Evaluation of Lactobacilli for Antagonistic Activity Against the Growth, Adhesion and Invasion of *Klebsiella pneumoniae* and *Gardnerella vaginalis*

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Abstract Urinary tract infections are the most common infectious diseases in babies and the elderly and are often acquired as nosocomial infections. The purpose of the present study was to identify strains of lactic acid bacteria (LAB) which could be used as alternatives to antibiotics for the treatment of urinary tract infections because of their ability to inhibit urinary tract pathogens (*Klebsiella pneumoniae* BCRC 10694 and *Gardnerella vaginalis* BCRC 17040). We screened 370 LAB strains using spent culture supernatants by inhibition zone assay to assess their antimicrobial effects. We studied the effect of heat, pH and enzyme treatment on the inhibitory activity of LAB strain supernatants. Anti-growth activity against urinary tract pathogens was evaluated by co-culture inhibition assay using seven LAB strains. Anti-adhesion and anti-invasion activities against urinary tract pathogens were evaluated by SV-HUC-1 uroepithelium cell culture. The results showed that the supernatants had good heat stability. However, antibacterial activity disappeared entirely at pH 7.0. After enzyme treatments, the supernatants showed first- or second-order inhibitory effects on *K. pneumoniae* BCRC 10694. The survival rate of urinary tract pathogens was 0–10.65% and pH of the culture medium decreased after co-culture with LAB strains for 4 h. In a competition assay, PM2 and RY2 inhibited urinary tract pathogens. PM68, PM78, PM201, PM206 and PM229 inhibited the invasion

of SV-HUC-1 cells by *G. vaginalis* BCRC17040. In conclusion, PM78, PM229 and RY2 showed the strongest inhibitory activity against urinary tract pathogens and could be suitable for use in the treatment of urinary tract infections.

Keywords Urinary tract infections · Lactic acid bacteria · Adhesion · Invasion · SV-HUC-1 uroepithelium cell

Introduction

Every year, an estimated one billion women are affected by genitourinary infections including urinary tract infections (UTIs) and bacterial vaginosis [1] caused by *Klebsiella pneumoniae* and *Gardnerella vaginalis*. Approximately 20–50% of urethral infections are reported to be nosocomial infections acquired in intensive care units; *K. pneumoniae* causes 6–17% of urethral infections in hospitals [1]. Most nosocomial urethral infections are associated with the use of catheters [1, 2]. A catheter may damage the protective urinary epithelial mucosa during insertion into the urethra, exposing the bacterial adhesion binding site. This increases the likelihood of bacterial adhesion and interference with the host defence system, resulting in an overactive bladder and incomplete urination, enabling the proliferation of residual microorganisms [3]. Patients who use catheters for > 7 days have a 25% higher risk of developing a UTI, 5% of which are potentially dangerous [2].

Klebsiella pneumoniae belongs to the Enterobacteriaceae family. It is a rod-shaped Gram-negative, non-motile facultative anaerobe. The bacterium is encapsulated by a layer of capsule polysaccharide (CPS). *K. pneumoniae* is present on aqueous surfaces, in soils and plants, and also on

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the mucosal surfaces of mammals, including the gut and respiratory tract of healthy humans [1]. *K. pneumoniae* is commonly implicated in nosocomial infections, with the elderly, newborn babies and immunocompromised patients being the most susceptible. The urethra and respiratory tract are the main sites of infection, but *K. pneumoniae* can proliferate in the gastrointestinal tract and invade the bloodstream, causing sepsis, pneumonia, urinary tract infections, purulent infections and other diseases [4].

Gardnerella vaginalis is a Gram-negative, non-capsulated, non-flagellated, non-sporulating and non-motile and is predominantly found in the vagina. *G. vaginalis* secretes adherent proteins during growth which facilitate its adsorption to vaginal epithelial cells [5]. It also secretes vaginolysin (VLY), a cholesterol-dependent cytotoxin which creates holes in cholesterol-containing vaginal epithelial cell membranes, increasing membrane permeability and resulting in the loss of K^+ ions [5–7]. At present, the treatment of a UTI usually involves a short-term course of antibiotics. Antibiotic treatment is highly effective, but the increasing antibiotic resistance of pathogens is a global issue of grave concern [8]. Urinary tract infections can often recur, and antibiotics can cause side effects (diarrhoea, depression, headache and kidney failure) and upset the balance of healthy intestinal and vaginal flora [9].

Microbial flora of the genitourinary tract in healthy women comprises 50–90% lactic acid bacteria (LAB) in the vagina and 38% LAB in the urethra [1]. LAB may interfere with the ability of pathogens to adhere to host cells and thus can prevent bacterial infections. Atassi et al. showed that *Lactobacillus helveticus* KS300 inhibits *G. vaginalis*, *Prevotella bivia*, uropathogenic *Escherichia coli* (UPEC) and *Salmonella typhimurium* [10]. Atassi et al. demonstrated that *L. helveticus* KS300 also inhibits the adhesion of *G. vaginalis* DSM4944 and UPEC IH1128 to HeLa cells and the ability of *S. typhimurium* to invade Caco-2 or TC7 cells [11]. Uehara et al. conducted clinical trials which confirmed the safety and efficacy of a LAB vaginal suppository to treat bacterial UTIs. Patients were fitted with pessaries containing *Lactobacillus crispatus* GAI 98322. The results revealed significant reduction in the number of recurrences of infection without any adverse complications ($p = 0.0007$). *L. crispatus* GAI 98322 is therefore effective in the treatment and exclusion of recurrent UTIs [12].

In the present study, we collected 370 LAB strains isolated from fruits and fermented plant products as well as reference strains from Bioresources Collection and Research Center (BCRC) (Hsin-Chu, Taiwan). We assessed the antibacterial activity of the LAB culture supernatant and its ability to inhibit pathogen adhesion and invasion. Our aim was to evaluate the potential of

indigenous LAB to replace antibiotics as an effective treatment for UTIs.

