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*Original Article*

# Evaluation of inhibitory activity of domestic probiotics for against invasion and infection by *Proteus mirabilis* in the urinary tract

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## Abstract

Introduction: Approximately 5% of men and 40%–50% of women have experienced urinary tract infections (UTI), which are the most common infectious diseases and nosocomial infections in humans. *Proteus mirabilis* is susceptible to most antibiotics, but antibiotic treatment usually causes side effects. In this research, lactic acid bacteria (LAB) was assessed for its inhibitory activity against a urinary tract pathogen.

Methodology: We studied the effect of pH adjustment, heat, and enzyme treatments on the inhibitory activity of LAB strains and their supernatants, using well-diffusion and co-culture assays. In the cell culture assay, anti-adhesion and anti-invasion activities against *P. mirabilis* were tested with SV-HUC-1 urothelial cells.

Results: LAB were able to adhere to the urothelial cells and inhibited *P. mirabilis* growth. LAB were also able to inhibit *P. mirabilis* adhesion to or invasion of SV-HUC-1 urothelial cells. Finally, in the competition assay, LAB showed inhibitory effects against *P. mirabilis*. LAB could also inhibit the invasion of *P. mirabilis* into urothelial cells.

Conclusions: Two LAB strains (PM206 and 229) exhibited antagonistic activity against *P. mirabilis* adhesion or invasion of urothelial cells in culture. In the future, probiotics may be used in food or urinary tract cleansing and could replace antibiotic treatments.

**Key words:** lactic acid bacteria; *Proteus mirabilis*; SV-HUC-1 urothelial cells; urinary tract infection; inhibitory activity; invasion.

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## Introduction

Urinary tract infection (UTI) is an infection or colonization of the urinary tract, including urethra, bladder, ureter and/or kidney, by microorganisms [1]. The most common bacterial pathogens detected in UTIs are *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis* and *Enterobacter cloacae* [2]. *P. mirabilis* is commonly known to cause urinary tract infections in catheterised patients [3].

*P. mirabilis* is distributed in soil, water and the human intestinal tract (HIT); it is a rod-shaped and facultatively anaerobic gram-negative bacterium belonging to Enterobacteriaceae [4]. The source of *P. mirabilis* in UTI is through cross-contamination of the periurethral area with the HIT flora [4]. Bacterial adhesion to the uroepithelium is an important step for colonization and infection [1]. Adherence of *P. mirabilis* occurs when the urine pH value rises to approximately 8.2, resulting in crystalline stone formation. *P. mirabilis* UTI can includes catheter-associated UTI, cystitis, prostatitis, acute pyelonephritis and urolithiasis. Urolithiasis is the key pathology of *P. mirabilis* UTI, characterised by the development of bladder and kidney stones [4,5].

Probiotics are live microorganisms that promote beneficial effects on host health. Owing to the property of probiotics to adhere to epithelial cells, they have been used to reduce or exclude the adhesion of pathogenic bacteria to epithelial cells; probiotics are the products of substances, such as organic acids, hydrogen peroxide or bacteriocins [6]. The antagonistic effects of LAB, such as *Lactobacillus murinus* against *P. mirabilis*, have been previously reported [3].

In our previous study, LAB strains and their fermented probiotic products were observed to exhibit potent zones of inhibition against uropathogenic *Escherichia coli* (UPEC). Moreover, LAB strongly adhered to SV-HUC-1 urothelial cells in culture. The growth of UPEC was inhibited after co-culture with LAB in human urine. The enhanced levels of lactic acid dehydrogenase, IL-6 and IL-8 were significantly reduced after treatments with LAB in UPEC-induced urothelial cells. In addition, oral administration of LAB

reduced viable UPEC in the urine of UPEC-challenged BALB/c mice [7]. The aim of the present study was to identify LAB which could inhibit *P. mirabilis* attachment to and invasion of cultured urothelial cells. LAB was screened for antimicrobial effects on UTI using a well-diffusion assay and for anti-growth activity against *P. mirabilis* using a co-culture inhibition assay. Anti-adhesion and anti-invasion activities against *P. mirabilis* were evaluated using cultured SV-HUC-1 urothelial cells.

