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Original article

ANTIBACTERIAL ACTIVITY OF LACTOBACILLI FROM BUFFALO MILK AND YOGHURT IN BANDAR-E GAZ, NORTH-WEST IRAN

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Summary

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A cross-sectional study was conducted from April to June 2016 to determine the inhibitory effect of *Lactobacillus* strains isolated from buffalo milk and yogurt in Bandar-e Gaz, Golestan province, North-West Iran. The raw milk and yogurt samples were collected and cultivated on MRS medium by anaerobical incubation at 37 °C for 48 h. The suspected colonies were identified on the basis of Gram's staining and conventional biochemical tests. The antibacterial activity of the cell-free supernatant extracted from *Lactobacillus* strains was determined using the agar well diffusion method against standard strains *Escherichia coli* ATCC 11303, *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 19115, *Listeria monocytogenes* ATCC 19111, *E. coli* and *S. aureus* isolates from local cheese samples. A total of 10 *Lactobacillus* spp. were isolated and identified as *L. plantarum*, *L. casei*, *L. acidophilus*. *L. plantarum* A1 and *L. acidophilus* R1 exhibited relatively strong inhibitory effect against *S. aureus* and *B. cereus*, respectively. Both isolates had no inhibitory effect against *L. monocytogenes. L. casei* B1 showed moderate inhibitory effects against *L. monocytogenes*. This study showed that *Lactobacilli* from buffalo dairy products had good inhibitory activity towards Gram-positive indicator organisms and were one of the best choices to control these pathogens in food products.

Key words: antibacterial activity, buffalo, *Lactobacillus*, milk, yoghurt

INTRODUCTION

In recent years, researchers in the field of food safety and regulatory agencies have faced a growing number of food-borne illness outbreaks. Overuse and misuse of antibiotics have led to the rapid emergence of antibiotic resistant bacteria. Thus, there is an urgent need to find novel and safe antibacterial substances as alternatives for antibiotics. Moreover, consumer demand for application of natural preservatives instead of chemical preservatives for safe and fresh food products is increasing day by day (Taheri *et al.*, 2011; Kasra-Kermanshahi & Mobarak-Qamsari, 2015).

Today, there is a general tendency towards use of natural preservatives in food and that is why lactic acid bacteria (LAB), due to the potential production of metabolites with antimicrobial activity, have received the attention of many researchers (Eid *et al*., 2016). They produce various compounds such as diacetyl, hydrogen peroxide, acetaldehyde, organic acids, bacteriocin and bacteriocin-like substances. Their antibacterial effects have also been attributed to the reduction in pH and competition for nutrients resources (Tesfaye, 2014). Therefore, studies relating to the antibacterial properties of these organisms to prevent, control and treat diseases and maintenaning health have become increasingly interesting (Chowdhury *et al*., 2012). In the past 20 years, antagonistic effect of LAB against many microorganisms including pathogens and spoilage organisms have been reported (Taheri *et al*., 2011; Kasra-Kermanshahi & Mobarak-Qamsari, 2015).

High amounts of LAB are present in dairy products. Many people consume different types of milk and milk products as an important source of protein (Forhad *et al*., 2015). Among them cow, sheep and goat milk are very common in Iran, whereas buffalo milk and traditionally made yoghurt from buffalo milk is more common in rural areas of the country (Hossein Alipour *et al.*, 2018). Compared to cow, sheep or goat milk, buffalo milk has a lower cholesterol content and a higher level of calcium. In addition to its nutrient content, it is also a source of antimicrobial metabolites such as lactic acid and bacteriocins (Forhad *et al.*, 2015). In Iran, most of research works have been focused on the identification of *Lactobacillus* strains in cow milk and their antibacterial activities, while buffalo milk has so far received very little attention. Therefore, the aim of this work was to isolate and identify different strains of *Lactobacillus* spp. from buffalo milk and yoghurt and determine their *in vitro* antibacterial activity against some common human pathogens.

