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Original Article

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 Aliabad-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

Background

In recent years, the rise of antibiotic resistance and declining discovery of new antibiotics have created a global health crisis. Overuse and misuse of antibiotics have led to the rapid emergence of antibiotic-resistant bacteria. Of particular concern, no new antibiotics have been approved for treating gram-negative pathogens in decades. Therefore, there is an urgent need to find novel and safe antibacterial substances as alternatives to antibiotics (1,2).

Today, lactic acid bacteria (LAB) due to the potential production of metabolites with antimicrobial activity including diacetyl, hydrogen peroxide, acetaldehyde, organic acids, bacteriocin, and bacteriocin-like substances have received the attention of many researchers (3). Moreover, their antibacterial effects have been attributed to the reduction in pH and competition for nutrients resources (4). Therefore, studies related to the antibacterial activity of these organisms have received much attention to prevent, control, and treat diseases (5). In the past 20 years, the antagonistic effect of LAB on many pathogenic microorganisms has been reported (1,2).

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 10^6 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 4. Growth Inhibition Zones (mm) Created by CFSs of *Lactobacillus casei* Isolates

Standard Strains and Isolates	*L. casei* C1-M	*L. casei* C2-M	*L. casei* C1-Y	*L. casei* C2-Y	*L. casei* C3-Y
E. coli control	13	11	10	11.1	10.2
E. coli E1	14.7	12	11.2	15.8	13.6
E. coli E2	12.3	12.1	9.5	11.3	10.5
E. coli E3	10.8	10.2	11.2	10.2	10
K. pneumoniae control	10.2	10	10.4	10	9.8
K. pneumoniae	10.8	13.1	12.2	10.6	10.7
K. pneumoniae	10.3	8.8	9.8	10.1	10.3
P. aeruginosa control	9.2	9.8	10	9	10.4
P. aeruginosa P1	10.3	10.2	11	10.2	10.8
P. aeruginosa P2	8.3	9	10.6	8.8	9.8

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. pneumoniae control	-	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-	-
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.

Table 5. Growth Inhibition Zones (mm) Created by CFSs of *Lactobacillus helveticus* and *Lactobacillus acidophilus* Isolates

Standard strains and isolates	*L. helveticus* H1-M	*L. helveticus* H1-Y	*L. helveticus* H2-Y	*L. acidophilus* A1-M	*L. acidophilus* A1-Y	*L. acidophilus* A2-Y
E. coli control	12	10.7	11.1	10.2	14	10
E. coli E1	15.3	16.2	9.8	14.5	12	10.3
E. coli E2	10.8	11.3	11.3	10.5	15.1	10.1
E. coli E3	10.2	10.2	10.2	10	11.2	10
K. pneumoniae Control	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-
P. aeruginosa control	9.4	8.3	10.2	10.8	11	10
P. aeruginosa P1	7.8	9.2	9	10.4	11.2	10.8
P. aeruginosa P2	7.7	9.6	8.8	9.8	7.8	10.1

-, 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

and *L. lactis* showed the highest inhibitory effects on drug-resistant *Acinetobacter baumannii* (19). A study from Pakistan showed the antibacterial effect of LAB on multi-drug-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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