## Methodology

*Bacteria and cell culture*

Seven LAB strains were identified as follows: *Lactobacillus paracasei* (PM2), *L. salivarius* (PM78), *L. plantarum* (PM206 and 229), *L. crispatus* (RY2) and *Pediococcus pentosaceus* (PM68 and 201) using the API 50 CHL assay. *P. mirabilis* BCRC 10725 was procured from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). LAB strains were stored at -80°C, inoculated in Lactobacilli MRS broth, and activated twice before incubation at 37°C for 24 hours. *P. mirabilis* was inoculated into nutrient broth (NB) at 37°C overnight.

The SV-HUC-1 urothelial cell BCRC 60358 was also purchased from the BCRC. SV-HUC-1 cells were cultured on 93% Ham's F12 medium supplemented with 10% foetal bovine serum, 1.176 g/ L sodium bicarbonate and 1% non-essential amino acid.

*The inhibition zone of LAB against P. mirabilis*

*P. mirabilis* cells were cultured in NB broth overnight, diluted to a concentration of $10^7$ CFU/mL, and spread on nutrition agar (NA). A volume of 100 μL supernatant (5,000×*g* for 10 minutes) of the 24-hourLAB culture was inoculated into a well on NA and the culture plates were incubated for 12 hours at 37°C. The diameters of the inhibition zones were measured using a previously described method [7].

Similar studies were conducted for heat-treated (100°C for 15 minutes) and enzyme-treated supernatants. To assess the sensitivity to enzyme treatment, spent LAB culture supernatants (SCS) were incubated for 1 hour at 37°C, with amylase (200 mg/mL), catalase (0.5 mg/mL), lactate dehydrogenase (250 mg/mL), pepsin (200 mg/mL), trypsin (200 mg/mL), or proteinase K (1 mg/mL),

*Antimicrobial assay of LAB and P. mirabilis co-culture*

The method used in the present study is a modification of the procedure described by Chapman *et al.* [8] and Tsai *et al.* [9]. For antimicrobial tests, broths inoculated with *P. mirabilis* ($10^7$ CFU/mL) and LAB ($10^9$ CFU/mL) were mixed (ratio of 1:100) in a sterile test tube. The experimental group contained 1 mL broth inoculated with *P. mirabilis*, 1 mL broth inoculated with LAB, 4 mL NB and 4 mL MRS broth. The control group contained 1 mL broth inoculated with *P. mirabilis*, 4 mL NB and 5 mL MRS broth. After incubation for 1, 2, 3 and 4 hours at 37°C, 1 mL of cultured broth was collected for serial dilution for the poured-plate method. NA plates were incubated overnight at 37°C before *P. mirabilis* colonies were counted. The survival rate of pathogens (%) = (number of *P. mirabilis* colonies after co-culture with LAB / number of *P. mirabilis* colonies after culture with MRS) × 100.

*Competitive adhesion assay of LAB and P. mirabilis on SV-HUC-1 urothelial cells*

The method was based on the procedure described by Gopal *et al.* [10] and Tsai *et al.* [9]. The cells ($6\times10^5$ cells/mL) were washed twice with PBS, transferred with 0.05% trypsin into a 24-well multi-dish containing fresh culture medium and incubated for 2 days at 37°C in a $CO_2$ incubator (Forma Direct Heat $CO_2$ Incubator, Thermo Scientific, Waltham, MA, USA). Then, the plate was washed with 1× PBS buffer. After removing the PBS buffer, 800 μL fresh medium without antibiotics, 100 μL broth inoculated with LAB ($10^8$ CFU/mL) and 100 μL broth inoculated with *P. mirabilis* ($10^6$ CFU/mL) were added to each well in the experimental group. MRS broth was added to each well in the control group. The competitive adhesion assay was performed as follows: (I) For the exclusion group, LAB were added and left to adhere for 1 hour before adding *P. mirabilis* and leaving to adhereefor 1 hour. (II) For the competition group, LAB and *P. mirabilis* were added simultaneously and left to adhere for 2 hours. (III) For the displacement group, *P. mirabilis* was added for 1 hour before adding LAB and leaving to adhere for 1 hour. The survival rate of *P. mirabilis* (%) = (number of *P. mirabilis* colonies after culture with LAB / number of *P. mirabilis* colonies in the control group) × 100.

