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Original Article

Antagonistic Effects of Lactobacilli spp. against Ciprofloxacin-Resistant Uropathogenic Escherichia coli Strains

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ABSTRACT

Background and Objectives: Recently, the use of probiotics for the treatment of Urinary Tract Infections has become more popular. The use of probiotics in therapy is useful as only a few side effects such as destruction of resistant bacteria or disturbance of the intestinal microbiota have been reported. The aim of this study was to evaluate the probiotic effects of lactic acid bacteria by co-aggregation of ciprofloxacin-resistant uropathogenic *Escherichia coli* strains using microbial techniques.

Materials and Methods: Three strains of *Lactobacillus plantarum*, *Lactobacillus casei and Lactobacillus acidophilus* were provided. Twenty isolates of uropathogenic *Escherichia coli* were collected from Shahid Labbafinejad hospital, Tehran. Eight samples with resistance to ciprofloxacin were selected using the disk diffusion method for the co-aggregation test. PCR was used to evaluate the presence of *qnrA* and *qnrS* genes in ciprofloxacin-resistant isolates. To evaluate the antimicrobial activity of complete culture and supernatants of lactobacilli, modified double-layer culture method and well diffusion methods were used, respectively. Co-aggregation of lactobacilli was evaluated by the co-aggregation test and microscopy test.

Results: Results showed that the eight human isolates were resistant to ciprofloxacin among other samples. Only one strain had both *qnrA* and *qnrS* genes simultaneously. *L. plantarum* with the average growth inhibition zone of 42 mm and with 65% of the co-aggregation had the best probiotic effects among all lactobacilli bacteria.

Conclusions: The probiotic lactobacilli had spectacular antimicrobial effects against the ciprofloxacin-resistant uropathogenic *Escherichia coli* strains. Also, lactobacillus spp. were aggregated with uropathogenic *Escherichia coli* strains and preventing from their adhesion to specific receptors on the Urethra, thus, the subsequent invasion to the host was prevented.

Keywords: Antimicrobial, Co-aggregation, *Lactobacillus acidophilus*, *Lactobacillus case*i, *Lactobacillus plantarum*

Introduction

Uropathogenic *E. coli* (UPEC) is one of the major concerns in the food industry. In some cases, the propagation of a single UPEC colony may occur within the population *via* contaminated food (1-3). The emergence of resistance to quinolones, as a type of antibiotics, among gram negative bacteria, limits the benefit of use of these antibiotics (4, 5). Among the quinolones, ciprofloxacin has the most effect against both gram-negative and gram-positive bacteria (6). Ciprofloxacin prevents from rebuilding, translating, and regeneration of bacterial DNA through the inhibition of the DNA gyrase in gram negative bacteria and topoisomerase IV in gram-positive bacteria (7,8). Resistance to ciprofloxacin is due to the mutation in the chromosome-dependent subunit A of DNA gyrase (9). Besides, the qnr genes are responsible for the plasmid resistance to quinolones by preventing the inhibition effect of ciprofloxacin on DNA gyrase and topoisomerase enzymes (10-12).

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On the other hand, lactic acid bacteria (LAB), capable of producing lactic acid, are gram positive and non-spore forming (13,14). LAB are widely used as the starters in the fermentation to produce dairy products. Lactobacillus spp. are considered as the most important genus of LAB (15,16), producing different compounds such as bacteriocin, lactic acid, and hydrogen peroxide, which prevent the growth of some pathogenic bacteria in food (17,18). Various diarrheal illnesses have been successfully treated by LAB as probiotic bacteria (19, 20). One of the characteristics of LAB is their co-aggregation with some pathogenic bacteria. Co-aggregation is the result of cell-to-cell recognition between two different bacterial strains. The co-aggregation properties of probiotic strains with pathogens prevent the adhesion of pathogenic bacteria through competing for binding sites in the urinary tract. Consequently, the use of LAB as the probiotics with the co-aggregation properties can be very useful and practical for the prevention of colonization of pathogens in body tissues (21). Therefore, the ability to co-aggregate is a desirable feature for probiotics in food safety (22).

