Lactobacilli From Buffalo Milk and Yogurt With Antibacterial Activity Against Gram-Negative Uropathogens

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Abstract

Background: This descriptive cross-sectional study was conducted to determine the antibacterial activity of Lactobacillus strains isolated from buffalo milk and yogurt in Alibabad-e Katul city, Golestan province, north-east of Iran.

Methods: Raw milk and yogurt samples were collected and cultured on MRS medium by incubating anaerobically at 37°C for 48 hours. The suspected colonies were identified on the basis of Gram staining, biochemical tests, and carbohydrates fermentation. The antibacterial activity of the cell-free supernatant (CFS) extracted from Lactobacillus strains was determined using the agar well diffusion method against standard strains of Escherichia coli ATCC 11303, Klebsiella pneumoniae ATCC 13883, and Pseudomonas aeruginosa ATCC 15442 as well as gram-negative uropathogens previously isolated from patients with urinary tract infections (UTIs). Three isolates of E. coli (E1, E2, and E3), two isolates of P. aeruginosa (P1 and P2), and two isolates of K. pneumoniae (K1 and K2) were used in this study.

Results: A total of 19 Lactobacillus strains were identified as L. plantarum, L. casei, L. acidophilus, and L. helveticus. Based on the results of antibacterial activity test, the isolates had the highest and lowest inhibitory effects on the E. coli and K. pneumoniae isolates, respectively. Among the isolates, only L. casei isolates showed inhibitory activity against K. pneumoniae isolates.

Conclusions: In this study, Lactobacilli from buffalo milk and yogurt demonstrated a good inhibitory activity against E. coli as a common cause of urinary tract infection. Therefore, further studies are recommended to elucidate their potential for being used as an alternative to antibiotic therapy.

Keywords: Buffalo milk, Yogurt, Lactobacillus, Antibacterial activity

There are lots of LAB in dairy products and many people consume different types of these products as a significant source of protein (6), among which cow, sheep, and goat yogurt and milk are very common in Iran. However, buffalo yogurt and milk are more common in rural areas of the country (7). Compared to other milks, buffalo milk has a low cholesterol content and a high level of calcium, in addition, it is also a source of antimicrobial metabolites such as lactic acid and bacteriocins (6). In Iran, most of the studies have focused on the identification of Lactobacillus strains in bovine dairy products and their antibacterial properties, while buffalo dairy products have received very little attention so far. Therefore, the aim of this study was to isolate and identify different strains of Lactobacillus spp. from buffalo yogurt and milk and determine their antibacterial activity against gram-negative standard strains and uropathogens isolated from patients with urinary tract infections (UTIs).

Materials and Methods

Isolation of Lactobacillus Species

This descriptive cross-sectional study was conducted from...
April to June 2016 to determine the inhibitory effect of Lactobacillus strains isolated from buffalo yogurt and milk collected in Aliabad-e Katul city, Golestan province, north-east of Iran. The raw milk and yogurt samples were collected in sterile screw-capped falcon tubes with ice packs and transported to the laboratory of Islamic Azad University, Gorgan Branch, Iran. Afterwards, 2 g of yogurt and 1 mL of milk were aseptically inoculated into MRS broth and incubated anaerobically at 37°C for 48 hours. Then, the enriched samples in MRS broth were cultured on MRS agar (Conda Pronadisa, Spain). The plates were incubated anaerobically at 37°C for 48 hours. Then, the suspicious colonies were tested using Gram staining and catalase reaction. Gram-positive and catalase-negative bacilli were purified by streaking on MRS agar and identified on the basis of biochemical tests including fermentation of galactose (Merck, Germany), maltose (Conda Pronadisa, Spain), fructose (Merck, Germany), sucrose (Merck, Germany), raffinose (Merck, Germany), sorbitol (Merck, Germany), lactose (Merck, Germany), rhamnose (Merck, Germany), and mannitol (Merck, Germany), as well as the ability to grow at 10°C and 45°C and in the presence of 6.5% NaCl according to Bergey’s Manual of Systematic Bacteriology (6,8). The isolates were stocked in MRS broth containing 20% glycerol (Oxoid, Canada) at −20°C until further used.