*LAB inhibition ofinvasion of P. mirabilis into urothelial cells*

In brief, according to Tsai *et al.* [9], 800 μL fresh medium without antibiotics, 100 μL broth inoculated with LAB ($10^8$ CFU/mL) and 100 μL broth inoculated with *P. mirabilis* ($10^6$ or $10^7$CFU/mL) were added to each well in the experimental group. In the exclusion

group, broth inoculated with LAB was added for reaction for 1.5 hours before adding broth inoculated with *P. mirabilis* for reaction for 1.5 hours. In the competition group, broths inoculated with LAB and *P. mirabilis* were added simultaneously for reaction for 3 hours. In the displacement group, broth inoculated with *P. mirabilis* was added for reaction for 1.5 hours before adding broth inoculated with LAB for reaction for 1.5 hours. The invasion rate of *P. mirabilis* (%) = (number of *P. mirabilis* colonies after culture with *Lactobacillus* / number of *P. mirabilis* colonies in the control group) × 100.

*Statistical analysis*

Statistical analysis (means ± standard deviation) was performed using the SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA) statistical software. A one-way analysis of variance (ANOVA) or independent sample t-test was used to determine the statistical significance ($p < 0.05$ indicates a significant difference).

## Results

*Effects of LAB supernatant on the inhibition of urethral pathogens after heating, pH adjustment and enzyme treatment*

When LAB supernatant was heated at 100°C for 15 minutes, the supernatant showed no effect on the antibacterial activity of *P. mirabilis* BCRC 10725, indicating that the bacteriostatic substance of the LAB supernatant was heat resistant. Seven LAB supernatants were detected with the pH values ranging 3.77 – 3.94; when the supernatant pH was adjusted to 7.0, the antibacterial activity was lost (Table 1).

The inhibition zone caused by LAB supernatant on *P. mirabilis* BCRC 10725 was reduced by treatments with the different proteolytic enzymes, α-chymotrypsin, pepsin, proteinase K and trypsin. However, it remained at the tertiary inhibitory level (Table1). The partial reduction antibacterial activity after treatment with proteolytic enzymes suggests that the inhibitory substances in the LAB supernatant contain proteins or peptides. LAB supernatant treated with α-amylase, catalase or L-lactic dehydrogenase also resulted in a decrease in bacteriostatic ability against *P. mirabilis* BCRC 10725.

*Antimicrobial assay of LAB and P. mirabilis co-culture*

When LAB strains and *P. mirabilis* were cultered together, the number of colonies of seven LAB strains increased with the co-culture time while the residual rate of *P. mirabilis* significantly decreased with the co-culture time. After 7 hours of co-culture, seven LAB strains could significantly inhibit the growth of the pathogens, resulting in a residual rate of 0.41% – 0.82%. All seven LAB strains tested were suitable for inhibition of growth of cultured urethral pathogens (Table 2).

*Competitive adhesion of LAB and P. mirabilis to urothelial cells*

The residues of *P. mirabilis* BCRC 10725 significantly decreased in the co-culture of seven LAB strains and *P. mirabilis* BCRC 10725; the inhibitory effect of strain RY2 was 0.68% and of PM2 was 0.58%. The inhibitory effects of PM2 and PM78 were the highest with remnant rates of 1.52% and 1.22%, respectively. Overall, the effect of PM2 was the greatest among all the strains. In the displacement group PM2 gave a residual rate of 3.10 %, while the inhibition rates of PM2 in the exclusion and competition groups were also among the highest again on the *P. mirabilis* BCRC 10725 (Table 3).

**Table 1.** Effect of heat（100°C, 15 min）, pH 7.0 and enzyme treatments on the antimicrobial activity of SCS of LAB strains against *Proteus mirabilis* BCRC 10725.

