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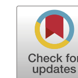
Original Article

Antimicrobial ability and mechanism analysis of *Lactobacillus* species against carbapenemase-producing *Enterobacteriaceae*



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Lactic acid;
In vitro activity

Abstract *Background:* This study aims to investigate the antimicrobial ability and mechanism analysis of *Lactobacillus* species against carbapenemase-producing *Enterobacteriaceae* (CPE).
Methods: Five *Lactobacillus* spp. strains and 18 CPE clinical isolates were collected. Their anti-CPE effects were assessed by agar well diffusion and broth microdilution assay, as well as time-kill test. Finally, the specific anti-CPE mechanism, especially for the effect of organic acids was determined using broth microdilution method.
Results: All of five *Lactobacilli* isolates displayed the potent activity against most CPE isolates with mean zones of inhibition ranging 10.2—21.1 mm. The anti-CPE activity was not affected by heating, catalase, and proteinase treatment. Under the concentration of 50% LUC0180 cell-free supernatant (CFS), lactic acid, and mix acid could totally inhibit the growth of carbapenem-resistant *Klebsiella pneumoniae* (CPE0011), and acetic acid could inhibit 67.8%.

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In contrast, succinic acid and citric acid could not inhibit the growth of CPE0011. While we decreased the concentration to 25%, only lactic acid and mix acid displayed 100% inhibition. In contrast, succinic acid, citric acid and acetic acid did not show any inhibitory effect.
Conclusions: Lactobacillus strains exhibit potent anti-CPE activity, and lactic acid produced by *Lactobacillus* strains is the major antimicrobial mechanism.
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Introduction

Lactobacillus is Gram-positive bacteria, and is also the most common probiotics. As probiotic, it has the beneficial properties of biological therapeutics and host immune-modulating biologicals. In addition to the benefit brought by probiotic, several recent studies showed the antimicrobial effect of *Lactobacillus*.[1–6] The proposed antimicrobial mechanisms of *Lactobacillus* includes nutrient competition, production of inhibitory compounds, immune-stimulation, competition for binding sites, the ability of lowering intestinal pH through the production of lactic acid, acetic acid, formic acid and other acids and the secretion of certain antimicrobial molecules, such as ethanol, fatty acid, hydrogen peroxide and bacteriocins.[7,8]

Previous studies has demonstrated the antibacterial effect of *Lactobacillus* against *Clostridium difficile*,[9] *Escherichia coli*,[10] *Shigella* spp.,[11] *Streptococcus mutans*,[12] *Pseudomonas aeruginosa*,[13] and *Staphylococcus aureus*.[14] Carbapenemase, including new Delhi metallo-β-lactamase (NDM), *Klebsiella pneumoniae* carbapenemase (KPC), and OXA-48 is the major antibiotic resistant mechanism of carbapenem-resistant *Enterobacteriaceae* (CRE).[15,16] The emergence of carbapenemase-producing Enterobacteriaceae (CPE) has become a global health threaten. Gene encoding carbapenemase are typically located on plasmids and horizontal transfer between species is one of the reasons for their rapid widespread dissemination. CPE can cause severe human infection and be associated with high morbidity and mortality; however, CPE infection is difficult-to-treat due to its high resistance to most of the antibiotics.[15–17] Because of its features to remain a potentially endogenous reservoir in the human gut and simultaneously resistance to carbapenems poses a great deal of threat to inhabitants.[15–17] It has been recorded over the last 10–15 years and again that asymptomatic fecal carriage of CPE introduces nosocomial infections.[18,19] Enforcing robust surveillance system, antibiotic stewardship and maintaining unprecedented hygiene are the need of the hour to curb CRE or even CPE.[19] Due to most CRE and even CPE was colonization, therefore, it is urgent to find some useful weapon to inhibit and control the growth and colonization of CRE in gut. Recently, our study[1] showed that some *Lactobacillus* strains exhibit anti- CRE activity and these antimicrobial effects was associated with lower pH. However, the mechanism of *Lactobacillus* against CPE remains unclear. Therefore, this study was conducted to investigate the antimicrobial effect of *Lactobacillus* against CPE, particularly the effect of pH and the role of different organic acid produced by *Lactobacillus*.

Material and method

Bacterial strains and culture conditions

Lactobacillus species

The five lactobacilli including LUC0180 (*Lactobacillus piracies*), LUC0219 (*Lactobacillus plantarum*), LYC0289 (*L. plantarum*), LYC0413 (*Lactobacillus rhamnosus*), and LYC1031 (*L. plantarum*) of Fifty-seven *Lactobacillus* isolates were collect as previously described.[1] In brief, all strains including nine species were obtained from Department of Food Science at the National Chiayi University in Chiayi, Taiwan. Species identification was performed by 16S rRNA gene sequencing. Sequences were compared with the NCBI GenBank database using the basic local alignment search tool (BLAST) search tool to find the closest matches. The five strains were selected because tolerance to pepsin, low pH and bile salts and better antimicrobial ability to carbapenem resistant Enterobacteriaceae.[1] The basic growth media for *Lactobacillus* were de Man-Rogosa-Sharpe (MRS; Oxoid Inc., Ogdensburg, NY, USA) final pH 6.5.