Therefore, the aim of the present study was to investigate the antagonistic effects of lactobacillus spp. against ciprofloxacin-resistant UPEC strains.

Materials and Methods

Bacterial strains: To confirm genus and species of *E.coli* strains, after cultivation in eosin methylene blue medium and observing the colonies with metallic luster, some conventional biochemical tests (indol, MR-VP, urea, simon citrate, motility and TSI) were carried out. Also, gram staining and microscopic observation confirmed the presence of *E. coli* strains in medium culture. *E. coli* ATCC 25922 was used as a control in this study and was purchased from the Persian Type Culture Collection. *Lactobacillus plantarum* (ATCC 136H3), *L. acidophilus* (ATCC 314), and *L. casei* (ATCC 25598) strains were provided by Pasteur Institute of Iran. To activate the bacterial cultures, lactobacilli strains were cultured in MRS broth and MRS agar medium under anaerobic conditions and were incubated at 37 °C for 72 h and UPEC strains were cultured under aerobic conditions and incubated at 37 °C for 24 h.

Antimicrobial drug susceptibility testing: The antimicrobial drug susceptibility profiles were performed by Kirby-Bauer method. A volume of 100 µl of an overnight culture of each UPEC isolate was plated and streaked on Mueller-Hinton agar medium. The routinely used 15 antibiogram discs, including Nalidixic acid, Amikacin, Ampicillin, Sulfamethoxazole / Trimethoprim, Ofloxacin, Cefoxitin, Norfloxacin, amoxicillin/clavulanate, Tobromicin, Gentamicin, Piperacillin/ tazobactam, Imipenem, Ciprofloxacin, Ceftizoxime, Nitrofurantoin (Padtan Teb, Iran) were placed on the surface of the inoculated plates. Then, the plates were incubated at 37°C for 24 h. The samples were evaluated by the presence or absence of the growth inhibition zone. The strains without any inhibition zone were considered as resistant to antibiotics (23, 24).

DNA extraction and polymerase chain reaction: To identify the resistance to ciprofloxacin in eight UPEC strains, *qnrA* and *qnrS* genes were analyzed by the PCR method. First, an optimized boiling method was used for DNA extraction (Table 1). UPEC strains were grown in Luria-Bertani Broth (Merck, Germany) at 37 °C overnight. Then, the bacteria were pelleted from 1.5 ml Luria-Bertani broth and suspended in sterile distilled water (200 µl) and incubated at 37 °C for 10 min and centrifuged. To amplify sequences of the *qnrA*, *qnrS* gens, specific primers were used. Detection of adhesion-encoding genes (*qnrA*, *qnrS*) was done by multiplex PCR (Bio-Rad, America). The reactions (25 µl) consisted of 2 µl templates DNA, 10 pmol l^{-1} of each primer, and 12.5 µl of a ready-to-use 2X PCR Master Mix Red by IBRC Taq DNA polymerase, with the amplification conditions of; initial denaturation at 94 °C for 10 min, followed by 35 DNA cycles of denaturation at 94°C for 2 min, annealing at a specific temperature and extesion at 72°C for 1 min. The PCR product (5 µl) underwent gel electrophoresis (Syngene G:BOX, America) on agarose (1% w. w-1) (Merck, Germany), followed by staining with ethidium bromide solution (Cinna colon, Iran). Amplified DNA elements of specific sizes were indicated by UV-induced fluorescence and the size of the amplicons was estimated by comparing with the 1 kb DNA ladder (Thermo Scientific, America) included on the same gel (25-27).

