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Antimicrobial Activity of *Lactobacillus* spp. Isolated From Fecal Flora of Healthy Breast-Fed Infants Against Diarrheagenic *Escherichia coli*

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Abstract

Background: Among the enteric pathogens, diarrheagenic *Escherichia coli* are important causes of diarrhea in children in both developing and industrialized countries. Some *Lactobacillus* species are commonly used as probiotics, with effects especially against acute diarrhea in childhood.

Objectives: The aim of this study was to explore antimicrobial activity of *Lactobacillus* strains isolated from fecal flora of healthy breast-fed infants against five diarrheagenic *E. coli* pathotypes such as enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC) enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC).

Materials and Methods: Fecal samples were collected from seven healthy breast-fed infants between 1 to 18 months of age in Tehran city, Iran. Identification of *Lactobacillus* isolates was performed by biochemical and 16S rRNA gene sequencing methods. An agar well diffusion assay was used for detection of antimicrobial activity of *Lactobacillus* isolates against five diarrheagenic *E. coli* pathotypes.

Results: A total of 20 *Lactobacillus* isolates were identified from stool samples. *Lactobacillus fermentum* was the most frequently isolated strain, followed by *L. plantarum* and *L. rhamnosus*. Seven *Lactobacillus* strains including *L. fermentum* (four isolates), *L. paracasei* (one isolate), *L. plantarum* (one isolate) and *L. rhamnosus* (one isolate) had a mild inhibitory activity against diarrheagenic *E. coli.* The mechanism of inhibitory activity of *Lactobacillus* strains appeared to be due to the production of organic acids or hydrogen peroxide.

Conclusions: Our findings show that *Lactobacillus* strains with human origin had a mild inhibitory activity against the diarrheagenic *E. coli*, and these strains may be useful as probiotic candidates in prevention of intestinal infections caused by diarrheagenic *E. coli*.

Keywords: Infant, inhibition, *Escherichia coli*, *Lactobacillus*

1. Background

Diarrheal disease is one of the leading causes of morbidity and mortality of infants in the developing world (1). A wide variety of microorganisms including bacteria, viruses, and parasites are the etiological agents of diarrhea (2). Among the bacteria, diarrheagenic *Escherichia coli* strains are most frequently associated with diarrhea in children in developing countries (3, 4). Diarrheagenic *E. coli* have been categorized to five major pathotypes: enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC) enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) (2).

Probiotic bacteria are defined as live microorganisms which when used in adequate amounts, have beneficial effects to the health of host (5). The emergence of bacterial pathogens with high resistance to antibiotics has caused scientists to suggest alternative disease prevention strategies such as the use of probiotics (6, 7). Some recent studies have documented the role of *Lactobacillus* in prevention and treatment of diarrheal infections caused by *Shigella* and *Salmonella* (8, 9). *Lactobacillus* species are found in plants, dairy products, as well as mouth, intestinal tract, and vagina of humans and many animals as normal flora (10, 11). Some studies reported that lactobacilli and bifidobacteria are dominant flora in the breast-fed infants (12). In separate studies, the antimicrobial activity of probiotic lactobacilli against diarrheagenic *E. coli* such as ETEC (13), EPEC (14), EHEC

(15), EIEC (16), and EAEC (17) has been previously studied, but no study has investigated the antimicrobial activity of probiotic lactobacilli against all diarrheagenic *E. coli* pathotypes together.

2. Objectives

For this reason, we decided to explore antimicrobial activity of *Lactobacillus* strains isolated from fecal flora of healthy breast-fed infants against five diarrheagenic *E. coli* pathotypes including EAEC, ETEC, EHEC, EPEC and EIEC.

3. Materials and Methods

3.1. Sample Collection

The study was conducted from April to December 2014. Fecal samples were collected from seven healthy breast-fed infants between 1 to 18 months of age at Farman Farmaian Health Care Center in Tehran city, Iran. The research ethics committee of Tehran university of medical sciences approved our study (No: 240.982) and informed parental consent was obtained.

