

Antimicrobial Activity of *Lactobacillus* Isolated From Kashk-e Zard and Tarkhineh, Two Iranian Traditional Fermented Foods

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Abstract

Background: Nowadays, microbial food safety is an increasing public health concern worldwide, especially in developing countries.

Objectives: The aim of this study was to apply established in vitro tests to evaluate the antimicrobial activity of the *Lactobacilli* isolated from two traditional Iranian fermented dairy-cereal based foods.

Materials and Methods: A total of 23 samples of Kashk-e Zard and 27 samples of Tarkhineh were collected from different regions of Iran. *Lactobacillus* spp. was isolated and identified by standard methods. The antibacterial effects of *Lactobacillus* isolates were implemented by well diffusion method. Kinetic study was conducted with a bacteriostatic activity in vitro in the presence of *Lactobacillus* supernatants.

Results: The results showed an antimicrobial activity of the *Lactobacillus* strains isolated from Kashk-e Zard and Tarkhineh against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella typhimurium* according to agar well diffusion test. In addition, kinetic study revealed a significant bacteriostatic activity of *Lactobacillus* supernatants.

Conclusions: Kashk-e Zard and Tarkhineh seemed to have probiotic properties, deserving further studies.

Keywords: Fermentation, Antimicrobial Activity, Probiotic, *Lactobacillus*

1. Background

A large variety of traditional fermented foods are globally produced and consumed. They are the product of a natural biotechnological process and produced by taking advantage of the natural microbiota associated with fresh food materials. Fermentation is one of the most practical, economic and widely applied empirical methods for preserving, often diversifying and enhancing organoleptic and nutritional quality of fresh foods (1, 2). These foods such as Kashk-e Zard and Tarkhineh are still mainly prepared at the household level and marketed through informal routes. They have a high nutrition value and are considered as a good source of bioavailability such as protein, vitamins and minerals (3). These fermented products are popular in the southeastern part of Iran (Sistan and Baluchestan province), produced by mixing cereal flour (mainly wheat flour), yoghurt, a variety of vegetables, salt and spices, followed by lactic and alcoholic fermentation in two steps of fermentation for several days.

Tarkhineh, Tarkhowana or Doowina in Kurdish is a unique product, made traditionally in west of Iran (Kurdistan, Kermanshah and Hamedan provinces). Wheat meal and sour dough are the main ingredients of this traditional product. Then, there is spontaneous fermentation

followed for 7-10 days in one step.

Nowadays, microbial food safety is an increasing public health concern worldwide, especially in developing countries (4). During fermentation, the growths of pathogens as well as other spoilage organisms are frequently inhibited through antimicrobial components produced by lactic acid bacteria (LAB) (5-7). Reports have shown that LAB-produced organic acids can work in combination to display a strong inhibitory activity against many foodborne pathogens such as *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens*, *Listeria monocytogenes*, and *Staphylococcus aureus* (8-12).

Dairy-cereal fermented foods often include LAB strains with probiotic properties. The mechanisms of health promoting effects of probiotic bacteria have been attributed to their anti-pathogenic properties (13). According to food and agriculture organization/world health organization (FAO/WHO), one of the most important parameters by which potentially new probiotic strains must be characterized is the production of antimicrobial substances under in vitro conditions (14). *Lactobacilli* could be considered among the most important of all LAB due to their role in various food and feed fermentations for the prevention of

food spoilage, intoxication and infection as well as production of several important metabolites. Additionally, they play important roles by acting as antagonists against other pathogens through the production of antimicrobials and bacteriocins (15, 16). These products, for their unique fermentation styles and production methods, could be a valuable source of native *Lactobacilli*, whereas no study has been done on any aspect of these invaluable foods.

2. Objectives

The main objective of this study was to determine the antimicrobial activity of the *Lactobacilli* spp. isolated from two traditional Iranian fermented foods, which are based on combination of both local cereal and dairy materials.