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Table 1. Acquired primer for PCR of qnr gene and Annealing temperature

Annealing temperature	Identified gen	Primer sequencing	Company	Size
56° for 30 sec	qnrA	5-AGA GGA TTT CTC ACG CCA GG-3 5-TGC CAG GCA CAG ATC TTG AC-3	Cinna Gen	562 bp
55° for 30 sec	qnrS	5-ACG ACA TTC GTC AAC TGC AA -3 5- TAA ATT GGC ACC CTG TAG GC - 3	Cinna Gen	417 bp

Antimicrobial effect of lactobacillus spp. complete cultures by modified double-layer method: To examine the antimicrobial effect of lactobacillus spp., first, 50 µl of the newly cultured probiotic lactobacilli in pasteurized milk were spotted in the center of MRS agar (Merck, Germany) plate. After 24 h of incubation and growth of lactobacilli, the melted Mueller-Hinton agar (Merck, Germany) was poured on palte, then it was cooled down to room temperature to get solid. Then, suspensions of UPEC strains with turbidity of 0.5 McFarland were cultured on the Mueller-Hinton agar and incubated at 37 °C for 24 h. This test was carried out separately for each UPEC strain and control sample. Then, the growth-inhibited zones of the samples were evaluated and reported as mm of the observed diameter (28).

Antimicrobial activity of cell free supernatants (CFS) against UPEC strains using agar well diffusion assay: Targeted colony of eight strains of UPEC were diluted using 0.1% w w-1 peptone water (Merck, Germany) to reach 0.5 McFarland Turbidity Standard. All targeted UPEC strains being used were freshly spread onto Muller Hilton Agar using Kirby Bauer technique. Then, 5 mm diameter size of wells were immediately made up in each plate. Overnight suspensions of inoculated lactobacilli in milk were centrifuged at 12000×g for 30 min. The isolated supernatants were filtered by sterile filter 0.25 µ. Then, 80 µl of obtained supernatants were transferred to each well separately. Each plate was controlled by adding sterilized peptone water. All plates were incubated aerobically at 37°C for 24 h. The inhibition zones were measured in all of the plates. *E. coli* ATCC 25922 was used as controls in the experiments. The experiments were carried out and repeated three times (29).

Co-aggregation of lactobacillus spp. with UPEC strains: *Lactobacillus* spp. strains were grown anaerobically. The culture were then harvested by using centrifugation at 10000× *g* for 10 min, and were washed twice with sterile PBS consisting of (g/L): KH_2PO_4, 0.34; K_2HPO_4, 1.21; NaCl, 8.0; pH 7.0 and re-suspended in PBS. Two mL of each *Lactobacillus* spp. suspensions was mixed with 2 mL of the UPEC strains suspensions for 10 s at least by a vortex mixer. Then, they were incubated (both aerobically and anaerobically) for 4 h at 37°C, the suspensions were measured using a Bioscreen C (DNV, Finland) at OD_{600nm} (T1800; Hitachi, Tokyo, Japan) with co-aggregation expressed as follows according to Handley *et al* (30):

$$Co-aggregation = \frac{[(A_x + A_y)/2] - A_{(x+y)}}{(A_x + A_y)/2} \times 100$$

Equation1: where A represents absorbance, x and y represent each of the two strains in the control tubes, and (x + y) represents their mixture (32, 33).

Statistical analysis: All experiments were carried out in triplicates. Statistical analysis was performed through analysis of variance (ANOVA) using IBM SPSS & Duncan Statistics Software version 19. A *p*-value ≤ 0.05 was considered to be statistically significant.

Results and Discussion

Identification of *E. coli* strains by biochemical analysis: *E. coli* strains were identified by biochemical analysis. The results indicated that all isolates were positive indole, positive MR-VP, negative urea, and negative simon citrate, Except for one strain, other strains were able to move, and in terms of TSI were acid / acid. Table 2 shows the results of performed biochemical tests on UPEC isolates producing urinary tract infections. Table 2 shows all of the biochemical tests for UPEC strains.

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Table 2. Identification of *E. coli* strains by biochemical analysis

Number of strain	Simon Sitrat	Urea	MR-VP	Indol	Motility	TSI
UPEC 1	-	-	+	+	+	A/A
UPEC 2	-	-	+	+	+	A/A
UPEC 3	-	-	+	+	+	A/A
UPEC4	-	-	+	+	+	A/A
UPEC5	-	-	+	+	-	A/A
UPEC6	-	-	+	+	+	A/A
UPEC7	-	-	+	+	+	A/A
UPEC8	-	-	+	+	+	A/A