3.2. Bacterial Isolation and Biochemical Identification

About one gram of each sample was cultured in Man, Rogosa, Sharpe broth (MRS broth, Scharlau, Spain) and incubated under anaerobic condition at 37°C for 48 - 72 hours, then was subcultured on MRS agar (Scharlau, Spain) plates and incubated under anaerobic condition at 37°C for 48 hours. Three to four colonies of each culture were selected for further characterization. Suspected colonies were tested by Gram stain and for catalase, fermentation of carbohydrates, hydrolysis of arginine, gas (CO_2) production from glucose and growth at different temperatures (15°C , 45°C) (18-20).

3.3. Polymerase Chain Reaction Amplification

Total chromosomal DNA was extracted according to a previously described method (21). In the present study, bacterial universal 16S primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1522R (5′-GCAGCAGTAGGGAATCTTC-3′) (Bioneer, Korea) were used for identification of *Lactobacillus* isolates at the species level. 16S rRNA gene PCR was performed as described previously (22). The PCR products were sequenced (Bioneer, Korea) and the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the determined sequences with the sequences deposited in NCBI GenBank. *L. acidophilus* ATCC 4356 was used as positive control.

3.4. Antimicrobial Activity

Antimicrobial activity was carried out according to the agar well diffusion assay as described previously (23). Diarrheagenic *E. coli* were cultured in Luria broth (Scharlau, Spain) for 24 hours, and then microbial density was adjusted to 107 CFU/mL and cultured on nutrition agar. *Lactobacillus* isolates were grown in MRS broth for 20 hours. Cell free culture supernatants (CFCS) were obtained by centrifuging the culture broth at 10000 g for 10 minutes and 100 μL of the CFCS was placed into the wells of the nutrition agar and the nutrition agar plates were incubated at 37°C for 14 - 15 hours. The diameter of the clear zones around each well was measured. *Lactobacillus* isolates with clear zones less than 11 mm, 11 to 16 mm, 17 to 22 mm and more than 23 mm were grouped as negative (−), mild (+), strong (++), and very strong (+++) inhibitor isolates, respectively. The *L. rhamnosus* GG was used as positive control and sterile MRS broth was used as negative control. The antimicrobial activity was tested against references strains, including EAEC 042, ETEC H10407, EHEC O157:H7 EDL933, EPEC E2348/69 and EIEC 4608-58.

Two major mechanisms of antimicrobial activity are production of organic acids, which reduce the pH and the production of hydrogen peroxide. The production of bacteriocins may be another mechanism of antimicrobial activity (24). For these reasons, the pH of CFCS was measured and changed to 6.5 with NaOH (Merck, Germany, 2.5M) and then catalase (1 mg/mL, Sigma-Aldrich, Germany) was added to CFCS and incubated at 25°C for 1 hour. The stability of the inhibitory activity of *Lactobacillus* isolates was also investigated by a heat-treatment (100°C, 15 minutes) and also by enzymatic treatment of the CFCS. In enzymatic treatment assay, three proteolytic enzymes including trypsin (Sigma-Aldrich, Germany), pepsin (Sigma-Aldrich, Germany) and proteinase K (Sigma-Aldrich, Germany) were added to the CFCS with final concentrations of 200 mg/mL, 200 mg/ml and 1 mg/mL, respectively. The CFCS was incubated at 37 °C for 1 hour before the agar well diffusion assay (25).

4. Results

4.1. Isolation and Identification of Lactobacillus Species

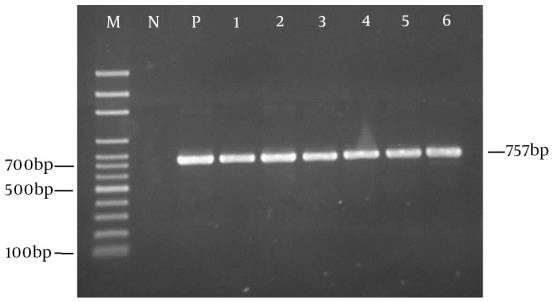
A total of 20 colonies were isolated from 7 stool samples (2 - 3 colonies per sample). All the isolates were Gram positive and catalase negative. Primary identification of 20 *Lactobacillus* isolates was performed on the basis of biochemical profiles (carbohydrate fermentations, arginine hydrolysis, CO_2 production and growth at different temperatures). Finally, the identification of the isolates was confirmed by sequencing of 16S rRNA gene (Figure 1). According to 16S rRNA sequencing, *L. fermentum* was the most frequently isolated species (10 isolates), followed by *L. plantarum* (3 isolates), *L. rhamno-*

sus (3 isolates), *L. paracasei* (2 isolates), *L. acidophilus* (1 isolate) and *L. brevis* (1 isolate).