3. Materials and Methods

3.1. Sampling, Enumeration and Isolation

A total of 23 samples of Kashk-e Zard from various regions of Sistan and Balouchestan province and 27 samples of Tarkhineh from Kurdistan, Hamedan and Kermanshah provinces of Iran were collected in sterile containers aseptically and brought to the laboratory. Ten grams of each sample was homogenized with 90 mL of peptone water in sterile bags by food mixer stomacher and ten-fold serial dilution was made to get a 10^{-7} dilution. Appropriate dilutions were surface-plate cultured on de Man Rogosa and Sharpe agar (MRS) and incubated at 37°C for 72 hours with 5% - 10% CO₂. After growth, colony forming units (CFU) were quantified using colony counter and representative morphotypes were isolated. Then, the selected colonies were cultured on MRS agar to obtain pure cultures. After initial identification, Gram-staining for microscopic and morphologic inspection, biochemical tests such as catalase activity, motility test in sulfide, indole, motility (SIM), indole production from tryptophan, carbohydrates fermentation pattern, nitrate reduction and gelatin hydrolysis tests were performed to confirm the presence of *Lactobacillus* genus in samples. Further approval of the isolates was carried out by cultivation at 15°C and 45°C in MRS broth and carbohydrates fermentation profiles (1, 9, 17).

3.2. Antimicrobial Activity of Isolates

The antagonistic activities of isolated *Lactobacillus* cultures were assessed against four intestinal pathogens as test organisms namely *E. coli* ATCC 700728, *S. aureus* ATCC 29737, *L. monocytogenes* ATCC 19115 and *S. typhimurium* ATCC 19430, using well diffusion test by detecting the presence of a growth inhibition zone of the indicator strain around the LAB, described by Schillinger and Lucke (18).

3.3. Kinetic Study of *Lactobacillus* Supernatants

The most isolated *Lactobacillus* strain (*L. casei*) was cultured in MRS broth medium and centrifuged in 12000 rpm for seven minutes for determining the kinetic study. Then, suspensions was prepared in 0.5 McFarland concentrations from the ones grown in CASO agar. One milliliter of this suspension was added to CASO broth medium in three separated parts. *L. casei* in concentrations of 10% and 20% was added to these suspensions at the same time. Then, optical densities (OD) of the target cultures were measured each two hours. There was one control with pathogen and without *Lactobacillus*.

4. Results

Average enumeration values of *Lactobacillus* in Kashk-e Zard and Tarkhineh were 2.84×10^5 CFU/mL (ranged from 0 to 2×10^6) and 4.55×10^5 CFU/mL (ranged from 0 to 1.18×10^7), respectively.

Isolates of Kashk-e Zard and Tarkhineh were identified as *Lactobacillus* spp. genus. All the isolates were gram-positive, rod-shaped, catalase-negative, carbohydrates fermentative with the ability to grow at different temperatures, non-motile, and indole and gelatin-negative. Based on the fermentation profile, seven and eight categories of *Lactobacillus* spp. were identified for Kashk-e Zard and Tarkhineh respectively, of which the most and the least frequent isolates belonged to *L. casei* (n = 20 and n = 28) and *L. acidophilus* (n = 2 and n = 1), respectively. *L. casei* was the predominant category of *Lactobacillus* spp.

Regarding isolates of Kashk-e Zard, the strongest growth inhibition against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. typhimurium* were respectively shown by *L. corustorum*, *L. plantarum*, and *L. casei*. Antagonistic activities of *L. alimentarius* against *E. coli* and *S. aureus*, and *L. rhamnosus* against *L. monocytogenes* and *S. typhimurium* were at the lowest levels (Table 1).

In the case of Tarkhineh, the highest antibacterial activities against *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* were suggested by species of *L. corustorum*, *L. plantarum*, *L. casei*, and *L. fermentum*, respectively. Similar to Kashk-e Zard, *L. alimentarius* and *L. rhamnosus* showed the smallest bactericidal effects as opposed to *E. coli* and *S. aureus*, and *L. monocytogenes*, and *S. typhimurium* (Table 1).