Materials and Methods

Bacterial Strains and Urethral Epithelial Cells

In total, 370 LAB strains were isolated from fruit, fermented plant products and obtained from BCRC. *K. pneumoniae* BCRC 10694, *G. vaginalis* BCRC 17040 and human urethral epithelial cells BCRC 60358 (SV-HUC-1) were purchased from BCRC. LAB preserved at $-80\text{ }^{\circ}\text{C}$ was inoculated into Lactobacilli MRS broth at $37\text{ }^{\circ}\text{C}$ for 24 h. *K. pneumoniae* BCRC 10694 was inoculated into Nutrient Broth at $37\text{ }^{\circ}\text{C}$ for 12 h. *G. vaginalis* BCRC 17040 was inoculated into Brain Heart Infusion Broth (BHI) and cultured at $37\text{ }^{\circ}\text{C}$ for 18 h. SV-HUC-1 cells were grown in Ham's F12 medium and supplemented with 10% FBS at $37\text{ }^{\circ}\text{C}$ in a humidified atmosphere of 95% air and 5% CO_2 . The culture medium was renewed each day. Cells were sub cultured weekly with 0.1% trypsin and 10 mM EDTA in PBS.

LAB Supernatant Inhibition Zone Test

The inhibition zone test was performed according to the method of Chapman et al. with modifications [13]. The bacterial broth of *K. pneumoniae* BCRC 10694 and *G. vaginalis* BCRC 17040 was diluted to 10^7 colony forming unit (CFU)/mL. A sterile cotton swab used to inoculate the broth of both strains onto the agar plate, and 9 mm diameter hole was made using a sterilize tip; 1 mL of LAB cultured for 18 h was centrifuged at 8000 rpm for 10 min and 100 μL of the supernatant was placed into the hole of the culture dish and then incubated at $37\text{ }^{\circ}\text{C}$ for 12 h. The diameter of the zone of inhibition was then measured.

In addition, to investigate the antibacterial characteristics of the LAB supernatant, the supernatant was heated to $100\text{ }^{\circ}\text{C}$ for 15 min, the pH was adjusted to 7.0 were different enzymes were added, and the inhibition zone test was the mean of three replicates. Decomposition enzymes included α -chymotrypsin, trypsin, pepsin, proteinase K, α -amylase, catalase and L-lactic dehydrogenase, each at a concentration of 1 mg/mL. Each enzyme (2 mg/mL) was mixed with the LAB supernatant in a 1:1 ratio and reacted at $37\text{ }^{\circ}\text{C}$ for 1 h before use.

Co-culture of LAB and Urethral Bacteria

This method was based on the description by Chapman et al. with modifications [13]. Bacteriostasis test was performed by adding 1:100 bacteria in a sterile test tube mixed

with bacteria of pathogen (10^7 CFU/mL) and LAB (10^9 CFU/mL). The mixtures were incubated at 37 °C, and at predetermined intervals 1 mL aliquots were removed at 1, 2, 3 and 4 h, a tenfold serially diluted and spread plated on nutrient agar or BHI agar were incubated overnight at 37 °C to determine the bacterial colony counts. Urethral pathogen white colonies were enumerated for CFU/mL and pH of the bacterial solution was detected at 1, 2, 3 and 4 h after the mixtures were incubated. The survival rate of urethral pathogenic bacteria (%) = (number of pathogenic bacteria after co-culture with LAB/number of pathogenic bacteria after culture without LAB) $\times$ 100.

Effect of LAB on Urethral Pathogen Adhesion

SV-HUC-1 Urothelial cells were inoculated into 24 well plates (6×10^5 cells/mL) and cultured in a CO₂ incubator at 37 °C for 2 days to allow the cells to attach and grow. After cell attachment was confirmed, the medium was removed and the cells were washed twice with 1 $\times$ PBS buffer; 800 μ L of SV-HUC-1 cells fresh medium, 100 μ L LAB (10^8 CFU/mL) and 100 μ L urethral bacteria (10^6 CFU/mL) were then added. MRS medium only was added to the control group. The adhesion test was conducted in three experimental modes: (1) Exclusion group: the LAB solution was cultured for 1 h, and then the urethral bacteria solution was added and incubated for a further hour. (2) Competition group: LAB and urethral bacteria were cultured together for 2 h. (3) Displacement group: the urethral bacteria solution was cultured for 1 h, and then the LAB solution was added and incubated for a further hour. The cells were incubated at 37 °C in a 5% CO₂ incubator, washed three times with 1 $\times$ PBS buffer in order to wash out the non-adhesion bacteria, trypsinated using trypsin–EDTA (0.25% trypsin, 1 mM EDTA) and a ten-fold serially diluted and spread plated on nutrient agar or BHI agar were incubated overnight at 37 °C to determine the bacterial colony counts. The urethral adhesion pathogen colonies were enumerated. Adhesion rate of urethral pathogenic bacteria (%) = (number of pathogenic bacteria detected by LAB culture/number of pathogenic bacteria in control group) $\times$ 100.