MATERIALS AND METHODS

Isolation of Lactobacillus species

A cross-sectional study was conducted from April to June 2016 to determine the inhibitory effect of *Lactobacillus* strains isolated from buffalo milk and yogurt collected in rural areas of Bandar-e Gaz, Golestan province, North-West Iran. The raw milk and yogurt samples were collected in sterile screw capped falcon tubes and transported to the laboratory with ice packs. Ten grams of yogurt samples and 10 mL of milk samples were aseptically transferred into 100 mL physiological peptone solution (PPS). After shaking for about half an hour, 10 mL of prepared suspensions were transferred to 200 mL of MRS broth and incubated anaerobically at 37 °C for 24 h. Then, 10 mL of the MRS broth were inoculated in phosphate buffered saline buffer (PBS, pH 3) and incubated at 37 °C for 2.5 hours. The bacterial cells were harvested by centrifugation at 10,000×g for 15 min and transferred into MRS broth (Conda Pronadisa, Spain) for enrichment. After incubation for 24 h at 37 °C, the cells were diluted up to ten logarithmic (10^{10}) fold with sterile physiological saline and 1 mL of each dilution was cultured on MRS agar (Conda Pronadisa, Spain). The plates were incubated anaerobically at 37 °C for 48 h. The colonies with different morphological appearance were isolated and purified on MRS agar medium. Then, the suspected colonies were identified on the basis of Gram's staining, biochemical tests including sugar fermentation of galactose, maltose, fructose, sucrose, raffinose, sorbitol, lactose, ramnose and mannitol, along with 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 (Whitman, 2009; Forhad *et al*., 2015; Narimani & Tarinejad, 2015). Majority of the sugars were supplied by Merck, Germany with exception of maltose (Conda Pronadisa, Spain). The isolates were subcultured onto MRS agar slants which were incubated at 37 °C for 24 h and preserved in 20% glycerol (Oxoid, Canada) at –20 °C until further used.

Antibacterial activity test

The antibacterial activity of each *Lactobacillus* isolate against standard strains was investigated by well diffusion method as described by Ivanova *et al*. (2000). In this method the isolated colonies were inoculated in MRS broth and incubated for 48 h at 150 rpm at 37 °C. After incubation, the whole broth was centrifuged at 10,000×g for 15 min and the supernatant were sterilised by passage through 0.45 μm Millipore filters. The standard strains used in this study included *Escherichia coli* ATCC 11303, *Staphylococcus aureus* ATCC 29213*, Bacillus cereus* ATCC 19115 and *Listeria monocytogenes* ATCC 19111 (provided by Tehran University, Faculty of Veterinary Medicine). In addition to standard strains, *E. coli* and *S. aureus* strains isolated in our previous study on local cheese samples (Rabinejad *et al*., 2020) were also used to evaluate the antibacterial activity of the *Lactobacillus* isolates. Fifty microliters of the cell-free supernatant were placed in 5 mm diameter 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 h. Based on the diameter (mm) of the clear inhibitory zone formed around the wells, antibacterial activity was estimated (Forhad *et al*., 2015; Eid *et al*., 2016). Inhibition zones <15 mm and ≥15 mm was considered to correspond to moderate and relatively strong activity, respectively (Karami *et al*., 2017). Antimicrobial tests were done in triplicate and the mean values were recorded.

RESULTS

A total of 16 LAB strains were isolated from 10 samples (including six milk and four yogurt samples), forming round, creamy white colonies on MRS agar plate. Morphological and biochemical characteristics were employed to identify the isolates (Table 1). Among the isolates, all were Gram-positive but 10 of them were rod-shaped and the rest were coccus-shaped. In the present study, rod-shaped lactobacilli isolates were used. Based on the results of sugar fermentation and different growth conditions, lactobacilli were identified as *L. plantarum* (n=4), *L. casei* (n=2) and *L. acidophilus* (n=4). *L. plantarum* isolates were specifically detected from the buffalo milk samples whereas *L. casei* isolates were detected in the buffalo yogurt samples. *L. acidophilus* isolates were identified in both samples.