Table 1 shows the growth inhibition power of cell-free culture supernatants obtained from the *Lactobacillus* isolates from Kashk-e Zard and Tarkhineh as attained and neutralized by NaOH. Overall, cell-free culture supernatants (CFCS) and neutralized CFCS of all the isolates suggested antimicrobial activity, although adjustment of pH to 6.5 led to the reduction of the average zone of inhibition.

Table 1. Zone of Inhibition for Cell-Free Culture Supernatants and Neutralized Cell-Free Culture Supernatants

	<i>E. coli</i>		<i>S. aureus</i>		<i>L. monocytogenes</i>		<i>S. typhimurium</i>	
	Mean \pm SD ^b , mm	Mean \pm SD ^a , mm	Mean \pm SD ^b , mm	Mean \pm SD ^a , mm	Mean \pm SD ^b , mm	Mean \pm SD ^a , mm	Mean \pm SD ^b , mm	Mean \pm SD ^a , mm
<i>L. acidophilus</i>								
CFCS	19.43 \pm 2.34	20.48 \pm 3.34	19.97 \pm 1.55	21.07 \pm 3.05	16.37 \pm 3.43	17.37 \pm 3.52	21.31 \pm 2.04	22.32 \pm 3.44
NCFCFS	17.42 \pm 1.38	19.42 \pm 2.08	18.40 \pm 3.01	19.34 \pm 4.11	12.99 \pm 2.32	13.87 \pm 2.25	19.68 \pm 3.54	21.08 \pm 3.58
<i>L. alimentarius</i>								
CFCS	14.47 \pm 4.21	15.57 \pm 4.42	17.36 \pm 2.33	19.06 \pm 3.13	19.32 \pm 3.21	20.30 \pm 4.01	17.32 \pm 3.61	18.02 \pm 3.58
NCFCFS	12.58 \pm 3.16	11.85 \pm 4.06	16.42 \pm 2.19	17.42 \pm 2.54	19.48 \pm 5.13	18.49 \pm 4.23	15.24 \pm 1.66	14.98 \pm 2.68
<i>L. casei</i>								
CFCS	27.60 \pm 2.88	24.70 \pm 6.08	20.00 \pm 2.44	21.20 \pm 3.04	22.60 \pm 5.88	23.61 \pm 4.78	25.00 \pm 2.24	24.24 \pm 3.14
NCFCFS	25.40 \pm 2.07	24.42 \pm 2.07	20.00 \pm 4.24	21.22 \pm 3.74	23.00 \pm 2.57	24.11 \pm 3.28	24.40 \pm 2.53	21.49 \pm 2.66
<i>L. corustorum</i>								
CFCS	31.27 \pm 2.58	30.47 \pm 4.08	21.16 \pm 3.16	25.18 \pm 3.34	20.35 \pm 3.25	21.42 \pm 3.73	25.15 \pm 2.10	24.19 \pm 3.18
NCFCFS	27.39 \pm 4.35	28.19 \pm 3.79	21.05 \pm 7.41	22.25 \pm 5.01	20.32 \pm 1.53	23.82 \pm 3.03	24.62 \pm 2.46	26.42 \pm 3.45
<i>L. lactis</i>								
CFCS	19.62 \pm 4.15	9.50 \pm 5.54	19.51 \pm 5.39	11.37 \pm 2.26	18.89 \pm 4.10	9.42 \pm 5.73	21.14 \pm 1.69	13.24 \pm 2.52
NCFCFS	18.88 \pm 2.70	3.31 \pm 4.74	19.11 \pm 2.05	11.93 \pm 3.37	17.40 \pm 3.47	8.53 \pm 3.47	20.59 \pm 3.42	12.26 \pm 1.14
<i>L. plantarum</i>								
CFCS	30.72 \pm 2.46	22.73 \pm 4.53	26.35 \pm 4.72	20.56 \pm 7.50	26.42 \pm 4.31	19.14 \pm 2.44	24.63 \pm 3.62	21.39 \pm 2.70
NCFCFS	27.61 \pm 2.41	21.37 \pm 2.71	26.21 \pm 3.26	20.12 \pm 2.11	24.49 \pm 3.52	20.13 \pm 2.32	24.06 \pm 3.53	20.48 \pm 2.62
<i>L. rhamnosus</i>								
CFCS	18.32 \pm 4.65	31.02 \pm 4.06	15.24 \pm 3.42	27.15 \pm 3.82	13.25 \pm 3.39	25.41 \pm 3.38	12.63 \pm 3.75	23.58 \pm 3.78
NCFCFS	14.58 \pm 6.22	27.35 \pm 4.12	16.20 \pm 2.39	25.71 \pm 3.55	12.18 \pm 5.94	23.51 \pm 3.86	9.39 \pm 7.27	23.54 \pm 3.89
Total average								
CFCS	26.82 \pm 3.03	19.41 \pm 5.12	21.42 \pm 3.45	16.14 \pm 3.56	18.49 \pm 4.23	14.21 \pm 3.09	16.98 \pm 2.68	12.99 \pm 3.78
NCFCFS	22.42 \pm 2.54	16.08 \pm 5.02	19.25 \pm 5.01	14.20 \pm 2.56	17.82 \pm 3.03	10.19 \pm 4.37	16.70 \pm 4.08	10.35 \pm 6.07