Effect of LAB on Urethral Pathogen Invasion

This test was conducted according to the method described of Kim et al. [14]; The SV-HUC-1 cells (6×10^5 cells/mL) were seeded in 24-well tissue culture plates and allowed to attach for at least 48 h before addition of bacteria. After incubation, cells were washed twice with PBS, and then 800 μ L of SV-HUC-1 cells fresh medium, 100 μ L LAB (10^8 CFU/mL) and 100 μ L urethral bacteria (10^6 CFU/mL) were mixed. Control group was mixed with

MRS medium only. Cells were then incubated for 2 h at 37 °C in a 5% CO₂ incubator; further, the cells were washed twice with 1 $\times$ PBS buffer and 1 mL gentamicin (200 μ g/mL) in order to kill non-invasion pathogenic bacteria. 24-well tissue culture plates were placed in a CO₂ incubator at 37 °C for 1 h to kill pathogenic bacteria which had adhered to the SV-HUC-1 cells. The mixed culture medium was then aspirated and cells were washed twice with 1 $\times$ PBS buffer; 1 mL of 1% Triton-X 100 was added for 10 min to lyse the SV-HUC-1 cells. The mixture in each well was mixed vigorously using a micropipette, serial dilution of the lyse SV-HUC-1 cells after 1% Triton-X 100 treatment and spread plated on nutrient agar or BHI agar were incubated overnight at 37 °C to determine the bacterial colony counts. The invasion rate of urethral pathogenic bacteria (%) = (number of pathogenic bacteria in LAB culture/number of pathogenic bacteria in control group) $\times$ 100.

Statistical Analysis

The experimental data were statistically analysed using SAS 9.4 software. All the values were analysed using one-way ANOVA or independent sample *t* test for mean difference significance analysis. Differences were considered significant when $p < 0.05$.

Results and Discussion

LAB Supernatant Inhibition Zone Test

In the *K. pneumoniae* BCRC 10694 inhibition zone test, 214 strains produced an inhibition ring ≤ 11 mm in diameter, 106 which produced an inhibition ring with a diameter of 12–16 mm and 50 produced a secondary inhibitory ring with a diameter of 17–22 mm (Table 1). In the *G. vaginalis* BCRC 17040 inhibition zone test, 352 strains produced inhibition rings ≤ 11 mm in diameter. Among them, 18 strains produced inhibition rings with diameters of 12–16 mm (Table 2).

Effect of Heat, pH Adjustment and Enzyme Treatment on the Antibacterial Activity of LAB Supernatant

In this experiment, bacteriostasis was categorised into four levels based on the size of the zone of inhibition [The inhibition zones ≤ 11 mm (–), 12–16 mm (+), 17–22 mm (++) and ≥ 23 mm (+++), were classified as strains of no; mild; strong and very strong inhibition, respectively]. Seven LAB strains had ++ inhibitory ability against *K. pneumonia* BCRC 10694. PM2, PM206, PM229

Table 1 Agar diffusion test showing the antagonistic activity of the spent culture supernatants of the lactic acid bacteria strains against *Klebsiella pneumonia* BCRC 10694

Strain	Inhibition zone (mm)
BCRC 10067, 10695, 10696, 10790, 11080, 11846, 11847, 12187, 12301, 13869, 14019, 14060, 14065, 14069, 14634, 14660, 14662, 14663, 14665, 14667, 14668, 14691, 14728, 15477, 17012, 17394	≤ 11
PM 3–5, 9, 11, 15–18, 21–27, 29, 30, 32, 35, 37, 39, 40, 41, 43–45, 47–49, 51–62, 64–66, 69, 72–75, 79, 82, 83, 85, 87–99, 102, 104–108, 111–113, 115, 118, 121–123, 126–133, 135, 136, 138, 141, 144–149, 151, 155, 159, 163, 165, 170–174, 177, 180–183, 185, 192–195, 199, 200, 202, 208, 210, 211, 218, 220, 225, 228	
FM 1–19, 21–25, 27, 28, 32, 34–51, 56–58, 61–66, 69–71	
Others TM39	
BCRC 10068, 10069, 10360, 10361, 11051, 11652, 11662, 12188, 12190, 12191, 12193, 12194, 12248, 12250, 12251, 12256, 12260, 12574, 12580, 12931, 12936, 12943, 12944, 14011, 14024, 14064, 14080, 14602, 14618, 14619, 14622, 14625, 14659, 14677, 14741, 15416, 16061, 16092, 17002, 17004, 17009, 17062, 17072, 17614, 17615, 17616, 17973, 17983, 80109	12–16
PM 1, 6, 8, 14, 20, 28, 31, 33, 34, 36, 42, 46, 50, 67, 70, 77, 78, 86, 100, 116, 117, 124, 125, 137, 139, 142, 175, 184, 186–188, 190, 191, 196–198, 201, 203, 207, 209, 213–216, 221–223, 227	
FM 55, 60, 67, 68	
Others En4, LA, LDL, LGA, MM1	
BCRC 14002, 14008, 14084, 14606, 14630, 14671, 14678, 14735, 14759, 15971, 16000, 17010, 17474	17–22
PM 2, 7, 10, 12, 19, 38, 63, 68, 71, 80, 81, 84, 101, 103, 110, 114, 119, 120, 134, 152, 153, 156–158, 160, 164, 166, 167, 176, 178, 204–206, 212, 229	
FM 59	
Others RY2	

The inhibition zones ≤ 11 mm: no inhibition; 12–16 mm: low inhibition; 17–22 mm: medium inhibition, and ≥ 23 mm: strong inhibition

Table 2 Agar diffusion test showing the antagonistic activity of the spent culture supernatants of the lactic acid bacteria strains against *Gardnerella vaginalis* BCRC 17040