TSI, Triple Sugar Iron Agar

Antimicrobial drug susceptibility testing: The effects of fifteen different antibiotics were tested on all twenty strains of UPEC. The results indicated that eight strains were resistant to ciprofloxacin antibiotic. In the present study, the resistance to ciprofloxacin was 40%, much greater than for other fourteen antibiotics. Therefore, the isolates showed high resistance to ciprofloxacin antibiotic. Since the large amount prescription of ciprofloxacin initially to treat urinary tract infections from 1962, resistance to this antibiotic is expected to be higher than other quinolone antibiotics. In a study by Nakhjavani *et al*, in 2007, on isolated *E. coli* strains from patients with urinary tract infections, resistance to ciprofloxacin was 40.2% (34). By comparing the results of this study with the research carried out by Nakhjavani *et al*, it was observed that the level of resistance to ciprofloxacin in UPEC strains has been almost stable in the recent years. In a study in Pakistan in 2011, resistance of *E. coli* isolates to ciprofloxacin was 36.45% (35). The high prevalence to ciprofloxacin resistance in this study may be due to excess consumption of ciprofloxacin without any official supervision in developing countries.

In United States in 2006, resistance of *E. coli* strains was reported 21% to quinolones and 12% to fluoroquinolones (36). The difference between the results of the study in the US and the present study in terms of the level of resistance may be due to more detailed health-monitoring programs and lack of availability of these types of antibiotics for all people in the US.

Identification of *qnr* genes in UPEC isolates: Electrophoresis of PCR product has been shown in Figure 1 to identify *qnrA* and *qnrS* genes, on the 1% agarose gel with a 1000 bp marker. These genes are responsible for resistance to ciprofloxacin (37). By using PCR method for isolates with phenotipic resistance to ciprofloxacin, it was determined that among thirteen isolates resistant to ciprofloxacin, only one isolate had both *qnrA* and *qnrS* genes and two isolates had only *qnrS* gene. In the present study, the prevalences of *qnrA* and *qnrS* genes in ciprofloxacin- resistant isolates were 7.7% and 23.1%, respectively.

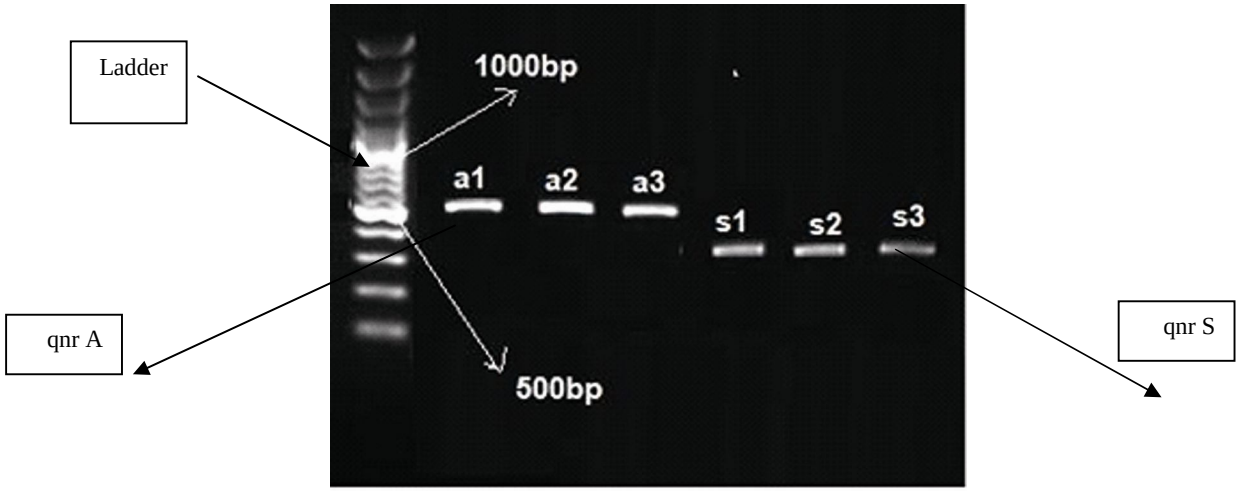

Figure 1. Electrophoresis of PCR product for *qnrA* and *qnrS* genes on 1% agarose gel in UPEC strain
s1,s2 ,s3 = *qnrS*
a1,a2,a3= *qnrA*