### Antibacterial Activity Test

The antibacterial activity of Lactobacilli strains against standard strains and gram-negative uropathogens was investigated by well diffusion method (9). In this method, the isolated colonies were inoculated in MRS broth and incubated for 48 hours at 37°C. Subsequently, the MRS broth was centrifuged at 10 000×g for 15 minutes to obtain cell-free supernatants (CFSs). The CFS was sterilized by passage through 0.45 μm Millipore filters. The standard strains used in this study included Escherichia coli ATCC 11303, Klebsiella pneumoniae ATCC 13883, and Pseudomonas aeruginosa ATCC 15442 (provided by the Faculty of Veterinary Medicine, Tehran University). In addition to standard strains, three isolates of E. coli (E1, E2, and E3), two isolates of P. aeruginosa (P1 and P2), and two isolates of K. pneumoniae (K1 and K2) which were previously isolated from patients with UTIs were included in this study (10). Then, 50 μL of the CFS was poured in each 5-mm-deep wells punched into the nutrient agar plates previously seeded with 10⁶ CFU/mL of the test bacteria pre-cultured in LB broth. The plates were then incubated at 37°C for 24 hours. Based on the diameter (mm) of the clear inhibitory zone formed around the wells, antibacterial activity was estimated (3,6). Inhibition zone <15 mm and ≥15 mm was considered moderate and relatively strong activity, respectively (11). Antimicrobial tests were done in triplicate and the mean values were recorded.

### Results

A total of 19 Lactobacillus strains were isolated from 10 samples (5 samples of milk and 5 samples of yogurt), forming round creamy white colonies on MRS agar plate. Morphological and biochemical characteristics were employed to identify the isolates (Table 1). Based on the results of sugar fermentation and different growth conditions, Lactobacilli were identified as shown in Table 2. Among the isolates, L. plantarum and L. casei were the most prevalent Lactobacilli.

According to the results of antibacterial activity test, L. plantarum P1-Y (16.8 mm) and L. helveticus H1-Y (16.2 mm) exhibited relatively strong inhibitory effects on E. coli E1 and did not show any inhibitory effect on the growth of K. pneumoniae isolates. Moreover, L. casei C2-Y showed a relatively strong inhibitory effect (15.8 mm) on E. coli E1 but weak activity on K. pneumoniae and P. aeruginosa isolates. L. acidophilus A1-Y had a relatively strong inhibitory effect on the growth of E. coli E2 (15.1 mm) but no inhibitory activity against K. pneumoniae isolates. Among the isolates, only L. casei isolates demonstrated inhibitory activity against K. pneumoniae isolates. However, all of the isolates had the highest and lowest inhibitory effects on the E. coli and K. pneumoniae isolates, respectively. The mean diameters of growth inhibition zones (mm) were given in Tables 3-5.

### Discussion

The study was designed for identification of Lactobacillus spp. from buffalo milk and yogurt samples and assessment of their antibacterial activity against gram-negative standard strains and uropathogens. Based on the morphological characteristics, 19 isolates from the samples were identified as Lactobacillus spp. The isolated bacteria were non-spore forming gram-positive rod-shaped facultative anaerobes which were indicated to be

| Table 1. Identification of Lactobacilli Based on Sugar Fermentation and Different Growth Conditions |
|-----------------------------------------------|
| Isolates | Growth at 10°C | Growth at 45°C | Growth at 6.5% NaCl | Galactose | Fructose | Raffinose | Lactose | Maltose | Sucrose | Sorbitol | Rhamnose | Mannitol |
|----------|----------------|----------------|-------------------|-----------|----------|-----------|---------|---------|---------|----------|----------|----------|
| L. plantarum | - | - | - | + | + | + | + | + | + | + | + | + |
| L. casei | - | + | - | + | + | + | + | + | + | + | + | + |
| L. acidophilus | + | - | - | + | + | + | - | + | + | + | + | + |
| L. helveticus | + | - | - | - | + | + | + | + | + | - | - | - |
the member of *Lactobacillus* spp. Based on the results, they were identified as *L. plantarum*, *L. casei*, *L. acidophilus*, and *L. helveticus*.

