



Antagonistic effect of isolated probiotic bacteria from natural sources against intestinal *Escherichia coli* pathotypes

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Abstract

Background: Probiotics are live microorganisms which are beneficial bacteria that are normal flora of the digestive system which, in determined amounts, show beneficial effects on host health, and can balance gastrointestinal microflora. Digestive tract diseases such as diarrhea are one of the major causes of child mortality in developing countries. Different pathotypes of *Escherichia coli* cause diarrhea that affects children, therefore reduction of these colonization strains in humans or animals can decline gastrointestinal disorders such as diarrhea.

Objective: The aim of this study was to determine the antimicrobial effect of probiotic bacterial strains isolated from different natural sources against 4 pathotypes of pathogenic *E. coli* using disk and well diffusion methods.

Methods: This cross-sectional study was conducted from December 2013 to July 2014 on Martyr Chamran University in Ahwaz city. A total of 13 probiotic colonies isolated from 20 samples of traditional dairy products including yogurt, cheese and milk, and 20 samples of vegetables including carrots and cabbages (red and white), of which 5 isolates were selected to determine the antimicrobial effect against 4 *Escherichia coli* pathotypes, randomly. The antimicrobial effect was evaluated using two methods: disk diffusion and well diffusion tests and measuring growth inhibition zones of probiotics against 4 pathotypes of pathogenic *E. coli*.

Results: Obtained results showed growth inhibition effects of all 5 probiotic strains against *Escherichia coli* pathotypes in both used methods. But in comparison *Lactobacillus plantarum* had higher growth inhibitory effects in both methods.

Conclusion: results of this study demonstrated high antimicrobial effect of probiotic bacteria against pathogenic *Escherichia coli* strains. It indicated a positive and beneficial role of probiotics in human health and prevention of illness.

Keywords: Probiotics, Antimicrobial effects, Pathogenic strains, Intestinal infections, *Escherichia coli*

1. Introduction

There are many reasons for the relationship between lifestyle and inducing most diseases and infections. In the past centuries, changing in living conditions with reduced physical activity, stresses of modern life, artificial nutrition and a normal life have increased susceptibility to infectious diseases in humans. Widespread use of synthetic antimicrobial agents caused resistance of infectious agents to these compounds (1-3). For this reason, prevention and treatment by natural antimicrobials appear to be essential. One of the vulnerable parts of the body to pathogens, is the digestive system. Different pathotypes of *Escherichia coli* are common pathogens of gastrointestinal tract diseases (1, 2). Use of antibiotics for treatment and prevention of this bacteria not only induces resistance to these

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compounds, but also causes disturbance of the normal flora of the digestive system leaving the body vulnerable to a variety of intestinal diseases such as diarrhea. Oral consumption of beneficial microorganisms such probiotics as dietary supplement can immunize people against pathogens (1, 4). Probiotics are live microorganisms that, in determined amounts, show beneficial effects on host health, and can balance gastrointestinal microflora (5). They promote the growth and balance of useful bacteria, reducing pathogenic microorganisms and therefore reduce gastrointestinal diseases (6). They are found in dry foods and different natural resources, and can be used in the treatment and prevention of many infectious diseases (2, 4). In fact, one of the beneficial effects of probiotics is to prevent and reduce gastrointestinal infectious diseases in the host (3). Lactobacillus, Enterococcus, Bifidobacterium, Saccharomyces, Clostridium, and Bacillus are the main genera of probiotics. Several mechanisms are used by probiotics to inhibit growth and multiplication of intestinal pathogens including production of antimicrobial substances, production of organic acids, activation of body's immune system, competition with pathogens in getting food and attachment to host cell's receptors (2, 3). *E. coli* pathotypes are one of these pathogens (7). Digestive tract disease such as diarrhea is one of the major causes of child mortality in developing countries. It's estimated that 3 to 5 million diarrhea cases have resulted in death. Different pathotypes of *E. coli* cause diarrhea that affect children. These pathotypes are divided into 6 categories based on different attributes (8). Several studies have shown that probiotics can inhibit growth of *Staphylococcus aureus*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Enterobacter spp.*, *Vibrio cholerae* and *Helicobacter pylori*, by competition and prevention of their attachment and colonization to host's intestinal cells, after taking orally (9,10). The aim of this study was to determine the antimicrobial effect of probiotic bacterial strains isolated from different natural sources against 4 pathotypes of pathogenic *E. coli* using disk and well diffusion methods.