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In China, resistance to ciprofloxacin was about 60% of the clinical isolates of *E.coli* during the period of 1997-1999 (38). Increasing the resistance to ciprofloxacin in Enterobacteriaceae is associated with an increase in the prevalence of PMQR genes, diversity of PMQR genes and the prevalence of mutations in *gyrA* and *parC* genes or both in positive PMQR strains (39). Corkille *et al*., in 2005, investigated the resistant of enterobacteria to ciprofloxacin and cefotaxime, related to bacteremia disease in the UK, found that prevalence of *qnrA* gene in the studied isolates was 32% (40). High prevalence of *qnrA* gene in the UK study compared to this study (7.7%) can be due to the fact that the UK study was carried out on isolates obtained from patients in ICU with high consumption of antibiotic. The findings of the present study also showed that some isolates without *qnrA* gene still have a phenotypic resistance to ciprofloxacin probably due to other qnr genes or other mechanisms of resistance to this antibiotic, such as mutation in ciprofloxacin targeted enzymes.

Antimicrobial effect of lactobacillus spp. complete culture by modified double-layer method: The antimicrobial effect of lactobacilli complete culture showed that *L. plantarum*, *L. casei*, and *L. acidiphylus* had maximum antimicrobial effect against UPEC strains with the growth-inhibited zone of 42 mm, 32 mm, and 28mm, respectively. The antimicrobial effect of complete culture of lactobacilli against UPEC strains and *E.coli* ATCC 25922 (control strain) has been shown in Figure 2.

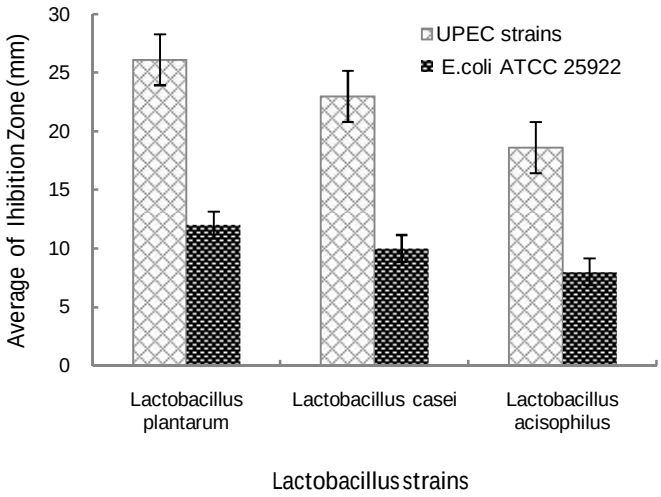

Figure 2. Antimicrobial effect of lactobacilli complete culture by modified double layer method (mm)

There are several methods for the evaluation of the antimicrobial properties of the complete culture of probiotics against pathogens. The best method is the one proposed by Mc given and Tagg in 1971, entitled as "Spot on-lawn method". In 2001, Maia changed its name to double-layer culture method (41). Then, this method was modified by Arbab Soleimani *et al*., in 2010 (modified double layer method). In this method, the antimicrobial effect of probiotic complete cultures is well visible in the second layer of culture medium despite the inhibition zone of UPEC strains (42). The results showed that antimicrobial effects of *L. plantarum* and *L. caesi* complete cultures against UPEC strains were more than *L. acidophilus*. It should be noted that the antimicrobial effects of complete culture of *L. plantarum* and *L. casei* were not significantly different.

Anas *et al*., announced that complete culture of *L. plantarum* had antimicrobial effect against *E. coli* and *Staphylococcus aureus*. This finding is in accordance with the findings of this study (43).

