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# Antifungal Activity and Improved Quality of Wheat Toast Bread by Iranian Lactobacillus Species.

**Jabbari Sharare[1], Mahmoodi Vajiheh[2], and Mahmoodi Elaheh[3]*.**

[1]Department of Food Science and Technology, Faculty of Agriculture, Varamin- Pishva Branch, Islamic Azad University, Varamin, Iran.
[2]Department of Food Science and Technology, Faculty of Agriculture, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.
[3]Department of Pathobiology, Alborz University of Medical Sciences, Karaj, Iran.

**ABSTRACT**

Human nutrition worldwide is largely dependent on bread. Quality of bread is dependent on the method of baking. Application of sourdough is a traditional way to improve quality and nutritional value of bread. Sourdoughs are a great source of diverse Lactic Acid Bacteria (LAB) with antifungal and antiropiness activities. Many researchers used different species of LAB to demonstrate their effect on bread quality. In this study we used 6 Iranian Lactobacillus strain at different fermentation conditions. Each strain and the most common fungi responsible for bread spoilage were grown routinely. Sourdoughs were prepared using each LAB, pH values & Titration of the Acidity (TTA) was determined and experimental and control breads were baked. Antifungal activity and bread sensory of each LAB was tested. *L. delbrueckii* and *L .fermentum* had the strongest antifungal activity with large clear zones and no growth around the colonies. The most increase in TTA was noted in samples prepared with Lactobacillus *delbrueckii* and *L. sanfranciscensis*. Final values for sourdoughs fermented with each of 6 strains were significantly higher comparing to that of common sourdough. Application of 10% w/w *L. delbrueckii* in sourdough resulted in best aroma, taste, and texture. The present study confirmed that addition of sourdough fermented by Iranian LAB caused a distinct change in the properties of the dough, specifically indicating that *L.delbrueckii* is a suitable starter for wheat sourdough and bread production. *L. delbrueckii* can successfully perform sourdough fermentation and produce good quality bread.

**Keywords:** LAB, sourdough, molds, Iran

***Corresponding author***

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## INTRODUCTION

Human nutrition worldwide is largely dependent on bread, constituting the main part of the human food basket. Quality of bread is dependent on the method of baking. Application of sourdough is a traditional way to improve the taste, texture, shelf-life, and nutritional value of bread (Lacaze et al., 2007; Randazzo et al., 2005). Use of sourdough fermentation dates back to the time of Pharaoh when his baker accidentally used the dough of previous day and found out that it helps produce a more porous and spongy bread that was more noticeable (Sanz-Penella et al., 2012; Wieser et al., 2007). This method was used for centuries in Iran and is still widely used in rural bread. However, in urban areas yeast and sodium bicarbonate are added to the dough to help fermentation and give volume.

Sourdoughs are a great source of diverse Lactic acid bacteria (LAB) and significant numbers of yeast species and strains. The oldest method for preparation of sourdough, which is a mixture of flour and water that is fermented, is based on spontaneous fermentation of flour natural flora. Alternatively, LAB developing in the dough may originate from a starter culture, a pre-ferment containing one or more known species of LAB (Messens and Vuyst, 2002).

Sourdough, which is rich in lactobacilli as well as baker's yeast, influences bread shelf-life depending on bacterial strain, fermentation conditions, and the process utilized (Katina, 2005). This is due to different factors especially low pH value, antimicrobial compounds, and LAB metabolites such as organic acids, exopolysaccharides and enzymes produced by LAB (Corsetti et al., 1998; Simsek et al., 2006). One of metabolic activities of sourdough LAB is the production of antibacterial/antifungal substances, anti-ropiness activities, and exopolysaccharides in dough, which potentially influences bread texture, staling and/or shelf-life (Corsetti et al., 1998; Corsetti et al., 1998c; Corsetti et al., 1996; Gobbetti, 1998; Hammes & Ga ¨nzle, 1998; Katina et al., 2002; Korakli et al., 2001; Korakli et al., 2003; Lavermicocca et al., 2000; Lavermicocca et al, 2003; Pepe et al., 2003). Many researchers have studied the effect of bacterial strain in sourdough on the quality and shelf-life of wheat bread (Dal Bello et al., 2007; Katina et al., 2002; Mentes et al., 2007). The loss of perceived freshness may generally be categorized into two groups including processes known as staling and those that are attributed to microbial spoilage (Pateras, 1998). Notably, fungi growth is usually blamed for microbial bread spoilage (Legan, 1993) while LAB preserves bread from fungal-mediated spoilage (Spicher, 1983; Brummer and Lorenz, 1991; Caplice and Fitzgerald, 1999; Katina et al., 2002). The anti-fungi activities of sourdough LAB have been previously studied (Corsetti et al., 1998b; Rocken 1996). These studies used sourdough containing different species of LAB to demonstrate the specific anti-fungi activities of the corresponding LAB. In this study, industrial bread was made by different sourdoughs containing one of 6 Iranian Lactobacillus at different fermentation conditions. Then anti-fungal activity and shelf-life duration was compared.