In this study, among the isolates, *L. plantarum* (42.1%) had the highest frequency followed by *L. casei* (26.3%). The CFSs of the isolates showed good and weak inhibitory effects on the *E. coli* and *P. aeruginosa* isolates, respectively. They did not have any inhibitory effects on *K. pneumoniae* isolates except for CFSs from *L. casei* isolates.

Similar studies have been undertaken in Iran and other countries. In agreement with the present study, in a study conducted by Dorri et al, the most commonly identified strains of *Lactobacilli* were *L. casei*, *L. acidophilus*, and *L. plantarum* (12). Moreover, the findings of Farahbakhsh et al and Naeemi et al are also consistent with the findings of the present study in which among *Lactobacilli* isolates, *L. plantarum* as a predominant isolate had the highest antibacterial activity against the test organisms (13, 14).

In another study, *L. casei* had the highest frequency in traditional dairy products followed by *L. acidophilus* in Gorgan, north-east of Iran (15). Forhad et al also isolated a total of four isolates including *L. fermentum*, *L. casei*, *L. Acidophilus*, and *Bifidobacterium longum* from buffalo milk in Bangladesh (6). In another study, Eid et al isolated *L. fermentum*, *L. Acidophilus*, and *L. pentosus* from buffalo milk, among which *L. pentosus* had the highest antibacterial activity against the indicator organisms (3).

In a study, Chowdhury et al isolated four *L. plantarum* strains that inhibited the growth of test pathogens to some extent but the highest and lowest inhibition zones were observed against *Bacillus cereus* and *Staphylococcus aureus*, respectively (5).

Hossein Alipour et al isolated *L. salivarius* from buffalo milk with the most and least inhibitory effect on *S. aureus* and *S. typhimurium*, respectively (7). The antagonistic activity of LAB isolated from traditional dairy products including *L. plantarum* and *L. fermentum* was studied against *E. coli* O157:H7 by Rahimpour Hesari et al. Among the isolates, the antagonistic activity of *L. plantarum* was greater than that of *L. fermentum* (16). Consistent with the findings of the present study, a study from Nepal showed the antibacterial effect of *Lactobacilli* isolated from dairy products on *E. coli*, *Salmonella paratyphi*, *Salmonella typhi*, *Pseudomonas* spp., *S. aureus*, *Proteus* spp., *Acinetobacter*

### Table 2. Different Lactobacilli Isolated From the Collected Milk and Yogurt Samples

| Isolates    | Milk | Yogurt |
|-------------|------|--------|
| *L. plantarum* | 5    | 3      |
| *L. casei*    | 2    | 3      |
| *L. acidophilus* | 1    | 2      |
| *L. helveticus* | 1    | 2      |
| Total        | 9    | 10     |