Strain	Inhibition zone (mm)
BCRC 10067, 10068, 10069, 10360, 10361, 10695, 10696, 10790, 11051, 11080, 11652, 11662, 11846, 11847, 12187, 12188, 12190, 12191, 12193, 12194, 12248, 12250, 12251, 12256, 12260, 12301, 12574, 12580, 12931, 12936, 12943, 12944, 13809, 14002, 14008, 14011, 14019, 14024, 14060, 14064, 14065, 14069, 14080, 14084, 14602, 14606, 14618, 14619, 14622, 14625, 14630, 14634, 14659, 14660, 14662, 14663, 14665, 14667, 14668, 14671, 14677, 14678, 14691, 14728, 14735, 14741, 14759, 15416, 15477, 15971, 16000, 16061, 16092, 17002, 17004, 17009, 17012, 17062, 17072, 17394, 17614, 17615, 17616, 17973, 17983, 80109	≤ 11
PM 1–75, 77–79, 81–83, 85–100, 102–108, 110–118, 120–139, 141, 142, 144–149, 151–153, 155, 156, 159, 163, 165, 167, 170, 171–174, 177, 180–188, 190, 191, 193–196, 198–218, 220–223, 225, 227–229	
FM 1–8, 10–19, 21–25, 27, 28, 32, 34–51, 55–67, 69–71	
Others En4, LA, LDL, LGA, MM1, RY2, TM39	
BCRC 17010, 17474	12–16
PM 80, 84, 101, 119, 157, 158, 160, 164, 166, 175, 176, 178, 192, 197	
FM 10, 68	

The inhibition zones ≤ 11 mm: no inhibition; 12–16 mm: low inhibition; 17–22 mm: medium inhibition, and ≥ 23 mm: strong inhibition

and RY2 had an inhibitory effect on *G. vaginalis* BCRC 17040 (Table 3).

After the LAB supernatant was heated to 100 °C for 15 min, the antibacterial activity against both pathogens remained unchanged, indicating that the antibacterial substance in the LAB supernatant is heat resistant. It has also

been reported that the LAB supernatant possesses antibacterial activity after heat treatment at 100 °C for 120 min or 121 °C for 20 min [15, 16]. This may be because of bacteriocin secreted by LAB, which possesses thermal stability and antibacterial properties; it effectively

Table 3 Effect of heat (100 °C, 15 min), pH 7.0 and enzyme treatments on the antimicrobial activity of spent culture supernatants of the lactic acid bacteria strains against urinary tract pathogens

Urinary tract pathogens	LAB strain	Inhibition zone diameter (mm)										
		SCS	H	pH = 7	Y	T	P	K	A	C	L	
<i>Klebsiella pneumonia</i> BCRC 10694	PM2	22	21	–	18	17	17	16	14	13	12	
	PM68	20	20	–	15	15	16	15	11	12	11	
	PM78	21	19	–	16	15	15	15	–	12	11	
	PM201	20	19	–	16	15	15	14	–	–	–	
	PM206	21	20	–	17	16	17	15	13	13	12	
	PM229	22	21	–	17	16	17	16	13	13	14	
	RY2	22	21	–	17	17	18	16	12	13	13	
<i>Gardnerella vaginalis</i> BCRC 17040	PM2	12	11	–	11	11	10	11	–	–	–	
	PM68	11	11	–	10	10	10	11	–	–	–	
	PM78	11	10	–	10	10	10	11	–	–	–	
	PM201	11	10	–	10	10	10	11	–	–	–	
	PM206	12	11	–	10	10	10	11	–	–	–	
	PM229	12	11	–	10	10	10	10	–	–	–	
	RY2	12	11	–	10	10	10	10	–	–	–	

The inhibition zones ≤ 11 mm: no inhibition; 12–16 mm: low inhibition; 17–22 mm: medium inhibition, and ≥ 23 mm: strong inhibition

SCS: spent culture supernatants of pH 3.77–3.94, H: 100 °C, 15 min, 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 4 The survival rates of urinary tract pathogens in co-culture with the lactic acid bacteria strains