^bTarkhineh.^aKashke Zard.

Kinetic study of intestinal pathogens was investigated by applying 10% and 20% CFCS for 24 hours. Time assay demonstrated a significant bacteriostatic effect of *L. casei* supernatants against all the pathogens. Although the concentration of 20% of *L. casei* supernatants inhibited the growth of all the investigated pathogens completely after 12 hours of incubation, concentration of 10% suggested its inhibitory effect on pathogen microorganisms after 24 hours. Applying 20% of supernatant had a higher bacteriostatic activity of *E. coli* in comparison with other pathogens, which was also confirmed by the results from agar well assay (Figures 1 and 2).

5. Discussion

Generally, several factors are involved in viability of probiotic cultures in fermented foods such as pH, acidity, the presence of other microorganisms, temperature, and oxygen content (17). Sengun et al. obtained 7 - 9.8 log CFU/mL for LAB in yoghurt and 1.3 - 9.4 log CFU/mL for cracked wheat in the study on Tarhana, similar fermented product to ours in Turkey (19). Tamime et al. reported a count of 1.02×10^6 CFU/mL of LAB for Kishk, fermented milk-cereal mixture (20).

The presence of organic acids in a food medium results in the reduction of the pH (6, 21-23). Reduced pH results in unfavorable growth conditions for a wide va-

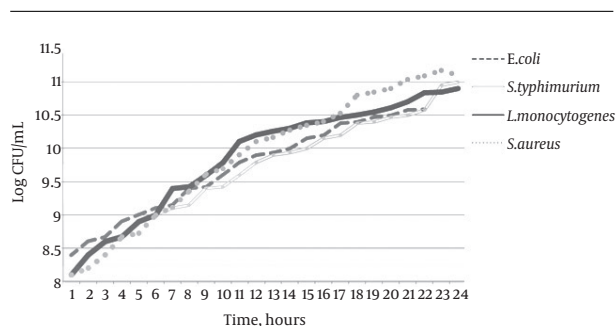


Figure 1. Bacteriostatic Kinetic Surviving Time of Supernatants of *Lactobacillus casei* (10%) Against *Escherichia coli* ATCC 700728, *Staphylococcus aureus* ATCC 29737, *listeria monocytogenes* ATCC 19115 and *Salmonella typhimurium* ATCC 19430