## MATERIALS AND METHODS

### *Bacterial strains and growth media*

Six strains of LAB were used in this study. These starters were *Lactobacillus plantarum subsp.plantarum (PTCC No: 1745), Lactobacillus sanfranciscensis (PTCC No: 1739), Lactobacillus sakei subsp.sakei (PTCC No: 1712), Lactobacillus reuteri (PTCC No: 1655), Lactobacillus fermentum (PTCC No: 1744)*, and *Lactobacillus delbrueckii subsp. delbruckii (PTCC No: 1333)* purchased from Iranian Research organization for Science and Technology (IROST) as vacuum dried cultures. Each strain was grown routinely in MRS broth medium (Merck) at 30ºC for 18h under aerobic conditions (De Man et al., 1960).

***Fungal cultures and preparation of the spore solution***

The most common fungi responsible for bread spoilage (*Aspergillus niger, Penicillium roqueforti*, and *Fusarium* graminearum) previously isolated from contaminated bread were purchased and used as target organisms for assay of antifungal activity in vitro. Fungi were cultivated on Saboura dextrose agar (Difco) at 25°C for roughly 18h until sporulation occurred. Spores were harvested in 0.2% peptone water (w/v). Conidial count was performed using haemocytometer chamber; a conidial suspension with a concentration of $10^6$ per /ml was prepared, and stored at 4°C until use.

***In vitro antifungal activity***

The inhibitory activity of each lactobacillus strain against fungi-mediated spoilage was determined using Magnusson and Schnurer (2001). Each lactobacillus was placed as cell spots on MRS agar in neutral pH at room temperature for 18h. To investigate antifungal activity, conidial suspension ($10^6$/1ml) was sprayed on the surface of plates and then the plates were incubated at room temperature for 12h. The inhibitory activity of mold growth was indicated by the absence of growth around the spot of lactobacillus culture. Alternatively, each lactobacillus was placed as line on MRS agar and then 100µl of conidial suspension was linearly cultured in perpendicular to the linear culture of lactobacillus.

***Sourdoughs preparation***

Each lactobacillus was inoculated in MRS broth and incubated in 30ºC for16h. When the incubation time was over, the culture media was centrifuged at 10000g for 10 min at 4ºC, and the supernatant was discarded. To activate the bacteria, the pellet was then transferred into sterile milk and incubated at 30ºC, until clot formation which usually takes for 12h. Then, the bacterial cell count was determined as $10^7$cfu/g. A mixture of 35gr of wheat flour and 65gr of water were inoculated with 20% of a $10^7$cfu/g of individual lactobacillus cells. The mixtures were allowed to ferment at the appropriate temperature until pH reached to 3.5-3.8.

***Total Titratable Acidity (TTA) measurement***

Ten gram of sourdough samples were homogenized with 90 ml of sterile distilled water (D.W). The titration of the acidity was done using 0.1 N NaOH to final pH 8.5. The TTA was reported in terms of ml 0.1 N NaOH (Katina, 2005).