| LAB strains | Inhibition zone diameter (mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SCS | H | pH=7 | Y | T | P | K | A | C | L |
| PM2 | 34,+++ | 33,+++ | - | 29,+++ | 29,+++ | 29,+++ | 28,+++ | 26,+++ | 26,+++ | 26,+++ |
| PM68 | 33,+++ | 31,+++ | - | 27,+++ | 29,+++ | 27,+++ | 27,+++ | 25,+++ | 26,+++ | 24,+++ |
| PM78 | 32,+++ | 30,+++ | - | 28,+++ | 28,+++ | 28,+++ | 27,+++ | 23,+++ | 25,+++ | 22,++ |
| PM201 | 30,+++ | 28,+++ | - | 28,+++ | 28,+++ | 27,+++ | 26,+++ | 23,+++ | 23,+++ | 26,+++ |
| PM206 | 33,+++ | 31,+++ | - | 29,+++ | 28,+++ | 29,+++ | 28,+++ | 26,+++ | 27,+++ | 26,+++ |
| PM229 | 33,+++ | 32,+++ | - | 29,+++ | 28,+++ | 29,+++ | 28,+++ | 26,+++ | 25,+++ | 23,+++ |
| RY2 | 33,+++ | 32,+++ | - | 29,+++ | 29,+++ | 30,+++ | 29,+++ | 26,+++ | 26,+++ | 27,+++ |

[1]The inhibition zones≦11 mm, 12~16 mm, 17~22 mm and ≧23mm, were classified as strains of no -, low +, medium ++ and strong +++ inhibition, respectively; [2]SCS: spent culture supernatants of pH 3.77-3.94; H: 100°C, 15min; pH 7: spent culture supernatants of pH 7.0; Y: α-chymotrypsin; T: trypsin; P: pepsin; K: proteinase K; A: α-amylase; C: catalase; L: L-lactic dehydrogenase.

**Table 2.** The survival rates of *Proteus mirabilis* BCRC 10725 in co-culture with the LAB strains.

| LAB Strains | Time (hrs) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | |
| | Log CFU/mL | % | Log CFU/mL | % | Log CFU/mL | % | Log CFU/mL | % |
| Control | 6.36±0.14 | $100^{Aa}$ | 6.71±0.19 | $100^{Aa}$ | 6.78±0.41 | $100^{Aa}$ | 7.34±0.33 | $100^{Aa}$ |
| PM2 | 5.77±0.06 | $32.88^{Ba}$ | 5.81±0.05 | $20.17^{Ba}$ | 5.33±0.50 | $5.03^{Bb}$ | 2.70±3.82 | $0.52^{Bb}$ |
| PM68 | 5.88±0.04 | $42.98^{Ba}$ | 5.77±0.01 | $17.99^{Bb}$ | 5.31±0.54 | $4.83^{Bb}$ | 2.70±3.82 | $0.52^{Bb}$ |
| PM78 | 5.90±0.08 | $44.78^{Ba}$ | 5.81±0.01 | $19.51^{Bb}$ | 5.61±0.00 | $13.25^{Bb}$ | 2.80±3.96 | $0.82^{Bb}$ |
| PM201 | 5.81±0.13 | $38.38^{Ba}$ | 5.86±0.06 | $21.37^{Bab}$ | 5.71±0.06 | $10.86^{Bbc}$ | 2.88±4.07 | $0.65^{Bc}$ |
| PM206 | 5.74±0.05 | $32.81^{Ba}$ | 5.78±0.07 | $20.67^{Bab}$ | 5.69±0.03 | $11.04^{Bab}$ | 2.88±4.07 | $0.71^{Bb}$ |
| PM229 | 5.77±0.11 | $37.69^{Ba}$ | 5.73±0.04 | $19.14^{Bab}$ | 5.60±0.01 | $8.50^{Bb}$ | 2.81±3.97 | $0.41^{Bb}$ |
| RY2 | 5.81±0.02 | $34.00^{Ba}$ | 5.79±0.13 | $18.85^{Bab}$ | 5.13±0.84 | $1.76^{Bab}$ | 2.90±4.11 | $0.57^{Bb}$ |

Bacteria counts are converted to Log CFU/mL; % means survival rates of *Proteus mirabilis* BCRC 10725; $^{A, B}$ Value in the same column with different letters indicate significant difference ($p < 0.05$); $^{a, b, c}$ Value in the same row with different letters indicate significant difference ($p < 0.05$).