Urinary tract pathogens	LAB strains	Time (h)							
		1		2		3		4	
		Log CFU/ mL	%	Log CFU/ mL	%	Log CFU/ mL	%	Log CFU/ mL	%
<i>Klebsiella pneumonia</i> BCRC 10694	Control	6.74 $\pm$ 0.15	100 ^{Aa}	7.37 $\pm$ 0.21	100 ^{Aa}	7.75 $\pm$ 0.33	100 ^{Aa}	8.61 $\pm$ 0.14	100 ^{Aa}
	PM2	6.40 $\pm$ 0.16	33.94 ^{Ba}	6.33 $\pm$ 0.04	6.36 ^{BCb}	5.82 $\pm$ 0.31	0.77 ^{Bb}	0.00 $\pm$ 0.00	0.00 ^{Bb}
	PM68	6.33 $\pm$ 0.10	28.43 ^{Ba}	6.31 $\pm$ 0.08	6.10 ^{BCb}	2.97 $\pm$ 4.19	0.40 ^{Bb}	0.00 $\pm$ 0.00	0.00 ^{Bb}
	PM78	6.25 $\pm$ 0.08	30.28 ^{Ba}	6.21 $\pm$ 0.19	5.19 ^{Cb}	3.04 $\pm$ 4.30	0.58 ^{Bc}	0.00 $\pm$ 0.00	0.00 ^{Bc}
	PM201	6.25 $\pm$ 0.06	30.25 ^{Ba}	6.26 $\pm$ 0.11	5.75 ^{BCb}	5.82 $\pm$ 0.29	0.77 ^{Bc}	0.00 $\pm$ 0.00	0.00 ^{Bc}
	PM206	6.24 $\pm$ 0.00	34.56 ^{Ba}	6.15 $\pm$ 0.12	6.00 ^{BCb}	5.76 $\pm$ 0.32	0.97 ^{Bc}	0.00 $\pm$ 0.00	0.00 ^{Bc}
	PM229	6.24 $\pm$ 0.01	34.70 ^{Ba}	6.11 $\pm$ 0.10	5.46 ^{Cb}	5.57 $\pm$ 0.22	0.61 ^{Bc}	0.00 $\pm$ 0.00	0.00 ^{Bc}
	RY2	6.14 $\pm$ 0.17	32.81 ^{Ba}	6.07 $\pm$ 0.18	8.26 ^{Bb}	5.20 $\pm$ 0.35	0.56 ^{Bc}	0.00 $\pm$ 0.00	0.00 ^{Bc}
	Control	6.24 $\pm$ 0.15	100 ^{Aa}	6.31 $\pm$ 0.13	100 ^{Aa}	6.54 $\pm$ 0.14	100 ^{Aa}	7.27 $\pm$ 0.25	100 ^{Aa}
<i>Gardnerella vaginalis</i> BCRC 17040	PM2	6.07 $\pm$ 0.02	98.84 ^{ABa}	6.10 $\pm$ 0.07	77.31 ^{Ba}	6.04 $\pm$ 0.06	36.64 ^{Bb}	5.95 $\pm$ 0.01	8.26 ^{BCc}
	PM68	6.00 $\pm$ 0.09	83.39 ^{ABa}	6.04 $\pm$ 0.08	67.30 ^{BCa}	6.00 $\pm$ 0.10	32.30 ^{Bb}	5.64 $\pm$ 0.18	4.57 ^{Dc}
	PM78	6.11 $\pm$ 0.06	106.65 ^{Aa}	6.12 $\pm$ 0.03	80.67 ^{Bb}	6.06 $\pm$ 0.12	39.70 ^{Ac}	6.05 $\pm$ 0.07	10.65 ^{Bd}
	PM201	6.25 $\pm$ 0.11	83.98 ^{ABa}	6.21 $\pm$ 0.12	44.62 ^{Db}	6.15 $\pm$ 0.11	24.08 ^{Bc}	6.01 $\pm$ 0.10	4.53 ^{Dd}
	PM206	6.20 $\pm$ 0.17	74.97 ^{Ba}	6.15 $\pm$ 0.22	38.69 ^{Db}	6.12 $\pm$ 0.15	22.88 ^{BCc}	6.06 $\pm$ 0.15	4.98 ^{CDd}
	PM229	6.26 $\pm$ 0.14	85.93 ^{ABa}	6.25 $\pm$ 0.11	49.36 ^{CDb}	6.17 $\pm$ 0.16	24.07 ^{BCc}	6.06 $\pm$ 0.12	4.87 ^{Dd}
	RY2	5.98 $\pm$ 0.12	50.16 ^{Ca}	5.78 $\pm$ 0.16	18.39 ^{Eb}	5.53 $\pm$ 0.26	6.76 ^{Cb}	4.89 $\pm$ 0.34	0.48 ^{Eb}

Bacteria counts are converted to Log CFU/mL

% means survival rates of *Klebsiella pneumonia* BCRC 10694 and *Gardnerella vaginalis* BCRC 17040

^{A,B,C,D,E}Values (%) in the same column (group) with different letters indicate significant difference ($p < 0.05$)

^{a,b,c,d}Values (%) in the same row (LAB strain) with different letters indicate significant difference ($p < 0.05$)

Fig. 1 The pH of **a** *Klebsiella pneumoniae* BCRC 10694 and **b** *Gardnerella vaginalis* BCRC 17040 in co-culture with the lactic acid bacteria strains

inhibits the growth of *E. coli*, *Streptococcus* spp., *Pseudomonas* spp. and other pathogens [16].

The pH value of the seven LAB strains was 3.77–3.94. When the pH value of the supernatant was adjusted to 7.0, the antibacterial effects of five of the strains were completely lost (Table 3), probably because organic acids in the antibacterial substance are ineffective under pH neutral conditions. After the LAB supernatant was treated with different proteolytic enzymes (α -chymotrypsin, trypsin, pepsin and proteinase K), its bacteriostatic effect on *K. pneumoniae* BCRC 10694 changed from medium inhibition to strong inhibition (it maybe the protein in the LAB supernatant digested to the small peptide that could enhance to inhibit the pathogen). All bacteriostatic effects on *G. vaginalis* BCRC 17040 were lost (Table 3). Proteolytic enzymes affected the bacteriostatic properties of LAB supernatant in all cases, indicating that the bacteriostatic substance is a protein or peptide.

Co-culture of LAB and Urethral Pathogen

During co-culture, the survival rate of urethral pathogens significantly decreased. The survival rates of pathogenic bacteria were 5.19 and 5.46% after 2 h of co-cultivation of *K. pneumoniae* BCRC 10694 with PM78 and PM229, respectively. After 3 h, the survival rate had decreased to 0.40 and 0.97%, respectively, and further decreased to 0% after 4 h (Table 4). All seven LAB strains completely inhibited the growth of *K. pneumoniae* BCRC 10694. *G. vaginalis* BCRC 17040 was most strongly inhibited by RY2; the survival rate after 4 h was 0.48% (Table 4).

Several researchers believe that metabolites secreted by LAB, including lactic acid, hydrogen peroxide and some proteins, can inhibit pathogenic bacteria [13, 17]. At low pH, the synergy between lactic acid and bacteriocins significantly inhibits *E. coli* [17]. However, at pH 6.0–6.5 the LAB metabolites of bacteriocins are inactivated, resulting in hydrogen peroxide degradation, which prevents LAB inhibition of pathogens, allowing *G. vaginalis* to overgrow and cause bacterial vaginosis.

We also measured the pH of the co-culture medium during this experiment. As shown in Fig. 1, the pH of both *K. pneumoniae* BCRC 10694 and *G. vaginalis* BCRC 17040 medium was approximately 5.5 at hour 0. With increasing co-culture time, the pH value of the culture medium gradually decreased and the survival rates of urethral pathogens also showed a downward trend, indicating that low pH inhibited urethral pathogens. The lowest pH value recorded was 4.19, measured at 4 h of PM68 and pathogen co-cultivation.