2. Material and Methods

2.1. Isolation and identification of bacteria

This cross-sectional study was conducted from December 2013 to July 2014 in Ahwaz city. To separate probiotic bacteria, 20 samples of traditional dairy products including yogurt, cheese, milk and 20 samples of vegetables including carrots and cabbages (red and white), were collected in May 2014 from different areas of Ahwaz city as well as 10 samples of Tarkhineh (traditional food rich of variety of specific probiotic) which were collected from Khorramabad (West of Iran). MRS Broth and MRS Agar mediums (Merck, Germany) were used for the separation of prepared samples. At first, 1 g of each sample (dairy, vegetables, Tarkhineh) were crushed in a sterile mortar and weighed, dissolved in 10 ml MRS Broth medium and incubated at 37 °C, then shaken at 140 rpm for 24 hours (11). Serial dilution was prepared (to 10⁻³), further purification was performed under sterile conditions by culturing in MRS Agar medium. All isolated colonies were identified using biochemical tests (gram staining, catalase test, oxidase test, growth at different temperatures and carbohydrate fermentations, motility) and molecular assessment (polymerase chain reaction test) (12). General primers were used to perform PCR test (Takapoozist, Iran). Primer's sequencing was shown in Table 1. To confirm genome replication and determine fragment size, PCR protocol was followed on agarose gel. Gel extraction kit was used for extraction of 1500-bp single bands. To determine sequencing, purified genoms were sent to the Takapoozist company. The results were arranged using BioEdit software and was evaluated in the National Center for Biotechnology Information (NCBI) website using Blast software. Phylogenetic trees of identified isolates were drawn using mega-4 software.

Table 1. Selected primers for polymerase chain reaction

Primers type	Primers sequences (5' to 3')	Product size (bp)
Forward primer (FP1)	5'-AGAGTTTGATCATGGCTCAG-3'	20 bases
Reverse primer (RP1)	5'-TACGGYTACCTTGTTACGACTT-3'	22 bases

2.2. Supernatant preparing of bacteria

After isolation and molecular identification of isolates, 5 strains of probiotic bacteria were randomly selected. All selected isolates were cultured in MRS Broth, and incubated at 37 °C for 24 hours to obtain 0.5 McFarland turbidity (1.5×10⁸ cfu/ml). Bacterial supernatant was prepared by centrifuge of isolates culture suspension for 25 min at 4 °C and 3500 rpm. Four pathogenic strains of *E. coli* including ETEC (Enterotoxigenic *E. coli*), EAEC (Enteroaggregative *E. coli*), EIEC (Enteroinvasive *E. coli*) and EPEC (Enteropathogenic *E. coli*) were provided of microbiology department of Tarbiat Modares University in Tehran. TSB medium was used for culturing strains at 37 °C.

2.3. Evaluation of antimicrobial activity of probiotic bacteria

Mueller-Hinton agar (MHA) was used to investigate antimicrobial activity of isolated probiotic bacteria. For this purpose, inhibitory and antagonistic effects of probiotic bacteria on 4 pathotype of *E. coli* was determined using two methods: Disk Diffusion Agar and Well Diffusion Agar methods. All tests were repeated twice.

2.4. Disk Diffusion Agar method

Sterile blank disks (8 mm diameter) were smeared with supernatant of 5 probiotic bacteria for 5 minutes. In order to dry the disks, they were placed at 37 °C for 15 minutes. The suspension of pathogenic bacteria was spread on plates containing MHA medium. Impregnated discs with supernatant were placed on MHA with a 2.5 cm distance. All plates were incubated at 37 °C and after for 24 hours' growth, inhibition zone of probiotics against pathotypes of *E. coli* were measured by a ruler.

2.5. Well Diffusion Agar method

Suspension of pathogen bacteria cultured in TSB were spread on plates containing MHA, then 10µl of each probiotic supernatant was spilled to punched wells in the plates. After incubation at 37 °C for 24 hours' growth, inhibition zone was measured (13). In comparison, antimicrobial effects of 5 antibiotics on 4 pathotype *E. coli* were investigated including: nalidixic acid, penicillin, cephalosporin, gentamicin and novobiocin. For this purpose, the suspension of pathogenic bacteria was spread on TSA plates and antibiotic discs were placed with a distance (2.5 cm) on them. After incubation at 37 °C for 24 hours' growth, inhibition zone were measured with a ruler.

3. Results

A total of 13 probiotic colonies isolated from samples which 5 isolates were selected to evaluate the antimicrobial effect against 4 *E. coli* pathotypes, randomly, were subjected to biochemical and molecular identification. Identified probiotics strains were *Lactobacillus plantarum*, *Lactobacillus Gasseri*, *Enterococcus faecium*, *Bacillus subtilis*, *Weissella paramesenteroides* (Figure 1). Obtained results showed growth inhibition effects of all 5 probiotic strains against *E. coli* pathotypes in both used methods. Average growth inhibition zone of 5 probiotic strain against 4 *E. coli* pathotypes in disk diffusion and well diffusion methods were 9-12 mm and 6-9 mm, respectively. In comparison, inhibition zones in disk diffusion were considerable. Results of growth inhibition zones of two disk diffusion and well diffusion methods were shown in Tables 2 and 3. Inhibition zone of used antibiotics against 4 pathotypes of *E. coli* was shown in Table 4. Results showed nalidixic acid that is commonly used to treat urinary tract infections, had significant inhibitory effect on the pathotype of ETEC (18 mm), EIEC (20 mm), EAEC (20 mm), but did not show inhibitory effects against EPEC strains.