### Table 3. Growth Inhibition Zones (mm) Created by CFSs of *Lactobacillus plantarum* Isolates

| Standard Strains and Isolates | *L. plantarum* P1-M | *L. plantarum* P2-M | *L. plantarum* P3-M | *L. plantarum* P4-M | *L. plantarum* P5-M | *L. plantarum* P1-Y | *L. plantarum* P2-Y | *L. plantarum* P3-Y |
|-------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| *E. coli* control             | 15.2                | 11                  | 10.7                | 10.4                | 14                  | 16                  | 11.1                | 10.6                |
| *E. coli* E1                  | 16                  | 12                  | 13.4                | 11.2                | 15.1                | 16.8                | 10.8                | 11                  |
| *E. coli* E2                  | 12.3                | 12.1                | 11.7                | 12.5                | 12                  | 14.2                | 11.3                | 10.8                |
| *E. coli* E3                  | 11.8                | 10.7                | 11.2                | 11.4                | 11.6                | 12.1                | 10.2                | 11.2                |
| *K. pneumonia* control        | -                   | -                   | -                   | -                   | -                   | -                   | -                   | -                   |
| *K. pneumonia*                | -                   | -                   | -                   | -                   | -                   | -                   | -                   | -                   |
| *K. pneumonia*                | -                   | -                   | -                   | -                   | -                   | -                   | -                   | -                   |
| *P. aeruginosa* control       | 10.3                | 10.2                | 10                  | 11.2                | 9.4                 | 6.8                 | 10.2                | 10.8                |
| *P. aeruginosa* P1            | 9.2                 | 9.8                 | 11                  | 10.4                | 7.8                 | 9.2                 | 10.1                | 9                   |
| *P. aeruginosa* P2            | 8.3                 | 9                   | 10.6                | 8.7                 | 7.7                 | 9.6                 | 8.1                 | 8.8                 |

* No inhibitory zone.
and no inhibitory effects on K. pneumoniae and Shigella spp (17). In a study, Lactobacilli including L. alimentarius, L. sake, and L. collinoides from traditional dairy samples showed moderate activity against S. aureus ATCC 6538, Bacillus subtilis ATCC 12711, and P. aeruginosa ATCC 27853, while L. collinoides and L. alimentarius had relatively strong activity against P. aeruginosa and Bacillus subtilis, respectively (11). In a research, among the LAB isolates from ewe milk, traditional yogurt and sour buttermilk, Pediococcus acidilactici had a great antibacterial activity against L. monocytogenes, S. aureus, and Salmonella enteritidis (18). In a study, L. plantarum and Lactococcus piscium from goat milk were the most common probiotic isolates

while L. lactis showed the highest inhibitory effects on drug-resistant Acinetobacter baumannii (19). A study from Pakistan showed the antibacterial effect of LAB on multidrug-resistant uropathogens including Candida albicans, P. aeruginosa, K. pneumoniae, Enterococcus faecalis, and E. coli. The growth inhibition zone was over 10 mm against all the uropathogenic test organisms, while L. fermentum and L. plantarum strains demonstrated great inhibitory activities against E. coli and E. faecalis (20).

In a study, L. casei and L. lactis isolates from yogurt showed better inhibitory effects on pathogenic bacteria and the highest and lowest inhibitory effect was observed on Yersinia enterocolitica and B. cereus (21). In a study by Kazemi Darsnaki et al, six LAB strains were isolated from yogurt and probiotic pills and L. acidophilus had the highest antibacterial activity against B. cereus (22). In a study from Egypt, the highest antagonistic activity was observed for Lactobacillus paracasei and L. helveticus against the tested pathogens followed by L. fermentum, while Bifidobacterium longum and L. lactis subsp. lactis showed weak or no antibacterial activity against the tested strains (23).

Considering the results of all studies including the present study, LAB from dairy products presented the inhibitory activity against gram-positive and gram-negative bacteria. Among the LAB, lactobacilli with satisfactory antagonistic activity against the pathogens have been shown to be one of the best alternatives to antibiotic therapy.

Conclusions
In this study, Lactobacilli had the highest antibacterial activity against the E. coli isolates as a common cause of UTIs; therefore, further studies are recommended to elucidate their potential for being used as an alternative to antibiotic therapy.

Conflict of Interests
The authors declare that they have no conflict of interests.

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Ethical Approval
Not applicable.

Authors’ Contribution
AAA; Data curation and formal analysis: AAA, SB, TD; Investigation: SB; Methodology and project administration: AAA; Supervision: AAA; Validation: AAA; Writing of original draft:AAA; Writing, reviewing, and editing: AAA, TD.

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