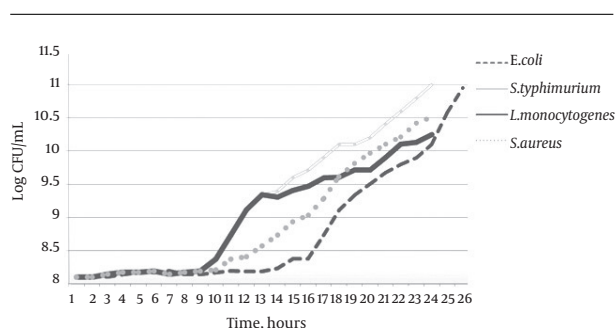


Figure 2. Bacteriostatic Kinetic Surviving Time of Supernatants of *Lactobacillus casei* (20%) Against *Escherichia coli* ATCC 700728, *Staphylococcus aureus* ATCC 29737, *listeria monocytogenes* ATCC 19115 and *Salmonella typhimurium* ATCC 19430

riety of pathogens and spoilage microbes, whereas LAB are more tolerant to lower pH environments (2). Therefore, decrease in zone of inhibition in our study can be attributed to the elimination of organic acids when CFCS was neutralized with NaOH (Table 1). However, growth inhibition can be associated to other antimicrobial agents such as bacteriocins. Numerous LAB synthesize bacteriocins have varying spectrums of inhibition on closely related Gram-positive bacteria and certain yeast strains (23). Inhibition of foodborne pathogens such as *L. monocytogenes* and *S. aureus* by bacteriocins has led to the realization of their potential roles as natural food preservatives (6, 23, 24). In a previous study, isolated *Lactobacillus* strains exhibited the highest zones of inhibition (15 mm) against *S. aureus* (25). Another investigation showed the antibacterial activity of bacteriocin-producing *Lactobacillus* species isolated from traditional milk products (26). Furthermore, antimicrobial-producing lactic acid bacterial isolates from raw barley and sorghum have been indicated by Hartnett and Vaughan (27). Inhibition zones between 0.5 - 13.0 mm by *Lactobacillus* strains in indigenous fermented food against the indicator organisms have

been reported by Sanni et al. (28). Researches of Savadog et al. showed inhibition diameters of lactic acid bacteria strains isolated from Burkina Faso fermented milk on indicators strains such as *E. coli*, *S. aureus* and *B. cereus*, which were between 8 - 12 mm. The largest diameter of 12 mm inhibition was obtained with *L. fermentum* on *Enterococcus faecalis*, and the smallest diameter was obtained with *Leuconostoc mesenteroides* on same indicators (29). In our study, isolates of Kashk-e Zard and Tarkhineh, had the highest and lowest growth inhibition diameters against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. typhimurium* by *L. corostorum*, *L. plantarum*, and *L. casei* and also by *L. alimentarius* and *L. rhamnosus*, respectively.

Traa et al. with adjacent culture supernatant of different *Lactobacillus* with pathogenic bacteria such as *L. monocytogenes*, *Salmonella* and *S. aureus* and examining the obtained turbidity at different intervals concluded that the turbidity levels studied reduced dramatically (30). In this study, concentrations of 10% and 20% of *L. casei* supernatants inhibited the growth of all investigated pathogens after 24 and 12 hours of incubation, respectively. It seems that with higher concentrations of supernatants, the antimicrobial compounds concentrations increased.

Lactobacilli are able to compete with target cultures when they are incubated together, but the degree of inhibition, as we observed, was bacterial strain-dependent, which was previously reported by Wei et al. (31). The bacteriocin-producing strains may be used as protective cultures to improve the microbial safety of foods (32-36), and they also play an important role in preservation of fermented foods, which is usually achieved by inhibition of contaminating spoilage bacteria such as *Pseudomonas* and pathogens such as *S. aureus*, *Salmonella* spp. and *L. monocytogenes* (37, 38). In conclusion, this study demonstrated bacteriostatic activities of *Lactobacillus* isolated from Kashk-e Zard and Tarkhineh against the most frequent foodborne target cultures is a desirable property.

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