***Dough preparation and baking of experimental bread***

At first, raw materials including wheat flour, water, salt, active dry yeast *S. Cerevisiae*, sugar, and oil were prepared and weighted. Then an amount of 0.4% NaCl, 0.25% the yeast *S. Cerevisiae* (0.25%), and 13% sourdough mixture was added to 100gr wheat flour and mixed until the dough was well-formed. Finally, the dough was rolled, covered in foil, and fermented at 30°C for 18h. The dough without LAB was used as control. Measurement of dough volume, which is an indicator of bread power, was calculated using the following formula in which Vf and Vi stand for final and initial volume of dough, respectively=Vf-Vi.
Finally, doughs were scaled into portions, molded, placed in tins, and baked at room temperature. The process was the same for control bread without addition of sourdough containing LAB.

***Antifungal activity tests***

Two groups of test (prepared using sourdough containing LAB) and control (prepared without LAB) was considered. Each group contained 54 loaves of bread dividing into 6 subgroups of 9 loaves. To determine antifungal activity of the lactobacilli in the bread slices, the conidial solution of *Aspergillus, Penicillium*, or *Fusarium* ($10^6$ conidia/ml, 1ml per 100g loaf) was sprayed one side of each slice. Each slice was then packed in poly ethylene plastic bag, sealed, and incubated at room temperature. During the monitoring procedure of the breads, a small slot was left open to ensure comparable aerobic conditions in each bag. The samples were examined until mould growth was detected. Half-life of bread was determined based on the time of mould growth detection.

***Testing of Bread sensory***

Evaluation of aroma and flavor of bread samples qualitatively was performed by the aid of some trained and untrained. Following cooling and cutting, samples were initially coded and then results of three independent assays are presented as mean values ± standard deviation (SD). Data were compared by the way analysis of Variance (ANOVA) and by Dunnett t-test. Statistical significance ($p < 0.05$) was assessed with the minitab-12software.

## RESULTS

***Comparison of in vitro antifungal activity of six lactobacillus species***

Analysis using overlay method was chosen to investigate antifungal activity of *L. plantarum* subsp. *plantarum*, *L.sanfranciscensis*, *L.sakei* subsp.*sakei*, *L.reuteri*, *L. fermentum*, and *L. delbrueckii* subsp. *delbruckii* against spoilage moulds. As indicated in table1: *L. delbrueckii* and *L .fermentum* had the strongest inhibitory effect with large clear zones and no growth around the colonies (Fig1). Clear zones of inhibition were recorded and scored as follows: −, no visible inhibition; +, visible inhibition only in the soft agar above the bacterial

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streak; ++, inhibition area per bacterial streak of 0.1 to 3.0% of the Petri dish; +++, inhibition area per bacterial streak of 3.0 to 8.0% of the Petri dish; or ++++, inhibition area per bacterial streak of >8.0% of the Petri dish (fig1).

**Table 1:** In vitro inhibitory activity of Iranian Lactobacillus species against common moulds

| | *L. plantarum subsp. plantarum* | *L.sanfranciscensis* | *L.sakei subsp.sakei* | *L.reuteri* | *L. fermentum* | *L. delbrueckii subsp. Delbruckii* |
|---|---|---|---|---|---|---|
| *Aspergillus niger* | *+++* | *+* | *+* | *++* | *++++* | *+++++* |
| *Penicillium roqueforti* | *+++* | *+* | *++* | *+++* | *++++* | *+++++* |
| *Fusarium graminearum* | *++* | *-* | *+* | *-* | *++* | *+++* |

–, no visible inhibition; +, visible inhibition only in the soft agar above the bacterial streak; ++, inhibition area per bacterial streak of 0.1 to 3.0% of the Petri dish; +++, inhibition area per bacterial streak of 3.0 to 5.0% of the Petri dish; ++++, inhibition area per bacterial streak of 5.0 to 8.0% of the Petri dish or +++++, inhibition area per bacterial streak of >8.0% of the Petri dish.