Carbapenemase producing *Enterobacteriaceae* (CPE)

Eighteen clinical strains, including six carbapenem-resistant *E. coli* (CREC), ten *K. pneumoniae* (CRKP) with different pulse field gel electrophoresis (PFGE) genotype and one *Enterobacter cloacae*, one *Morganella morganii* strains were isolated from Chi Mei Medical Center. Species confirmations were performed using the matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (microflex LT, Bruker Daltonics, Bremen, Germany). Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI, USA) were used for bacterial pathogens. The β-Lactamase genes were detected as previously described.[20–23] Briefly, PCR and sequencing were applied to detect and identify the main CPE (KPC, NDM, IMP, VIM, and OXA–48) and ESBL (SHV, TEM, and CTX-M) and AmpC (CMY, DHA)-encoding genes. Amplicons were purified with PCR clean-up kits (Roche Diagnostics, GmbH, Penzberg, Germany) and were sequenced on an ABI PRISM3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA). The *Lactobacillus* spp. and CPE isolates were stored at −80 °C in

Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) before use.

Pulsed-field gel electrophoresis (PFGE)

PFGE for the *E. coli* and *K. pneumoniae* isolates were performed as described previously.[21] In brief, bacterial DNAs were digested using XbaI (New England Biolabs, Beverly, MA, USA). Electrophoresis was carried out for 22 h at 14 μC, with pulse times ranging from 2 s to 40 s at 6 V/cm, using a Bio-Rad CHEF MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The PFGE patterns were visually examined and interpreted according to the criteria of Tenover et al.[24]

Cell-free supernatant (CFS) preparation

The culture supernatants of Lactobacilli were grown in MRS broth (pH 6.5) for 24 h at 37 °C and, then, centrifuged (10,000×*g* for 20 min, 4 °C). The supernatants were sterilized by filtration through a 0.22 μm cellulose acetate filter (Millipore, Billerica, MA, USA) and stored at −80 °C until used.

Well diffusion assay

The well diffusion method was as previously described,[25] MH agar plates were swabbed on the surface with CPE bacterial cultures (McFarland 0.5). Then, 6 mm diameter wells were prepared and 100 μl CFS were loaded in the wells. After 24-h incubation at 37 °C, inhibition zones were recorded. All tests were done in triplication and twice.

Broth microdilution assay

Broth microdilution assay was carried out as previously described,[26] with modifications. Overnight cultures of CPE bacteria were inoculated into fresh MHB media and seeded into 96-well plates (BD Discovery Labware, Bedford, MA, USA). The CFSs were diluted with De Man, Rogosa and Sharpe (MRS) broth and used at different percentages (i.e., 3.13, 6.25, 12.5, 25, 50%) in the final 200 μl volume. Plates were incubated at 37 °C for 24 h and viable pathogenic bacteria was monitored by serial dilution and plating. The inhibition percentage was compared with original culture number. All tests were done in triplication.

Time-kill test in Lactobacilli and CPE co-cultures

The CRKP (CPE0011) and *Lactobacillus* strains were cultured in their respective broth medium at 37 °C for 24 h. The cultures were centrifuged at 10,000×*g*, 22 °C for 10 min to collect the cell pellet. Then, CPE were inoculated at 1×10^6 CFU/ml and lactobacilli at 1×10^5, 1×10^6,