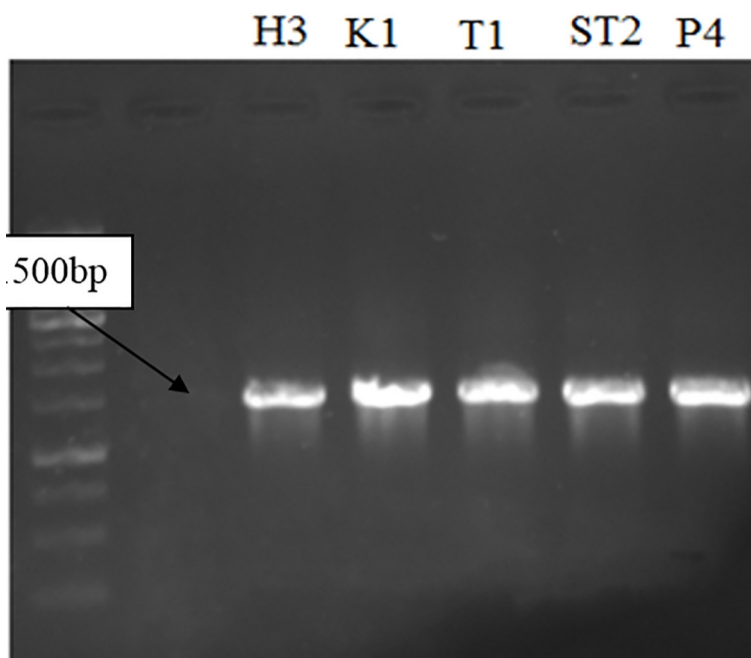


Figure 1. Electrophoresis pattern of PCR products of selected probiotic bacteria

Table 2. Growth Inhibition zone (mm) of selected probiotic strains against 4 pathotypes of *E. coli* (Disk diffusion method)

Probiotic	Pathogen			
	EAEC	EIEC	ETEC	EPEC
<i>L. plantarum</i>	10	9	12	10
<i>L. gasseri</i>	9	9	10	9
<i>E. fascium</i>	10	9	9	9
<i>B. subtilis</i>	-	-	-	10
<i>W. paramesenteroides</i>	10	9	10	-

Table 3. Growth Inhibition zone (mm) of selected probiotic strains against 4 pathotypes of *E. coli* (well diffusion method)

Probiotic	Pathogen			
	EAEC	EIEC	ETEC	EPEC
<i>L. plantarum</i>	7	9	-	9
<i>L. gasseri</i>	-	-	6	-
<i>E. fascium</i>	-	-	-	-
<i>B. subtilis</i>	6	-	-	9
<i>W. paramesenteroides</i>	7	-	-	-

Table 4. Growth inhibitory zone of selected antibiotics against 4 pathotypes of *E. coli* (Antibiogram)

Antibiotic	Pathogen			
	EAEC	EIEC	ETEC	EPEC
Novobiocin	-	-	-	10
Penicillin	-	-	-	-
Nalidixic acid	20	20	18	-
Cephalosporins	-	-	-	-
Gentamycin	15	-	-	16

4. Discussion

According to obtained results of this study selected probiotics, particularly Lactobacillus strains, had good inhibitory effects against *E. coli* pathotypes. Probiotics such as Lactobacillus genera are natural inhabitants of the intestine that play an important role in human health by avoiding intestinal infection and pathogens elimination. They produce lactic acid and other organic acids that reduce intestinal PH as well as various metabolites to prevent the growth of many pathogenic bacteria (14). In this study, produced metabolites by selected probiotics which were isolated by centrifugation were able to prevent the growth of pathogenic pathotypes of *E. coli*. Isolated Lactobacilli showed the highest inhibitory effect in both methods. Similar results were obtained in another study. Boris and coworkers showed inhibitory effects of isolated Lactobacilli of dairy products against a range of pathogens (15). Coconnier investigated Lactobacillus acidophilus inhibitory effects against pathogen bacteria (16). In the history of microbiology, most human studies are focused on human and animal pathogenic organisms, while fewer studies are done on beneficial micro-organisms inhabitant in the human body, which causes a poor understanding of internal flora living in the body. The human body is surrounded by a biological system, important microorganisms in or on the body that are human microbiome (17). The human gastrointestinal tract is colonized by various microorganisms which some of these micro-florae are responsible for microbial balance in the host (18). There is co-existence between the host and intestinal microflora (17). The gastrointestinal tract is considered as one of the largest links between the outside environment and the inside of the human body, and animals that use probiotic microorganisms cause beneficial effects on host microflora (19). Probiotics promote the growth of benefits in bacteria and reduce pathogenic microorganisms that result in decline of gastrointestinal diseases. Other beneficial effects of probiotics are anti-allergic effects, anti-cancer, reduction of harmful fats and cholesterol and increasing host immune response (20). It is well established that some intestinal disorders such as irritable bowel syndrome, inflammatory bowel disease and antibiotics caused diarrhea could be more depending on the type of intestinal flora. Probiotics consumption has been recognized as an efficient strategy to deal with these diseases (14).

5. Conclusions

At present, with increasing antibiotic resistance and side effects of chemical drugs, an alternative treatment is necessary for this problem. Probiotics and their produced metabolites can be widely used as treatments to various disorders.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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