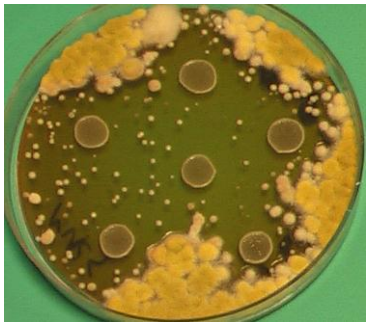

**Fig. 1:** Clear zones of inhibition around the LAB on MRS agar Petri dish.

***Determination of TTA***

The results of total titratable acidity showed that TTA values of fermented products using each of individual Lactobacillus increased with increased storage time and temperature. The most increase in TTA at 30°C after 18 hours of fermentation and the least TTA at 30°C after 20 hours of fermentation were noted in samples with Lactobacillus *delbrueckii* and *L. sanfranciscensis*, respectively. TTA of sourdough prepared by *L.delbrueckii* ranged between 10.5-11. Final values for sourdoughs fermented with 6 strains were significantly higher comparing to that of common sourdough. The decline of pH was concomitant with the increase of acid production in fermented sourdough (data was not shown). This was supported by increasing value of TTA value in fermented samples which indicated the presence of organic acids in liquid sourdough after 24 hours fermentation. These findings are in agreement with Gobbetti et al. (1995a; 1995b).

***Preservative effects on the shelf-life***

The ability of 6 lactobacillus isolate to ferment sourdough were investigated and compared with the common dough isolate *S.cervisiea* against all of spoilage mould testing (table1). The delay rate of fungal growth on bread prepared without or with individual Lactobacillus was investigated. Our results indicate that all of the 6 Lactobacillus are potent anti-mould agents. Remarkably, mould growth was observed on the common bread from day 1 of the storage time. Whereas, LAB-prepared breads still inhibited fungal growth until day 12 and *L. delbrueckii*-prepared breads had even more shelf-life.

***Evaluation of baking test***

Loaf volume in breads produced using each of the 6 lactobacillus strain was significantly greater than that of common bread. In addition, water activity or moisture measurement was significantly different between bread prepared using sourdough containing LAB and control bread prepared using sourdough without LAB. Notably, bread prepared using sourdough containing highest concentration of *L. delbrueckii* (10% w/w) had better aroma, taste, and texture. There were no significant differences between breads with regards to loaf volumes when compared to either the non-acidified treatments.

## DISCUSSION

Bread microbial spoilage remains responsible for economic losses of bread (Gray and Bemiller, 2003). The most frequent cause of microbial spoilage in bread is mould growth. Common fungi responsible for bread spoilage belong to the genera Penicillium*, Aspergillus, Monilia, Mucor, Endomyces, Cladosporium, Fusarium*, and *Rhizopus* (Legan, 1993; Poute and Tsen, 1987). In addition to the economic losses associated with spoilage of this nature, another concern is mycotoxins, produced by the fungi that may cause public health problems (Legan, 1993). Biopreservation, i.e. control of one organism by another, is one of the methods applied to prevent or minimize microbial spoilage of bread. In this regard, more interest has been attracted towards LAB, as they are generally considered safe. Antifungal activity of Lactobacillus has been attributed to a wide range of compounds including lactic acid, PLA, and antifungal peptides as the major components responsible for this activity (Corsetti et al., 1998; Corsetti et al., 1998b; Corsetti et al., 1998c; Dal Bello et al., 2007; Lavermicocca et al., 2000; Lavermicocca et al., 2003; Simsek et al., 2006; Strom et al., 2005). In addition, sourdough improves the quality of wheat bread (Barber et al., 1992; Hammes and Ga¨nzle, 1997) including aroma, taste, nutritive value and shelf-life of the bread (Clarke and Arendt, 2005) via the metabolic products of the yeasts and LAB. Application of sourdough in bulk production of bread in the industry needs stringent control of pH and Titratable acidity in sourdough and the resulting bread. It is achieved by controlling of sourdough fermentation and regulation of fermentation condition including time, temperature, and flour extraction degree (Erwang et al., 2011).