**Table 3.** Effect of LAB strains in the survival of *Proteus mirabilis* BCRC 10725 from colonizing SV-HUC-1 cell line.

| LAB Strains | Exclusion | | Competition | | Displacement | |
|---|---|---|---|---|---|---|
| | Log CFU/mL | % | Log CFU/mL | % | Log CFU/mL | % |
| Control | 5.25±0.08 | $100^{Aa}$ | 5.72±0.00 | $100^{Aa}$ | 4.82±0.01 | $100^{Aa}$ |
| PM2 | 3.01±0.19 | $0.58^{Cb}$ | 3.90±0.08 | $1.52^{Cb}$ | 3.31±0.09 | $3.10^{Da}$ |
| PM68 | 3.41±0.02 | $1.42^{BCa}$ | 4.12±0.37 | $2.97^{BCa}$ | 3.45±0.17 | $4.41^{CDa}$ |
| PM78 | 3.13±0.28 | $0.80^{BCb}$ | 3.80±0.14 | $1.22^{Cb}$ | 3.51±0.01 | $4.89^{CDa}$ |
| PM201 | 3.66±0.26 | $2.91^{Ba}$ | 4.29±0.03 | $3.69^{Ba}$ | 3.85±0.16 | $11.18^{BCa}$ |
| PM206 | 3.44±0.24 | $1.73^{BCa}$ | 4.17±0.13 | $2.89^{BCa}$ | 3.89±0.27 | $12.91^{Ba}$ |
| PM229 | 3.37±0.30 | $1.55^{BCb}$ | 4.08±0.11 | $2.31^{BCab}$ | 3.46±0.06 | $4.39^{CDa}$ |
| RY2 | 3.08±0.05 | $0.68^{Ca}$ | 4.05±0.01 | $2.15^{BCb}$ | 3.54±0.00 | $5.23^{CDc}$ |

Bacteria counts are converted to Log CFU/mL; % means survival rates of *Proteus mirabilis* BCRC 10725; $^{A, B, C, D}$ Value in the same column with different letters indicate significant difference ($p < 0.05$); $^{a, b, c}$ Value in the same row with different letters indicate significant difference ($p < 0.05$).

**Table 4.** Effect of LAB strains on invasion to SV-HUC-1 cell line by *Proteus mirabilis* BCRC 10725 ($10^6$ CFU/ mL).

| LAB Strains | Exclusion | | Competition | | Displacement | |
|---|---|---|---|---|---|---|
| | Log CFU/mL | % | Log CFU/mL | % | Log CFU/mL | % |
| Control | 2.62±0.12 | $100^{Aa}$ | 2.89±0.02 | $100^{Aa}$ | 2.73±0.01 | $100^{Aa}$ |
| PM2 | 0.00±0.00 | $0.00^{Cb}$ | 0.85±0.00 | $0.91^{Db}$ | 1.35±0.07 | $4.16^{Da}$ |
| PM68 | 0.00±0.00 | $0.00^{Cb}$ | 0.54±0.09 | $0.46^{DEb}$ | 1.11±0.10 | $2.40^{Ea}$ |
| PM78 | 0.00±0.00 | $0.00^{Ca}$ | 0.00±0.00 | $0.06^{Ea}$ | 0.35±0.49 | $0.55^{Fa}$ |
| PM201 | 0.00±0.00 | $0.00^{Cb}$ | 1.22±0.06 | $2.15^{Cb}$ | 1.65±0.05 | $8.35^{Ca}$ |
| PM206 | 0.00±0.00 | $0.00^{Ca}$ | 0.69±0.30 | $0.70^{Da}$ | 0.24±0.34 | $0.27^{Fa}$ |
| PM229 | 0.00±0.00 | $0.00^{Cb}$ | 0.84±0.09 | $0.90^{Da}$ | 0.24±0.34 | $0.27^{Fab}$ |
| RY2 | 1.39±0.12 | $6.41^{Ba}$ | 1.83±0.01 | $8.68^{Ba}$ | 1.77±0.01 | $11.02^{Ba}$ |

Bacteria counts are converted to Log CFU/ mL; % means survival rates of *Proteus mirabilis* BCRC 10725; $^{A, B, C, D, E, F}$ Value in the same column with different letters indicate significant difference ($p < 0.05$); $^{a, b}$ Value in the same row with different letters indicate significant difference ($p < 0.05$).