According to the results of antibacterial activity test, *L. plantarum* A1 exhibited relatively strong inhibitory effect against *S. aureus* (16.2 mm) and did not show any inhibition effects on the growth of *B. cereus* and *L. monocytogenes*. *L. casei* B1 showed moderate inhibitory effects (15 mm) against *L. monocytogenes*. *L. acidophilus* R1 had a relatively strong

Table 1. Identification of lactobacilli based on sugar fermentation and different growth conditions

	L. plantarum	*L. casei*	*L. acidophilus*
Growth at 10°C	–	–	+
Growth at 45°C	–	+	–
Growth at 6.5% NaCl	–	–	–
Galactose	+	+	+
Fructose	+	+	+
Raffinose	+	+	+
Lactose	+	+	–
Maltose	+	+	+
Sucrose	+	+	+
Sorbitol	+	+	+
Rhamnose	+	+	+
Mannitol	+	+	+

Table 2. Mean diameter of growth inhibition zones (mm) caused by cell-free supernatant of *Lactobacillus* isolates

Isolates	*E.coli*	*E.coli* ATCC 11303	*L.monocytogenes* ATCC 19111	*B. cereus* ATCC 19115	*S.aureus*	*S.aureus* ATCC 29213
L. plantarum A1	12.4	12.3	–	–	16	16.2
L. plantarum A2	–	–	11.2	10.2	11	11
L. plantarum A3	10.3	10.5	9.8	9.5	12.1	12.3
L. plantarum A4	11	11.2	–	11.2	10.2	10.3
L. casei B1	9	9	15	13.2	11.1	11
L. casei B2	10	9.8	11	–	12	12
L. acidophilus R1	10.1	10.3	–	16.5	11.3	11.5
L. acidophilus R2	–	–	10.4	11.2	10.2	10.3
L. acidophilus R3	9.5	9.2	–	12	11.1	11.2
L. acidophilus R4	–	–	10.3	12.3	11.1	11.3

inhibitory effect on the growth of *B. cereus* (16.5 mm) but exhibited no inhibitory effect against *L. monocytogenes*. The mean diameters of growth inhibition zones (mm) are given in Table 2. Also, there was no difference between mean diameters of growth inhibition zones of the cell-free supernatants of all *Lactobacillus*isolates against the standard strains or isolates from the tradition cheese samples.

DISCUSSION

The study was designed for identification of *Lactobacillus* spp. from buffalo milk and yoghurt samples and assessment of their antibacterial activity against some human pathogenic bacteria. Based on the morphological characteristics, 10 isolates from the samples were identified as *Lactobacillus* spp. The isolated bacteria were non-spore forming Gram-positive rod-shaped facultative anaerobes indicating them as members of *Lactobacillus* spp. Based on the results, they were identified as *L. plantarum, L. casei* and *L. acidophilus*.

In this study, the cell-free supernatants of 3 *Lactobacillus* isolates showed good inhibitory effect against the tested pathogenic bacteria. In a study in Bangladesh, Forhad *et al.* (2015) isolated a total of four strains including *L. fermentum*, *L. casei*, *L. acidophilus* and *Bifidobacterium longum* from buffalo milk. Eid *et al.* (2016) also isolated *L. fermentum*, *L. acidophilus* and *L. pentosus* from buffalo milk; among them *L. pentosus* had the highest antibacterial activity against the indicator organisms. In another study, four *L. plantarum* that inhibited growth of test pathogens to some extent were isolated but maximum and minimum zones of inhibition were observed against *Bacillus cereus* and *Staphylococcus aureus*, respectively (Chowdhury *et al*., 2012). In the study by Naeemi *et al.* (2019) *L. plantarum* was the commonest lactic acid bacterium from bovine colostrum that showed the highest antibacterial activity against the test organisms. In the study of Koohsari *et al.* (2019) *L. casei* had the highest frequency in traditional dairy products followed by *L. acidophilus* in Gorgan (North-east of Iran). In a similar study in Jahrom, *L. casei*, *L. acidophilus* and *L. plantarum* were the most common lactobacilli (Dorri *et al*., 2013).