Antimicrobial activity of lactobacillus spp. supernatants against UPEC strains using agar well diffusion assay: The antimicrobial effect of lactobacilli supernatant against UPEC showed that *L. plantarum*, *L. casei*, and *L. acidiphylus* had maximum antimicrobial effect against UPEC strains with the growth-inhibited zone of 32 mm, 29 mm, and 20 mm, respectively. The antimicrobial effects of lactobacilli supernatant against UPEC strains and *E.coli* ATCC 25922 (control strain) are shown in Figure 3.

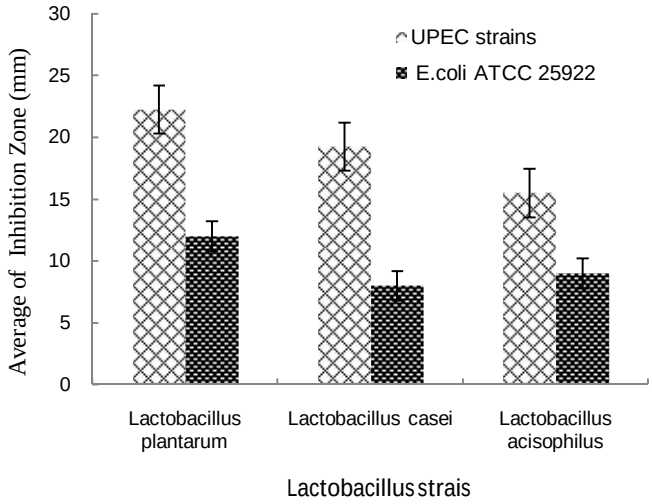

Figure 3. Antimicrobial effect of lactobacilli supernatants against UPEC strains by well diffusion method (mm)

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The antimicrobial effect of cells-free supernatants (CFSs) derived from probiotic bacteria can be due to two reasons: The first reason is the production of lactic acid or acetic acid by probiotics resulting the decrease of pH of the culture. Pathogenic bacteria are naturally sensitive to acidic conditions and are destroyed in acidic conditions. The second reason is the production of bacteriocin as an antimicrobial compound by probiotics (13).

Ogunbanwo *et al*. showed that supernatants resulted from the two probiotics of *L. plantarum* and *L. brevis* can inhibit the growth of *E. coli*, *Bacillus cereus* and *Yersinia enterocolitica* (44). The results of that study are similar to this research regarding the antimicrobial effect of *L. plantarum* against *E. coli*.

Several recent reports have documented the various antibacterial activities of CFSs of lactobacilli strains. In contrast, Hawaz observed that, filtered supernatants from some of the lactobacilli strains did not exhibit any inhibition against *Staphylococcus*, *E. coli*, and *Klebsiella* sp. (45). Recently, Jose *et al*. reported that the lactobacilli supernatant could inhibit the growth of *E. coli* (46). Additionally, few lactobacilli isolates from dairy products had antagonistic activity against *Listeria* sp. Also, other researchers reported high antagonistic activity against *B.cereus* as well. Furthermore, Rao *et al.*, showed that different strains of *L. plantarum* and *L. pentosus* have significant antimicrobial activities against *B. subtilis*, *Pseudomonas aeruginosa*, and *S. aureus* and other pathogenic bacteria (47).

Co-aggregation of lactobacillus spp. with UPEC: The results showed that the co-aggregation of *L. plantarum* with UPEC strains was higher than other present lactobacilli and the average co-aggregation was 41.5%. While, the average co-aggregations of *L. casei* and *L. acidophylus* with UPEC strains were 30.2 % and 34.2 %, respectively. Co-aggregation percent of lactobacilli strains and UPEC strains are shown in Figure 4.

One of the capabilities of the probiotic lactobacilli is that they can trap and accumulate with pathogenic bacteria, thus, the activity of pathogenic bacteria is stopped and inhibited. Cell aggregation between the microorganisms of the same strain (auto-aggregation), or between genetically different strains (co-aggregation) have significant importance in several ecological niches (48). There are several reports stating that probiotic lactobacilli can create a significant defense mechanism for the host against the pathogenic strains through cellular accumulation mechanism with pathogens. In additions, lactobacilli may act against pathogens by the production of antimicrobial agents such as organic acids, hydrogen peroxide, and bacteriocin (49). But, the mechanisms of cellular accumulation with pathogens have not been recognized yet. Cesena *et al.*, (50), Jankovic *et al.*, (48), and Collado *et al*., (51), described several methods for measuring the cellular accumulation of probiotics. The results confirmed that lactobacilli could accumulate with tested UPEC strains with high percentages and thus, inhibited the growth of UPEC strains.