4.2. Antimicrobial Activity

Antimicrobial activity assay showed that seven *Lactobacillus* isolates (35%) had inhibitory activity against diarrheagenic *E. coli* (Table 1). *L. fermentum* S1 and *L. fermentum* S2 were obtained from a boy at the age of thirty-four days. *L. fermentum* S16 was isolated from a girl with four months of age. *L. fermentum* S8 was isolated from a boy with eight months of age. *L. paracasei* S14 was isolated from a girl at the age of three days. *L. plantarum* S17 was isolated from a girl at twelve months of age and *L. rhamnosus* S19 was isolated from a boy at ten months of age.

Figure 1. 16S RNA Gene Polymerase Chain Reaction Products (757 bp)



Lane M, 100 bp DNA marker (Sinaclon, Iran); Lane N, Negative control; Lane P, Positive control (*L. acidophilus* ATCC 4356); Lanes 1 - 6, *Lactobacillus* isolates. For the negative control PCR was conducted without adding DNA.

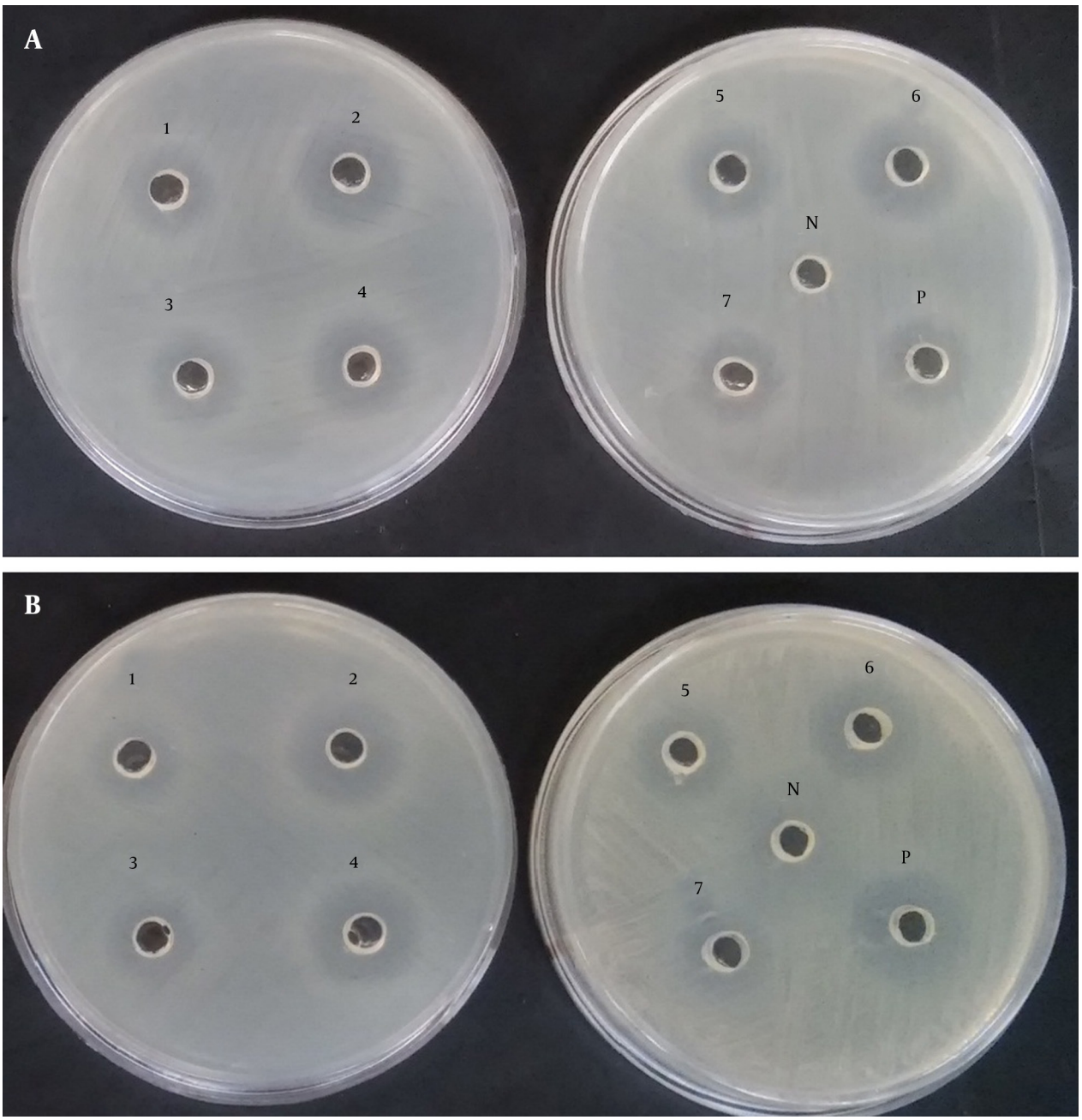
Table 1. Antimicrobial Activity of Cell Free Culture Supernatants of the *Lactobacillus* Isolates Against Diarrheagenic *Escherichia coli*[a]