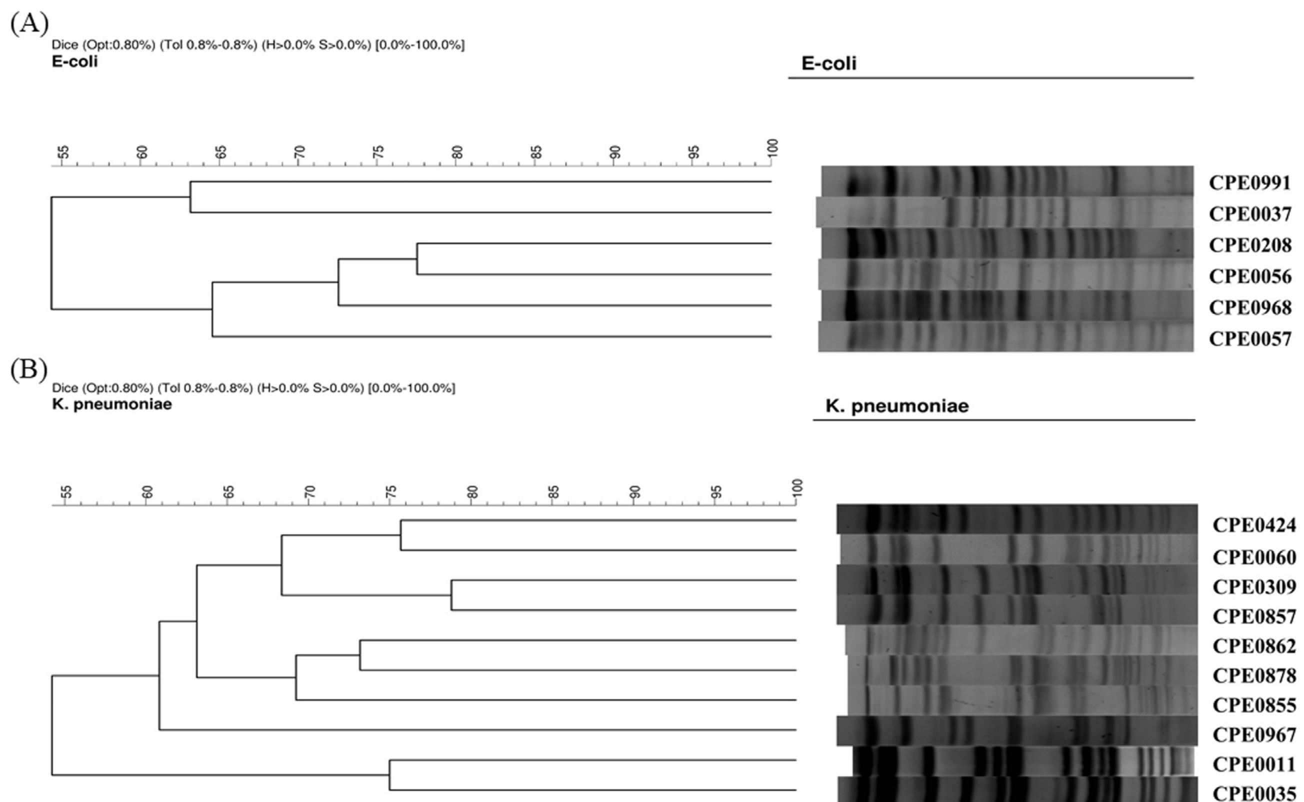

Figure 1. (A) PFGE of 6 isolates of carbapenemase-producing *E. coli* and (B) PFGE of 10 isolates of carbapenemase-producing *K. pneumoninae*. PFGE patterns of both species isolates after restriction with XbaI enzyme. PFGE settings: Similarity coefficient Dice. Optimization: 0.8%, Tolerance: 0.8%, Active zones: [0.0—100.0%]. Isolates that had <80% similarity on the PFGE profiles were considered different types.

Table 1 Gene expression in eighteen CPE isolates.

Isolates	Species	Carbapenemase					Amp-C		ESBL		
		KPC	NDM	IMP	OXA-48	VIM	DHA	CMY	SHV[b]	TEM[b]	CTX-M
CPE0037	*E. coli*	2	—	—	—	—	—	2[a]	—	1	3
CPE0056	*E. coli*	2	—	—	—	—	—	—	—	—	—
CPE0057	*E. coli*	2	1	—	—	—	—	2	—	1	—
CPE0208	*E. coli*	—	5	—	—	—	—	2	—	1	—
CPE0968	*E. coli*	—	1	—	—	—	—	—	—	1	55
CPE0991	*E. coli*	—	5	23	—	—	—	—	—	—	14
CPE0011	*Klebsiella pneumoniae*	2	—	—	—	—	—	—	12	—	65
CPE0035	*Klebsiella pneumoniae*	2	—	—	—	—	—	—	12	1b	65
CPE0060	*Klebsiella pneumoniae*	3	—	—	—	—	—	—	11	—	—
CPE0309	*Klebsiella pneumoniae*	17	—	—	—	—	—	—	12	—	—
CPE0424	*Klebsiella pneumoniae*	17	—	—	—	—	—	—	1	—	—
CPE0855	*Klebsiella pneumoniae*	—	1	—	—	—	1	146	5	1b	15
CPE0857	*Klebsiella pneumoniae*	17	—	—	—	—	—	—	12	—	—
CPE0862	*Klebsiella pneumoniae*	—	1	—	—	—	1	—	11	1b	15
CPE0878	*Klebsiella pneumoniae*	—	1	—	—	—	1	—	1	1b	15
CPE0967	*Klebsiella pneumoniae*	—	1	—	—	—	1	—	31	1	55
CPE0961	*Enterobacter cloacae*	—	1	—	—	—	—	—	12	—	—
CPE1124	*Morganella morganii*	17	—	—	—	—	—	—	—	—	—

[a] CMY-2 with insertion.
[b] SHV-1, TEM-1 and TEM-2 belong narrow-spectrum beta-lactamases, others belong ESBLs.

