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

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Summary


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 anaerobic 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 extract 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 maintaining 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 yoghurt collected in rural areas of Bandar-e Gaz, Golestan province, North-West Iran. The raw milk and yoghurt 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, rhamnose 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 sub-cultured 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 10⁶ 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
Antibacterial activity of lactobacilli from buffalo milk and yoghurt in Bandar-e Gaz, North-West Iran

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

<table>
<thead>
<tr>
<th></th>
<th>L. plantarum</th>
<th>L. casei</th>
<th>L. acidophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 10°C</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 45°C</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Growth at 6.5% NaCl</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>E.coli ATCC 11303</th>
<th>E.coli ATCC 19111</th>
<th>L.monocytogenes ATCC 19115</th>
<th>B. cereus ATCC 19115</th>
<th>S.aureus ATCC 29213</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum A1</td>
<td>12.4</td>
<td>12.3</td>
<td>−</td>
<td>−</td>
<td>16</td>
</tr>
<tr>
<td>L. plantarum A2</td>
<td>−</td>
<td>−</td>
<td>11.2</td>
<td>10.2</td>
<td>11</td>
</tr>
<tr>
<td>L. plantarum A3</td>
<td>10.3</td>
<td>10.5</td>
<td>9.8</td>
<td>9.5</td>
<td>12.1</td>
</tr>
<tr>
<td>L. plantarum A4</td>
<td>11</td>
<td>11.2</td>
<td>−</td>
<td>11.2</td>
<td>10.2</td>
</tr>
<tr>
<td>L. casei B1</td>
<td>9</td>
<td>9</td>
<td>15</td>
<td>13.2</td>
<td>11.1</td>
</tr>
<tr>
<td>L. casei B2</td>
<td>10</td>
<td>9.8</td>
<td>11</td>
<td>−</td>
<td>12</td>
</tr>
<tr>
<td>L. acidophilus R1</td>
<td>10.1</td>
<td>10.3</td>
<td>−</td>
<td>16.5</td>
<td>11.3</td>
</tr>
<tr>
<td>L. acidophilus R2</td>
<td>−</td>
<td>−</td>
<td>10.4</td>
<td>11.2</td>
<td>10.2</td>
</tr>
<tr>
<td>L. acidophilus R3</td>
<td>9.5</td>
<td>9.2</td>
<td>−</td>
<td>12</td>
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<tr>
<td>L. acidophilus R4</td>
<td>−</td>
<td>−</td>
<td>10.3</td>
<td>12.3</td>
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</tr>
</tbody>
</table>

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 Naemi 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 Kohosari 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 (Lactococcus 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 effect 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, Lactobacillus 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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