Effect of LAB on Urethral Pathogen Adhesion

The inhibitory effects of Probiotics by pathogens were evaluated in (1) Exclusion group: lactobacilli were allowed to adhere to SV-HUC-1 cells first and each of the pathogens was added later. (2) Competition group: between pathogens and LAB for adhesion on the surface of SV-HUC-1 cells. (3) Displacement group: pathogens were allowed to adhere to SV-HUC-1 cells first and lactobacilli was added later.

Seven strains of LAB competed with *K. pneumoniae* BCRC 10694 to inhibit adhesion to the epithelial SV-HUC-1 cells. In the exclusion group, seven strains of lactic acid bacteria significantly inhibited the growth and adhesion of pathogens. The inhibitory effect of PM2, PM68, PM229 and RY2 was the strongest in the competition group with an adhesion rate of 0.11–0.17%. In the displacement group, the inhibitory effect of PM2, PM68, PM78, PM201, PM229 and RY2 was significant and the adhesion rates were 0.76–2.95%. PM2, PM68, PM229 and RY2 had the strongest inhibitory effects in all three groups (Table 5).

Table 5 Effect of Lactic acid bacteria strains in the survival of urinary tract pathogens from colonizing SV-HUC-1 cell line

Urinary tract pathogens	Strains	Exclusion		Competition		Displacement	
		Log CFU/mL	%	Log CFU/mL	%	Log CFU/mL	%
<i>Klebsiella pneumonia</i> BCRC 10694	Control	6.76 ± 0.23	100 ^{Aa}	6.43 ± 0.08	100 ^{Aa}	5.35 ± 0.01	100 ^{Aa}
	PM2	3.67 ± 0.11	0.09 ^{Bb}	3.46 ± 0.12	0.11 ^{Cb}	3.24 ± 0.02	0.76 ^{Ca}
	PM68	3.78 ± 0.05	0.11 ^{Ba}	3.59 ± 0.24	0.17 ^{Ca}	3.38 ± 0.28	1.15 ^{Ca}
	PM78	4.08 ± 0.11	0.24 ^{Ba}	4.25 ± 0.00	0.66 ^{BCa}	3.76 ± 0.33	2.86 ^{Ca}
	PM201	3.85 ± 0.20	0.12 ^{Bb}	4.50 ± 0.22	1.33 ^{Bab}	3.82 ± 0.02	2.92 ^{Ca}
	PM206	4.19 ± 0.06	0.28 ^{Bb}	4.31 ± 0.39	0.99 ^{BCb}	4.81 ± 0.16	29.22 ^{Ba}
	PM229	3.83 ± 0.12	0.14 ^{Bb}	3.58 ± 0.15	0.15 ^{Cb}	3.58 ± 0.05	1.71 ^{Ca}
	RY2	3.72 ± 0.34	0.09 ^{Bb}	3.57 ± 0.15	0.14 ^{Cb}	3.82 ± 0.08	2.95 ^{Ca}
<i>Gardnerella vaginalis</i> BCRC 17040	Control	4.52 ± 0.41	100 ^{Aa}	5.26 ± 0.09	100 ^{Aa}	4.35 ± 0.14	100 ^{Aa}
	PM2	3.30 ± 0.73	6.80 ^{Eb}	4.19 ± 0.00	8.61 ^{Dab}	3.55 ± 0.14	16.18 ^{Ca}
	PM68	3.66 ± 0.52	13.87 ^{DEa}	4.35 ± 0.03	12.37 ^{CDa}	3.54 ± 0.05	15.70 ^{Ca}
	PM78	3.98 ± 0.55	29.61 ^{BCa}	4.81 ± 0.10	35.96 ^{Ba}	3.66 ± 0.29	21.04 ^{Ca}
	PM201	3.91 ± 0.52	25.02 ^{CDb}	4.64 ± 0.00	24.51 ^{BCb}	3.96 ± 0.13	41.46 ^{Ba}
	PM206	4.11 ± 0.50	39.25 ^{Ba}	4.78 ± 0.28	34.92 ^{Ba}	3.92 ± 0.38	40.18 ^{Ba}
	PM229	3.55 ± 0.62	11.42 ^{Ea}	4.48 ± 0.15	16.84 ^{CDa}	3.55 ± 0.02	16.23 ^{Ca}
	RY2	3.04 ± 0.70	3.67 ^{Ea}	4.08 ± 0.52	8.40 ^{Da}	3.22 ± 0.01	7.80 ^{Ca}

Bacteria counts are converted to Log CFU/mL

% means survival rates of *Klebsiella pneumonia* BCRC 10694 and *Gardnerella vaginalis* BCRC 17040

^{A,B,C,D,E}Values (%) in the same column (group) with different letters indicate significant difference ($p < 0.05$)

^{a,b}Values (%) in the same row (LAB strain) with different letters indicate significant difference ($p < 0.05$)

The adhesion rate of *G. vaginalis* BCRC 17040 was also significantly decreased by seven LAB strains. In the exclusion group, PM2, PM229 and RY2 inhibited adhesion most strongly; the adhesion rate was 6.80, 11.42 and 3.67%, respectively. In the competition group, the inhibitory effect of PM2 and RY2 was the strongest, with adhesion rates of 8.61 and 8.40%, respectively. In the displacement group, the inhibitory effect of PM2, PM68, PM78, PM229 and RY2 was most remarkable, with adhesion rates of 7.80–21.04%. Overall, PM2 and RY2 inhibited adhesion most effectively (Table 5).