**Table 5.** Effect of LAB strains on invasion to SV-HUC-1 cell line by *Proteus mirabilis* BCRC 10725 ($10^7$CFU/ mL).

| LAB Strains | Exclusion | | Competition | | Displacement | |
|---|---|---|---|---|---|---|
| | Log CFU/mL | % | Log CFU/mL | % | Log CFU/mL | % |
| Control | 3.32±0.34 | $100^{Aa}$ | 3.13±0.11 | $100^{Aa}$ | 3.70±0.37 | $100^{Aa}$ |
| PM2 | 1.00±0.00 | $0.55^{Ca}$ | 2.24±0.09 | $12.95^{Ba}$ | 3.09±0.12 | $32.48^{Ba}$ |
| PM68 | 0.50±0.71 | $0.42^{Ca}$ | 1.50±0.71 | $4.78^{Ca}$ | 2.94±0.03 | $20.70^{Ba}$ |
| PM78 | 1.00±0.00 | $0.55^{Ca}$ | 1.50±0.28 | $2.86^{Ca}$ | 2.84±0.16 | $19.14^{Ba}$ |
| PM201 | 0.65±0.92 | $0.83^{Ca}$ | 1.60±0.00 | $3.04^{Ca}$ | 2.90±0.04 | $17.95^{Ba}$ |
| PM206 | 1.30±0.00 | $1.11^{Ca}$ | 1.39±0.12 | $1.96^{Ca}$ | 2.44±0.30 | $9.04^{Ba}$ |
| PM229 | 0.50±0.71 | $0.42^{Ca}$ | 0.00±0.00 | $0.00^{Ca}$ | 2.24±0.09 | $3.79^{Ba}$ |
| RY2 | 2.56±0.31 | $17.36^{Ba}$ | 1.78±0.10 | $4.72^{Ca}$ | 2.82±0.00 | $15.57^{Ba}$ |

Bacteria counts are converted to Log CFU/ mL; % means survival rates of *Proteus mirabilis* BCRC 10725; $^{A, B, C}$ Value in the same column with different letters indicate significant difference ($p < 0.05$); $^{a}$ Value in the same row with different letters indicate significant difference ($p < 0.05$).

*LAB Inhibition of P. mirabilis invasion into urothelial cells*

Seven LAB strains were able to inhibit *P. mirabilis* BCRC 10725 invasion of SV-HUC-1 urethral epithelial cells. The residual rates of *P. mirabilis* BCRC 10725 in antimicrobial (1:100) tests are shown in Table 4. In the prevention group, RY2 could inhibit the pathogen invasion of cells, and the remaining 6 LAB strains could completely inhibit the pathogen's invasion of the cultured cells. In the competition group, the greatest inhibition was by PM78, with a residual rate of 0.06%. In the treatment group, PM78, PM206 and PM229 showed the greatest inhibition rates of the invasive effect, with residual rates of 0.27% to 0.55%.

The residual rates of *P. mirabilis* BCRC 10725 in antimicrobial (1:10) tests are shown in Table 5. These results indicate that the strains PM2, 68, 78, 206 and 229 could inhibit the pathogen's invasion, with residual rates of approximately 0.42% to 1.11% for exclusion. The inhibitory rates of PM68, 78, 201, 206, 229 and RY2 were approximately 0% – 4.78% for competition. The residual rates of seven LAB strains in the treatment group were 3.79% – 32.48% for displacement.

## Discussion

The present study investigated the inhibition of adherence and invasion of *P. mirabilis*, particularly analysed from the perspective of inhibition by exclusion, in which LAB adhering to SV-HUC-1 urothelial cell receptors excluded the attachment of *P. mirabilis*. Several proteins are known to be involved in the adhesion process of *P. mirabilis*, such as ambient-temperature fimbriae, *P. mirabilis* fimbriae (PMF), mannose-resistant *Proteus*-like (MR/P) fimbriae and uroepithelial cell adhesion (UCA)/non-agglutinating fimbriae [1,4,5].

Peerbooms *et al.* [11] have found the best invasive activity of cells of *P. mirabilis* in the pH range of 7.5 – 8.0, with the number of invasive bacteria ranging from $4.70 \times 10^3$ to $5.32 \times 10^3$ CFU per well. The researchers have speculated that *P. mirabilis* hydrolyses urea to increase the pH at the time of cell invasion. *P. mirabilis* secretes haemolysin, which is cytotoxic to human proximal tubular epithelial cells, and promotes *P. mirabilis*-induced pyelonephritis during UTIs; the haemolysin activity is positively correlated with invasive cell capacity [12]. Coker *et al.* [5] reported that *P. mirabilis* can secrete haemolysin into the eukaryotic cell membrane to form holes resulting in $Na^+$ loss as a result of cell damage.