Farahbakhsh *et al.* (2013) evaluated the antibacterial activity of lactobacilli isolated from traditional yogurt in Rafsanjan (South of Iran). Among the isolates, the greatest antibacterial activity was observed for *L. plantarum*. In a study conducted by Hossein Alipour *et al.* (2018) *L. salivarius* isolated from buffalo milk had the strongest and the least inhibitory effect on *S. aureus* and *S. typhimurium*, respectively. The antagonistic activity of LAB isolated from traditional dairy products against *E. coli* O157:H7 was investigated (Rahimpour Hesari *et al*., 2017). The isolates included *L. plantarum* and *L. fermentum*; the antagonistic activity of *L. plantarum* was greater than that of *L. fermentum*. A study from Nepal showed antibacterial effect of lactobacilli isolated from dairy products against *E. coli*, *Salmonella paratyphi*, *Salmonella typhi*, *Pseudomonas* spp., *S. aureus*, *Proteus* spp., *Acinetobacter* and no inhibitory effects against *Klebsiella pneumoniae* and *Shigella* spp. (Saud *et al*., 2020). In a study in Ahvaz, lactobacilli including *L. alimentarius*, *L. sake* and *L. collinoides* isolated from traditional dairy samples showed moderate activity (inhibition zone <15 mm) against *S. aureus* ATCC 6538, *Bacillus subtilis* ATCC 12711, and *P. aeruginosa* ATCC 27853 except for *L. collinoides* and *L. alimentarius* that had relatively strong activity (inhibition zone ≥15 mm) against *P. aeruginosa* and *Bacillus subtilis*, respectively (Karami *et al*., 2017). Iranmanesh *et al.* (2012) isolated LAB from ewe milk, traditional yoghurt and sour buttermilk. Among the isolates, *Pediococcus acidilactici* had a great antibacterial activity against *L. monocytogenes*, *S. aureus* and *Salmonella enteritidis*. Slozilova *et al.* (2014) examined the anti-listerial activity of six individual LAB strains (*Lactococus lactis* subsp. lactis CCDM 416 and NIZO R5, *L. plantarum* HV 11 and DC 1246, *P. acidilactici* HV 12, and *Enterococcus mundtii* CCM 1282) and one starter culture (DELVO-ADD 100-X DSF). The strains were found effective in the suppression of at least one studied *L. monocytogenes* strains. In a later study, *L. plantarum* and *Lactococcus piscium* were the commonest probiotic isolates from goat milk. The highest inhibitory effects against drug-resistant *Acinetobacter baumannii* was exhibited by *L. lactis* (Fozouni *et al.*, 2019). Sikarchi *et al.* (2018) studied inhibitory effects of probiotic bacteria from camel milk on clinical isolates of drug-resistant *Helicobacter pylori*. Among the isolates, *Lactobacillus plantarum* had the highest abundance. *L. plantarum, L. fermentum* and *L. casei* showed satisfactory inhibitory effects against the *H. pylori* isolates, but *L. plantarum* with inhibition zone of 20.3 mm exhibited the highest inhibitory effect. A study from Pakistan showed antibacterial effecst of LAB against multi-drug-resistant uropathogens, viz. *Candida albicans*, *P. aeruginosa*, *K. pneumoniae*, *Enterococcus fecalis*, 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 significant inhibitory activities against *E. coli* and *E. faecalis*, with a growth inhibition zone up to 28 mm (Manzoor *et al.*, 2016). Among the LAB isolates from yoghurt, *L. casei* and *L. lactis* showed better inhibitory effects against pathogenic bacteria. The maximum and minimum inhibitory effect was observed in *Yersinia enterocolitica* and *B. cereus* (Kiaie *et al.*, 2006). Kazemi Darsnaki *et al.* (2010) isolated six LAB from yogurt and probiotic pills among which the highest antibacterial activity was observed for *L. acidophilus* against *B. cereus*. In a study from Egypt, *Lactobacills paracasei* and *L. helveticus* exhibited the highest antagonistic activity against the tested pathogens followed by *L. fermentum*, while *Bifidobacterium longum* and *L. lactis* subsp. *lactis* showed weak or no activity against the tested strains (Gad *et al.*, 2016).

Considering the results of all studies including the present research, LAB from dairy products demonstrated inhibitory activity towards Gram positive and Gram negative indicator organisms. Among the LAB, lactobacilli with considerable good antagonistic activity against the most important pathogens were shown to be one of the best choices for their control. Hence, they have a great potential for application in the food industry to prevent growth of food borne pathogens in food products and for control of diseases.

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