The adherence of pathogens to the surface of epithelial cells constitutes the first step of infection [18]. LAB are important microorganisms in the vagina because they interfere with pathogen colonisation, thus assisting in the exclusion of urinary tract infections. Therefore, a decrease in vaginal lactobacilli may increase the likelihood of recurrent genitourinary infections [19]. Boris et al. [19] reported that incubation of *Lactobacillus acidophilus* with *Candida albicans* and *G. vaginalis* and vaginal epithelial cells for 30 min revealed a residual rate of 24.44 and 23.19% of *C. albicans* and *G. vaginalis*, respectively. The researchers noted that *L. acidophilus* has a higher affinity than *C. albicans* and *G. vaginalis* for vaginal epithelial cell surface receptors and that lactobacillus accumulates on

pathogenic bacteria, hindering pathogen access to cell surface receptors and thus preventing *C. albicans* and *G. vaginalis* from adhering to vaginal epithelial cells.

Effect of LAB on Urethral Pathogen Invasion

Pathogenic bacteria (10^7 or 10^6 CFU/mL) and LAB (10^8 CFU/mL) were inoculated in a ratio of 1:10 or 1:100 into SV-HUC-1 cells to evaluate the ability of LAB to inhibit the invasion of cells. In a 1:100 ratio completely inhibited the growth and invasion of both pathogens tested. In a 1:10 bacterial ratio, seven LAB strains completely inhibited the invasion of *K. pneumonia* BCRC 10694 into the SV-HUC-1 urothelial cells (Table 6). PM2 and RY2 in the exclusion group inhibited *G. vaginalis* BCRC 17040 invasion; the invasion rate was 2.83 and 34.36%, respectively (Table 6). RY2 in the competition and treatment groups invaded the cells with an invasion rate of 17.61 and 26.79%, respectively.

LAB inhibit pathogen invasion of epithelial cells, thus maintaining the integrity of cell structure and function and preventing clinical infection [20]. LAB have been shown to inhibit urethral pathogen adhesion and invasion into intestinal epithelial cells [21]. Reid and Burton [22] indicated that colonisation of the vagina with *Lactobacillus*

Table 6 Effect of Lactic acid bacteria strains on invasion to SV-HUC-1 cell line by urinary tract pathogens

Urinary tract pathogens	Strains	Exclusion		Competition		Displacement	
		Log CFU/mL	%	Log CFU/mL	%	Log CFU/mL	%
<i>Klebsiella pneumonia</i> BCRC 10694	Control	1.40 ± 0.28	100 ^A	2.73 ± 0.17	100 ^A	2.16 ± 0.15	100 ^A
	PM2	0	0 ^B	0	0 ^B	0	0 ^B
	PM68	0	0 ^B	0	0 ^B	0	0 ^B
	PM78	0	0 ^B	0	0 ^B	0	0 ^B
	PM201	0	0 ^B	0	0 ^B	0	0 ^B
	PM206	0	0 ^B	0	0 ^B	0	0 ^B
	PM229	0	0 ^B	0	0 ^B	0	0 ^B
	RY2	0	0 ^B	0	0 ^B	0	0 ^B
<i>Gardnerella vaginalis</i> BCRC 17040	Control	2.73 ± 0.01	100 ^A	2.68 ± 0.01	100 ^A	2.81 ± 0.10	100 ^A
	PM2	0.74 ± 1.04	2.83 ^C	0	0 ^C	0	0 ^C
	PM68	0	0 ^C	0	0 ^C	0	0 ^C
	PM78	0	0 ^C	0	0 ^C	0	0 ^C
	PM201	0	0 ^C	0	0 ^C	0	0 ^C
	PM206	0	0 ^C	0	0 ^C	0	0 ^C
	PM229	0	0 ^C	0	0 ^C	0	0 ^C
	RY2	2.26 ± 0.08	34.36 ^B	1.19 ± 0.19	17.61 ^B	2.23 ± 0.00	26.79 ^B

Bacteria counts are converted to Log CFU/mL

% means survival rates of *Klebsiella pneumonia* BCRC 10694 and *Gardnerella vaginalis* BCRC 17040

^{A,B,C}Values (%) in the same column (group) with different letters indicate significant difference ($p < 0.05$)

GR-1, B-54 and RC-14 can reduce the risk of bacterial vaginosis and urinary tract infections and maintain normal microbial flora. LAB strains were shown in this study to effectively inhibit the invasion of pathogens in the SV-HUC-1 urothelial cells, which may decrease the incidence of recurrent urinary tract infections or bacterial vaginosis.

Conclusion

In this study, we showed that some LAB supernatants have an inhibitory effect on the growth of *K. pneumonia* BCRC 10694 and *G. vaginalis* BCRC 17040 and their ability to adhere to and invade SV-HUC-1 urothelial cells. The antibacterial substances in the LAB supernatant demonstrated heat resistance but no bacteriostatic effect at pH 7.0, presumably because of the lactic acid metabolism of LAB. The antibacterial activity of the supernatant was affected by the proteolytic enzymes, catalase and α -amylase, which indicates that the antibacterial substances comprised protein or peptides and hydrogen peroxide.

Seven strains of LAB were co-cultured with pathogenic bacteria. During co-cultivation, the survival rate of pathogens and pH of the culture medium gradually decreased. The pH of the culture medium was lowest (4.19) after 4 h of co-culture with PM68. PM78 and PM229 effectively inhibited *K. pneumonia* BCRC 10694, and RY2 had the

strongest inhibitory effect on *G. vaginalis* BCRC 17040. Seven LAB strains decreased the adhesion of pathogens to urethral epithelial cells. PM2 and RY2 exhibited the strongest anti-adhesion activity on *K. pneumonia* BCRC 10694 and *G. vaginalis* BCRC 17040. Seven LAB strains inhibited the invasion of SV-HUC-1 urothelial cells by *K. pneumonia* BCRC 10694 (10^6 and 10^7 CFU/mL) and PM68, PM78, PM201, PM206 and PM229 inhibited *G. vaginalis* BCRC 17040 (10^7 CFU/mL). The results suggest that LAB (PM78, PM229 and RY2) or LAB supernatants could be used in the exclusive or therapeutic treatment of genitourinary infections.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