Heating the LAB supernatant did not affect its bacteriostatic ability, indicating that the bacteriostatic substance is heat resistant. LAB supernatant has been reported to retain antibacterial activity following heating to 100°C for 120 minutes or 121°C for 20 minutes [13,14]. This may be a small peptide in LAB, a bacteriocin, which is thermostable and has bacteriostatic ability; further, it is effective in inhibiting the growth of *E. coli*, *Pseudomonas* spp. and *Streptococcus* spp. [15].

The pH values of the seven LAB supernatants were 3.77 – 3.94. When the pH value of the supernatant was adjusted to 7.0, the antibacterial activity of the urethral pathogen was completely lost, probably because (A) in the presence of acidic substances, the resulting acidic environment inhibits pathogen growth; and (B) in the presence of antibacterial substances, in a neutral environment, antibacterial activity is lost, and at pH 7.0 organic acid loss affects antibacterial activities. Gopal *et al.* reported that the secretion of lactic acid and small peptides by LAB has a synergistic effect on the inhibition of bacteria; therefore, under acidic conditions, small peptides can effectively inhibit bacteria [10].

Jack *et al.* have reported that bacteriocin produces a protein or peptide molecule that is biologically activated by bacterial metabolism [16]. Some bacteriocins lose their antibacterial activity following treatment with lipase or α-amylase, indicating that bacteriocins contain carbohydrates or lipids, and biological activity requires partial carbohydrate or lipid participation to be activated; therefore, the size of the inhibition zone of the pathogen is affected by α-amylase treatment. As LAB lack catalase, hydrogen peroxide accumulates in the supernatant, as indicated by the results in the experimental group, in which the supernatant of hydrogen peroxide is one of the antibacterial substances. Annuk *et al.* [17] have reported that hydrogen peroxide has a strong oxidative effect that can damage the basic structure of microbial cell proteins and damage cells, inhibiting the bacteria. In addition, L-lactic dehydrogenase treatment of LAB supernatant results in catalytic lactic acid (lactate) oxidation to produce pyruvate, along with the transfer of hydrogen to the coenzymes $NAD^+$ and NADH. Because the bacteriostatic ability of the supernatant then declines, it is speculated that lactic acid is one of the antibacterial substances.

Osset *et al.* [18] have demonstrated that *Lactobacillus* strain 11 was able to inhibit the adherence of half of *S. saprophyticus* by competition and exclusion but not by displacement. *P. mirabilis* shows a number of putative virulence factors, including the secreted haemolytic toxin HpmA, which contributes to

the host cell invasion and cytotoxicity [19]. *P. pentosaceus* strain 40 has been used to evaluate its putative probiotic properties in a mouse model of *Salmonella* infection. *P. pentosaceus* modulates cell-mediated immune responses by upregulating the gene expression of proinflammatory cytokines IFN-γ and TNF-α in the small intestine [20]. *In vitro,* vaginal mucosa VK2/E6E7 cells have a potential function in the local immune responses against *Candida albicans* that can be promoted by *L. crispatus* [21].

Probiotic properties, such as acid, bile tolerance and inhibition of pathogens were used for screening of LAB from fermented fruit and vegetable products. Two strains (*P. pentosaceus* MP12 and *L. plantarum* LAP6) have been assayed against *Salmonella* invasion in mouse spleen and liver [22]. *L. paracasei* CIDCA 8339 and *L. kefiri* CIDCA 83102 can inhibit *Salmonella* invasion in Caco-2/TC-7 enterocytes [23]. Similar to the above-mentioned results, in our study the LAB adhered to the urethral epithelial cells to form a protective layer and to reduce the adhesion of *P. mirabilis* to urothelial cells, when the LAB and pathogenic bacteria compete for space on urethral epithelial cell surfaces.

## Conclusions

We conclude from the present study that LAB can inhibit *P. mirabilis* through antimicrobial activity, primarily by blocking adhesion to and invasion of urothelial cells. These strains may therefore be promising to use for safe treatment and prevention of UTI.

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