CFCS of the *Lactobacillus* Isolates	ETEC H10407	EAEC 042	EHEC EDL933	EIEC 4608-58	EPEC E2348/69
CFCS with no treatment					
L. rhamnosus GG	+[b]	+	+	+	+
L. fermentum S1	+	+	+	+	+
L. fermentum S2	+	+	+	-	+
L. fermentum S8	+	+	+	+	+
L. fermentum S16	+	+	+	+	+
L. paracasei S14	+	-	+	-	-
L. plantarum S17	+	+	+	+	+
L. rhamnosus S19	+	+	+	+	+
Heat (100°C, 15 min) or enzymatic treatment					
L. rhamnosus GG	+	+	+	+	+
L. fermentum S1	+	+	+	+	+
L. fermentum S2	+	+	+	-	+
L. fermentum S8	+	+	+	+	+
L. fermentum S16	+	+	+	+	+
L. paracasei S14	+	-	+	-	-
L. plantarum S17	+	+	+	+	+
L. rhamnosus S19	+	+	+	+	+
CFCS adjusted pH 6.5 and catalase (1 mg/mL)					
L. rhamnosus GG	-	-	-	-	-
L. fermentum S1	-	-	-	-	-
L. fermentum S2	-	-	-	-	-
L. fermentum S8	-	-	-	-	-
L. fermentum S16	-	-	-	-	-
L. paracasei S14	-	-	-	-	-
L. plantarum S17	-	-	-	-	-
L. rhamnosus S19	-	-	-	-	-

[a] Abbreviation: CFCS, cell free culture supernatants.
[b] Interpretation of zone diameter of inhibition: –, less than 11 mm; +, 11 - 16 mm; ++, 17 - 22 mm; and +++, more than 23 mm.

Our *Lacobacillus* isolates, like positive control strain, had a mild inhibitory activity against the diarrheagenic *E. coli*. Cell free culture supernatants of all the *Lactobacillus* isolates with no treatment inhibited the growth of both ETEC H10407 and EHEC O157:H7 EDL933 (Figure 2). Among the seven *Lactobacillus* isolates, five isolates including three *L. fermentum* (S1, S8 and S16), *L. plantarum* S17 and *L. rhamnosus* S19 inhibited all the diarrheagenic *E. coli*. Heat-treatment or enzymatic treatment (trypsin, pepsin, proteinase K) of the CFCS did not affect the antimicrobial activity of *Lactobacillus* isolates, but when the CFCS was adjusted to pH 6.5 and treated with catalase, the antimicrobial activity of the CFCS was disappeared.

Figure 2. Antimicrobial Activity of Cell Free Culture Supernatants of *Lactobacillus* Isolates



A, Antimicrobial activity with no treatment against ETEC H10407; B, EHEC O157:H7 EDL933. Well 1, *L. fermentum* S1; Well 2, *L. fermentum* S2; Well 3, *L. fermentum* S8; Well 4, *L. fermentum* S16; Well 5, *L. paracasei* S14; Well 6, *L. plantarum* S17; Well 7, *L. rhamnosus* S19; Well P, positive control (*L. rhamnosus* GG); Well N, negative control (sterile MRS broth).