In this study, antifungal activity of 6 Iranian species of Lactobacillus was screened. We employed the two testing systems, i.e. agar plates and bread slices. Our results demonstrated

the ability of 6 Iranian species of Lactobacillus in decreasing the time of mould-mediated spoilage, improving the sensory quality of bread, and increasing shelf-life. Our results specially revealed that *L.delbrueckii* acts more robust than other species and could keep the bread mould-free for average of 12 days. In the second place, *L.plantarum* was effective against mould-mediated bread spoilage. Our results is consistent with the previous studies (Corsetti and Settanni, 2006; Katina et al., 2006; Gül et al., 2005; Arendt et al., 2007). According to their results sourdough regulates α-amylase activity of flour and decreases bread staling by changing starch crystallization (Rollan et al., 2010). In our study, increasing of fermentation time and concentration of LAB leads to increasing sourdough acidity. In addition, increasing temperature up to 30-35°C and concentration of LAB up to $3\times10^7$ CFU/ml causes maximum acidity in dough. This is because the optimum growth of LAB is 30-35°C and increase of LAB leads to pH decrease. During fermentation, the number of yeast increases up to 24 hour, while yeast growth and thereby volume decreases from 30 hours on.

In addition, in vitro antifungal activity revealed that *L. delbrueckii* and *L.plantarum* had large clear zones and no growth around the colonies indicating the strongest inhibitory effect. It is noteworthy to mention that fungi have optimal conditions for growth on agar plates. There is no competitive organisms and abundance of nutrients on agar plates. On the other hand, bread is a stressful environment for fungal growth as it has reduced availability of nutrients, reduced water activity, and/or presence of other competitive moulds/bacteria. Thus, the reduced growth of Lactobacillus on bread slices is presumably due to the suboptimal growth conditions.

In summary, the *present* study confirmed that addition of sourdough fermented by Iranian LAB caused a distinct change in the properties of the dough, specifically indicating that *L.delbrueckii* is a suitable starter for wheat sourdough and bread production. *L. delbrueckii* can successfully perform sourdough fermentation and produce good quality bread. The formation of organic acids (lactic and acetic), alcohols (acetoin, aldehydes, ketones) and various carbonyl compounds during fermentation are due to the presence of LAB and yeasts which contributed to the decrease of pH and enhanced the aroma of the bread. These findings are in agreement with Gobbetti et al. (1995a; 1995b) who reported that non- volatile compounds including organic acids produced by homo- and hetero-fermentative LAB would decrease pH and contribute to a pleasant aroma of the bread dough.The oldest method for preparation of sourdough, which is a mixture of flour and water that is fermented, is based on spontaneous fermentation of flour natural flora. Alternatively, LAB developing in the dough may originate from a starter culture, a pre-ferment containing one or more known species of LAB (Messens and Vuyst, 2002).

Sourdough, which is rich in lactobacilli as well as baker's yeast, influences bread shelf-life depending on bacterial strain, fermentation conditions, and the process utilized (Katina, 2005). This is due to different factors especially low pH value, antimicrobial compounds, and LAB metabolites such as organic acids, exopolysaccharides and enzymes produced by LAB (Corsetti et al., 1998; Simsek et al., 2006). One of metabolic activities of sourdough LAB is the production of antibacterial/antifungal substances, anti-ropiness activities, and

exopolysaccharides in dough, which potentially influences bread texture, staling and/or shelf-life (Corsetti et al., 1998; Corsetti et al., 1998c; Corsetti et al., 1996; Gobbetti, 1998; Hammes & Ga ¨nzle, 1998; Katina et al., 2002; Korakli et al., 2001; Korakli et al., 2003; Lavermicocca et al., 2000; Lavermicocca et al, 2003; Pepe et al., 2003). Many researchers have studied the effect of bacterial strain in sourdough on the quality and shelf-life of wheat bread (Dal Bello et al., 2007; Katina et al., 2002; Mentes et al., 2007). The loss of perceived freshness may generally be categorized into two groups including processes known as staling and those that are attributed to microbial spoilage (Pateras, 1998). Notably, fungi growth is usually blamed for microbial bread spoilage (Legan, 1993) while LAB preserves bread from fungal-mediated spoilage (Spicher, 1983; Brummer and Lorenz, 1991; Caplice and Fitzgerald, 1999; Katina et al., 2002). The anti-fungi activities of sourdough LAB have been previously studied (Corsetti et al., 1998b; Rocken 1996).

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