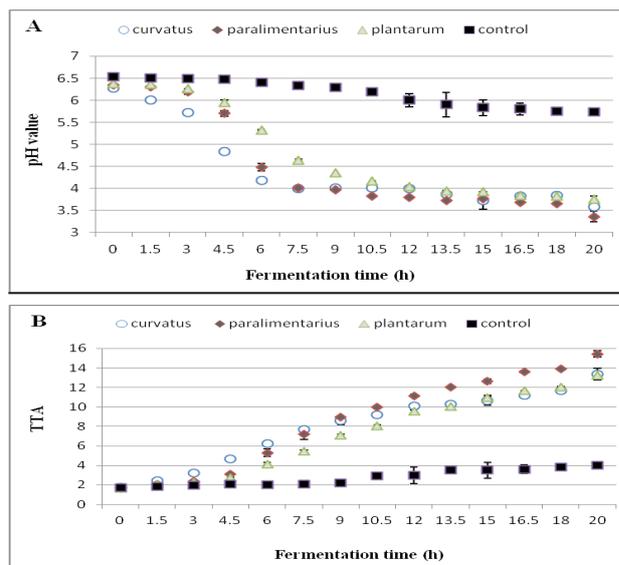


Figure 3. pH (A) and TTA (B) changes during sourdough fermentation with three *Lactobacillus* strains isolated from Iranian traditional sourdoughs

in MRS broth containing sucrose and also showed proteolytic activity. *Lactobacillus plantarum* and *L. paralimentarius* showed the highest diameter halo of degradation (14, 12 mm). *Lactobacillus curvatus* had weak proteolytic activity compared to other strains. It might be due to the fact that the level of proteolytic activity of lactic acid bacteria on wheat flour fractions is strain dependent (Clarke and Arendt, 2005). However, EPSs produced by *Lactobacilli* can affect dough rheology, water absorption of the dough, loaf volume and bread staling (Arendt *et al.*, 2007; Zotta *et al.*, 2008). Proteolytic activity of *Lactobacillus* strains are also reported to affect the mechanical properties of the dough and bread quality (Pepe *et al.*, 2004). A large number of *Lactobacilli* such as *L. plantarum*, *L. brevis*, *L. fermentum*, *L. sakei*, *L. casei*, *L. paracasei*, *L. hilgardii* and *L. helveticus* have also been known as EPSs producers (Sawadogo-Lingani *et al.*, 2007).

Acidification properties

The acidification properties of three *Lactobacillus* strains were determined in MRS broth. Figure 2 shows the acid production ability of the strains after 9 h and 24 h incubation at 30°C in MRS broth. The decrease in pH ranged from 0.61 to 1 at 9 h and from 1.29 to 1.69 at 24 h. *Lactobacillus curvatus* and *Lactobacillus paralimentarius* generated a significant drop in pH after 9 h of incubation. At the end of incubation time, *L. paralimentarius* had the highest value of decrease in pH while *L. plantarum* and *L. curvatus* showed similar levels acid production. Zotta *et al.* (2008) reported that the majority of *L. plantarum* group isolates (including *L. plantarum*, *L. paraplantarum* and *L. pentosus*) had the highest acid production

Table 2. Group differentiation of *Lactobacilli* isolated from the sourdoughs based on phenotypic properties

| Characteristic | A (n=37) | B (n=5) | C (n=1) | D (n=1) | E (n=3) | F (n=1) | G (n=1) | H (n=1) |
|-------------------|-------------|------------|------------|------------|------------|------------|------------|------------|
| Growth at 15°C | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Growth at 45°C | 73 | 20 | 100 | 100 | 100 | 100 | - | 100 |
| Growth in 2% NaCl | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Growth in 4% NaCl | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Growth in 6% NaCl | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Acid from Glucose | 100 | 80 | 100 | 100 | 100 | 100 | 100 | 100 |
| Lactose | 100 | 60 | 100 | 100 | 100 | - | 100 | - |
| Melibios | 78 | 80 | 100 | 100 | 100 | 100 | - | 100 |
| Arabinose | 95 | 100 | 100 | - | 100 | 100 | - | 100 |
| Fructose | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cellobios | 100 | 100 | - | 100 | 100 | - | - | 100 |
| Sucrose | 97 | 80 | 100 | 100 | 100 | 100 | 100 | 100 |
| Trehalose | 100 | 80 | 100 | 100 | - | 100 | 100 | 100 |
| Melezitose | 95 | 100 | 100 | 100 | 67 | 100 | 100 | 100 |
| Salicine | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Rhamnose | 65 | - | - | 100 | 33 | 100 | - | - |
| Raffinose | 51 | 100 | 100 | 100 | 33 | - | - | - |
| Ribose | 100 | 100 | 100 | 100 | 100 | 100 | 100 | - |
| Mannose | 100 | 100 | 100 | 100 | 100 | 100 | 100 | - |
| Manitol | 100 | 80 | 100 | - | 100 | - | 100 | - |
| Xylose | 70 | - | 100 | - | - | 100 | - | - |
| Galactose | 100 | 100 | - | 100 | - | 100 | - | - |

The percentage of isolates which were positive for any given test is shown
- : Negative reaction

ability after 24 h incubation.

Three *Lactobacillus* strains were also tested in sourdough fermentation. As shown in Figure 3, the pH value of the samples inoculated with each individual *Lactobacillus* strain decreased over the incubation period from 6.3 to <4. Similar results have been also reported with lactic acid bacteria starters by Robert *et al.* (2006). The drop in pH was much faster for the samples inoculated with *L. curvatus* and *L. paralimentarius* after 6 h of incubation. A stable final pH was reached after 13 h. The sample inoculated with *L. paralimentarius* had the lowest pH and the highest TTA at the end of incubation time. As expected, pH and TTA values of the control sample varied slightly during the fermentation period (Figure 3). Dough acidification due to the production of lactic and acetic acids is an important metabolic activity in bread making. The acidification of the sourdough causes changes in dough characteristics and also the activity of cereal and bacterial enzymes (Clarke and Arendt, 2005). Decrease in pH is essential for optimal swelling and baking of bread and also for making proper sensory properties in bread (Arendt *et al.*, 2007). Production of organic acids during fermentation is effective in preventing bacterial spoilage of wheat bread (Pepe *et al.*, 2004) and also in improving its nutritional properties (Zotta *et al.*, 2008).

Conclusions

In conclusion, the microbial composition of Iranian sourdoughs can be differed from other sourdoughs due to type of flour, fermentation conditions and process technology. Our study showed that *L. plantarum*, *L. curvatus* and *L. paralimentarius* were important members of Iranian traditional

sourdoughs microflora. All three *Lactobacillus* strains were able to produce EPS in MRS broth medium containing sucrose. They showed some differences in acidifying and proteolytic activities. The application of these strains as starter cultures for sourdough bread making should be evaluated.

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