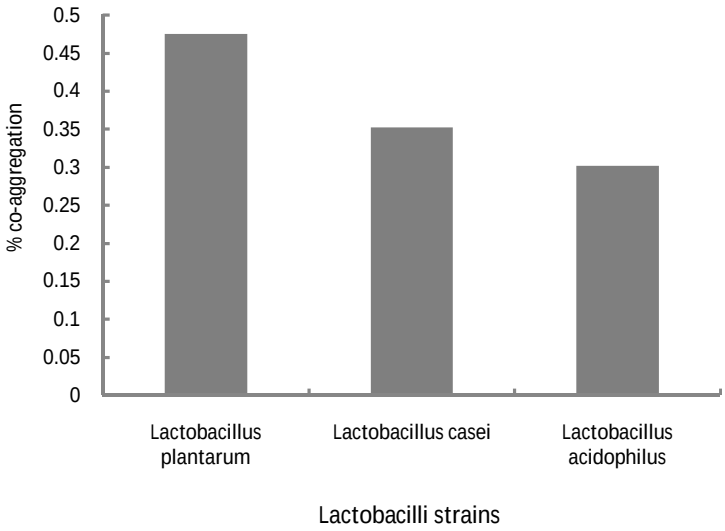

Figure 4. Co-aggregation percentage of lactobacilli with UPEC strains

Arbab Soleimani *et al.*, found that co-aggregation effect of *L. casei* with UPEC strains was higher than *L. acidophilus* (52). This finding was similar to the findings from this study. This study has found 61% co-aggregation for *L. casei* and 46% co-aggregation for *L. acidophilus*, indicating more co-aggregation effects than the present research.

The relationship between the lactobacilli co-aggregation and their antimicrobial power has been proposed in recent years (52). According to the present study, as the co-aggregation of probiotic Lactobacilli increased, their antimicrobial power of complete culture increased.

Furthermore, the cell surface characteristics of bacteria probably plays an important role in the co-aggregation. It was suggested that lactobacilli

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adherence is related to surface hydrophobicity, proteins or some other compounds such as carbohydrate and lipoteichoic acid (53). In a study, heat and pepsin had significant effects on the co-aggregation of *L. acidophilus* S1 with *E. coli* ATCC 11229, indicating that a proteinaceous surface component mediates the co-aggregation. However, sodium periodate did not have any effect on the co-aggregation, showing that carbohydrates are not involved in the co-aggregation process (54).

Moreover, it has been proved that the presence of *fim* gene expression, attributing to the presence of fimbriae in *E.coli* strains is important for the generation of co-aggregation with probiotic *L. casei* (55). It should be pointed out that *fim* gene was also identified in UPEC strains capable of co-aggregating with lactobacilli in this research.

Conclusions

It can be concluded that the complete cultures and supernatants resulted from *L. plantarum* ATCC 136H3, *L. casei* ATCC 25598, and *L. acidophilus* ATCC 314 had inhibitory effects against UPEC strains. However, *L. plantarum* ATCC 136H3 showed higher co-aggregation (41.5%) with UPEC strains among all lactobacilli. In general, the antimicrobial effect of complete culture of lactobacilli against UPEC strains was more than their supernatants, probably due to production of antimicrobial metabolites and acidic conditions or direct competition with pathogens for binding to the present receptors on the surface of the cells.

It can be also stated that co-aggregation can be associated with the antimicrobial agents of complete culture. Further research is required to better understand the antimicrobial mechanism of lactobacilli complete culture to treat the urinary tract infection (UTI) and prevent from the formation of a strain resistant to antibiotics. Therefore, application of probiotics in food stuff inhibits the growth of pathogens and prevents from food spoilage. Therefore, the application of probiotics as food preservatives is recommended.

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