5. Discussion

Among the enteric pathogens, diarrheagenic *E. coli* are important causes of diarrhea in both developing and industrialized countries (2). Increasing antimicrobial resistance among the diarrheagenic *E. coli* has been reported in several studies (26, 27). Although antibiotics are useful in a wide variety of bacterial infections, emergence of antibiotic resistant bacteria necessitates the development of novel therapeutic and preventive approaches (28, 29). The prevention and treatment of bacterial infections with probiotics is an interesting field of current biomedical research. *Lactobacillus* strains with probiotic potential are used in food industry and also used as biotherapeutic agents (9).

In the present study, the most frequently isolated *Lactobacillus* species from fecal flora of infants were *L. fermentum*, followed by *L. plantarum* and *L. rhamnosus*. Arici et al. (30) reported that the most frequently isolated *Lactobacillus* species in the feces of infants and children less than 2 years of age are *L. rhamnosus*, *L. paracasei* and *L. fermentum*, respectively. In our study, in accordance with Arici et al. (30) *L. rhamnosus* and *L. fermentum* were isolated from infant faces, but in Arici et al. study (30) *L. rhamnosus* was the most recovered species. In another study by Mirlohi et al. (31) the *L. acidophilus* and *L .plantarum* were the most recovered species from the feces of healthy infants between 1 to 19 months. Variations in lactobacilli flora in infant feces may be due to differences in feeding (breast or formula) and the geographical zone. Also, variations in methodology may account for the differences, since identification of lactobacilli by traditional biochemical methods is very difficult (32, 33).

In our study, four *Lactobacillus* species including *L. fermentum*, *L. paracasei*, *L. plantarum* and *L. rhamnosus* had antimicrobial activity against diarrheagenic *E. coli*. In previous studies by Tsai et al. (34) and Lin et al. (17) have been previously reported that *L .acidophilus* RY2, *L. salivarius* MM1 and *L. paracasei* En4 isolated from healthy infant feces significantly inhibit the growth of EAEC and ETEC. The *L. paracasei* En4 in these studies, in accordance with our study had antimicrobial activity against EAEC and ETEC. Also, in another study by Michail and Abernathy (35), antimicrobial activity of *L. plantarum* 299v strain with intestinal flora origin against EPEC has been shown. Other *Lactobacillus* species with intestinal flora origin, such as *L. acidophilus* LB (36), *L. casei* DN-114 001 (14), *L. fermentum* (9) and *L. helveticus* R0052 (37) have been previously shown to inhibit infection by diarrheagenic *E. coli* such as EHEC or EPEC within the intestinal epithelial barrier.

When the CFCS of our *Lactobacillus* was adjusted to pH 6.5 and treated with catalase, the antimicrobial activity was disappeared, but heating or protease treatment did not destroy the antimicrobial activity of CFCS. However, some bacteriocins may be resistant to heat, these findings suggest that the production of bacteriocins or bacteriocin-like compounds did not involve in the mechanism of antimicrobial activity. The inhibition of diarrheagenic *E. coli* growth appeared to be due to the production of organic acids or hydrogen peroxide produced by the *Lactobacillus* strains. Several studies have reported that a pH-dependent mechanism was involved in the antimicrobial activity of *Lactobacillus* strains (38, 39).

In conclusion, our findings suggest that *Lactobacillus* strains with human origin have a mild inhibitory activity against the diarrheagenic *E. coli* and these strains may be useful as probiotic candidates in prevention of intestinal infections caused by diarrheagenic *E. coli*.

Footnotes

Authors' Contribution: Abolfazl Davoodabadi: concept and design of the study and doing experiments; Mohammad Mehdi Soltan Dallal: concept and design of the study, critical revision, final approval of the study, and obtaining funding for the study; Elahe Lashani: doing experiments; Maryam Tajabadi Ebrahimi: analysis and interpretation of data, critical revision and administrative and technical or logistical support.

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