- Salvatorea S, Salvatoreb S, Cattonia E, Siestoc G, Seratia M, Soricea P, Torellad M (2011) Urinary tract infections in women. Eur J Obstet Gynecol 156:131–136. <https://doi.org/10.1016/j.ejogrb.2011.01.028>
- Dobrindt U, Hacker J (2010) Uropathogens and virulence factors. In: Naber KG, Scaeffler AJ, Heyns CF, Matsumoto T, Shoskes

- DA, Johansen TEB (eds) Urogenital infections, 1st edn. International Consultation on Urological Diseases (ICUD) and European Association of Urology, Arnheim (ISBN: 978-90-79754-41-0)
3. Jacobsen SM, Stickler DJ, Mobley HL, Shirliff ME (2008) Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin Microbiol Rev 21:26–59. <https://doi.org/10.1128/CMR.00019-07>
 4. Damian M, Usein CR, Palade AM, Ceciu S, Cosman M (2009) Molecular epidemiology and virulence characteristics of *Klebsiella pneumoniae* strains isolated from hospital-associated infections. Open Epidemiol J 2:69–78
 5. Harwich MDJ, Alve JM, Buck GA, Strauss JF, Patterson JL, Oki AT, Girerd PH, Jefferson KK (2010) Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. BMC Genom 11:375–386. <https://doi.org/10.1186/1471-2164-11-375>
 6. Schwebke JR, Muzny CA, Josey WE (2014) Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. J Infect Dis 210:338–343. <https://doi.org/10.1093/infdis/jiu089>
 7. Vick EJ, Park HS, Huff KA, Brooks KM, Farone AL, Farone MB (2014) *Gardnerella vaginalis* triggers NLRP3 inflammasome recruitment in THP-1 monocytes. J Reprod Immunol 106:67–75. <https://doi.org/10.1016/j.jri.2014.08.005>
 8. Su CJ (2008) Female lower urinary tract infection. JTUA 19:12–20
 9. Lamb N (2011) Can probiotics reduce urinary tract infections? Clinical Use Probiotics. <https://doi.org/10.1097/CCM.0b013e31826a409c>
 10. Atassi F, Brassart D, Grob P, Graf F, Servin AL (2006) *Lactobacillus* strains isolated from the vaginal microbiota of healthy women inhibit *Prevotella bivia* and *Gardnerella vaginalis* in coculture and cell culture. FEMS Immunol Med Microbiol 48:424–432. <https://doi.org/10.1111/j.1574-695X.2006.00162.x>
 11. Atassi F, Brassart D, Grob P, Graf F, Servin AL (2006) *In vitro* antibacterial activity of *Lactobacillus helveticus* strain KS300 against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. J Appl Microbiol 101:647–654. <https://doi.org/10.1111/j.1365-2672.2006.02933.x>
 12. Uehara S, Monden K, Nomoto K, Seno Y, Kariyama R, Kumon H (2006) A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection. Int J Antimicrob Agents 28:30–34. <https://doi.org/10.1016/j.ijantimicag.2006.05.008>
 13. Chapman CMC, Gibson GR, Todd S, Rowland I (2013) Comparative *in vitro* inhibition of urinary tract pathogens by single- and multi-strain probiotics. Eur J Nutr 52:1669–1677. <https://doi.org/10.1007/s00394-013-0501-2>
 14. Kim SH, Wei CI (2007) Invasiveness and intracellular growth of multidrug-resistant salmonella and other pathogens in Caco-2 cells. J Food Sci 72:72–78. <https://doi.org/10.1111/j.1750-3841.2006.00268.x>
 15. Yang E, Fan L, Jiang Y, Doucette C, Fillmore S (2012) Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. AMB Express 2:2–48. <https://doi.org/10.1186/2191-0855-2-48>
 16. Zhou F, Zhao H, Bai F, Piotr D, Liu Y, Zhang B (2014) Purification and characterisation of the bacteriocin produced by *Lactobacillus plantarum*, isolated from Chinese pickle. Czech J Food Sci 32:430–436. <https://doi.org/10.17221/270/2013-CJFS>
 17. Poppi LB, Rivaldi JD, Coutinho TS, Astolfi-Ferreira CS, Piantino Ferreira AJ, Mancilha IM (2015) Effect of *Lactobacillus* sp. isolates supernatant on *Escherichia coli* O157:H7 enhances the role of organic acids production as a factor for pathogen control. Pesqui Vet Brasil 35:353–359. <https://doi.org/10.1590/S0100-736X2015000400007>
 18. Machado A, Salgueiro D, Harwich M, Jefferson KK, Cerca N (2013) Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. Anaerobe 23:1–4. <https://doi.org/10.1016/j.anaerobe.2013.07.007>
 19. Boris S, Suárez JE, Vázquez F, Barbés C (1998) Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun 66:1985–1989 (PMCID: PMC108154)
 20. Wine E, Gareau MG, Johnson-Henry K, Sherman PM (2009) Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. FEMS Microbiol Lett 300:146–152. <https://doi.org/10.1111/j.1574-6968.2009.01781.x>
 21. Mapple LJ, Tchórzewska MA, Cooley WA, Woodward MJ, La Ragione RM (2011) Lactobacilli antagonize the growth, motility, and adherence of *Brachyspira pilosicoli*: a potential intervention against avian intestinal spirochetosis. Appl Environ Microbiol 77:5402–5411. <https://doi.org/10.1128/AEM.00185-11>
 22. Reid G, Bruce AW (2003) Urogenital infections in women: can probiotics help? Postgrad Med J 79:428–443 (PMCID: PMC1742800)