Table 2 Zone of inhibition (mm) of five *Lactobacillus* isolates against eighteen carbapenemase-producing *Enterobacteriaceae* by well diffusion assays.

No. / Lactobacilli	CPE0011	CPE035	CPE0037	CPE0056	CPE0057	CPE0060	CPE0208	CPE0309	CPE0424
LUC0180	19.0 ± 1.7	19.6 ± 0.5	15.8 ± 0.6	13.7 ± 0.5	15.2 ± 0.7	16.5 ± 0.8	13.7 ± 0.8	16.8 ± 0.3	17.0 ± 0.8
LUC0219	18.6 ± 0.8	20.9 ± 0.3	15.3 ± 0.8	13.4 ± 1.5	15.0 ± 0.9	16.1 ± 0.3	14.9 ± 0.7	16.5 ± 0.6	15.4 ± 0.5
LYC0289	18.4 ± 0.6	21.1 ± 1.3	16.2 ± 0.3	15.4 ± 1.9	15.7 ± 0.4	18.6 ± 0.6	15.9 ± 0.4	18.6 ± 0.8	16.3 ± 1.2
LYC0413	18.2 ± 0.8	20.0 ± 0.2	14.7 ± 0.6	14.0 ± 1.9	15.9 ± 0.2	15.9 ± 0.5	15.6 ± 0.5	18.8 ± 0.7	15.3 ± 0.4
LYC1031	19.8 ± 0.6	20.1 ± 0.5	15.6 ± 0.6	13.6 ± 1.7	15.5 ± 0.8	20.0 ± 0.8	15.5 ± 0.9	16.4 ± 0.4	15.7 ± 1.1
No. / Lactobacilli	**CPE0855**	**CPE0857**	**CPE0862**	**CPE0878**	**CPE0961**	**CPE0967**	**CPE0968**	**CPE0991**	**CPE 1124**
LUC0180	18.3 ± 0.6	19.4 ± 0.5	17.0 ± 0.1	17.9 ± 0.3	10.7 ± 0.5	17.8 ± 0.7	15.8 ± 0.6	14.7 ± 0.3	15.3 ± 0.7
LUC0219	18.3 ± 0.5	19.1 ± 0.3	17.4 ± 0.5	17.4 ± 0.3	10.8 ± 0.2	17.8 ± 0.6	15.1 ± 0.8	14.5 ± 0.4	15.7 ± 0.2
LYC0289	16.8 ± 0.4	19.4 ± 0.8	18.2 ± 0.6	17.9 ± 0.4	10.8 ± 0.3	18.7 ± 1.1	16.2 ± 0.4	15.4 ± 0.5	16.1 ± 0.3
LYC0413	15.3 ± 0.5	16.6 ± 0.3	16.2 ± 0.7	17.9 ± 0.8	13.7 ± 0.4	17.6 ± 0.4	16.7 ± 1.3	14.1 ± 0.5	13.6 ± 0.4
LYC1031	17.6 ± 0.6	18.2 ± 0.9	18.6 ± 0.6	18.3 ± 0.8	10.2 ± 0.9	18.9 ± 0.4	16.1 ± 1.1	12.8 ± 0.6	15.5 ± 0.4

1×10^7 or 1×10^8 CFU/ml into mono-cultures or 1×10^6 CFU/ml pathogenic bacteria co-culture with different CFU/ml lactobacilli in tubes containing 10 ml of MRS-MH broth (1:1).[27] Mono-cultures and co-cultures were incubated at 37 °C for 48 h. Samples were collected at 0, 2, 4, 8, 24 and 48 h for the determination of viable cell count and pH measurements. A 0.5 ml culture medium of each sample was used to prepare serial dilutions that were poured 100 μl onto each the appropriate agar plate; MRS agar was used for *Lactobacillus* spp., while MacConkey Agar was used for CPE. Plates were incubated at 37 °C for 24 h and colonies were counted. The assay detection limit was 100 CFU/ml.[28] All tests were done in triplication.

Antimicrobial mechanism analysis

Broth microdilution method with different treatment culture supernatant of LUC0180 was used to analysis the passible antimicrobial mechanism. CFS-A were untreated CFSs, CFS-B were heating at 121 °C for 15 min, CFS-C were catalase digestion (1 mg/ml; Sigma–Aldrich Corporation, USA) at 22 °C for 2 h to eliminate the hydrogen peroxide, CFS-D were digestion at 37 °C for 2 h with 2 mg/ml proteinase K, then, inactive proteinase K activity at 100 °C for 2 min, and CFS-E were neutralized with 2 M NaOH to pH 6.5. MRS pH 3.79 (equal to culture supernatant of LUC0180) and MRS pH 6.5 were also tested in this study for control. All

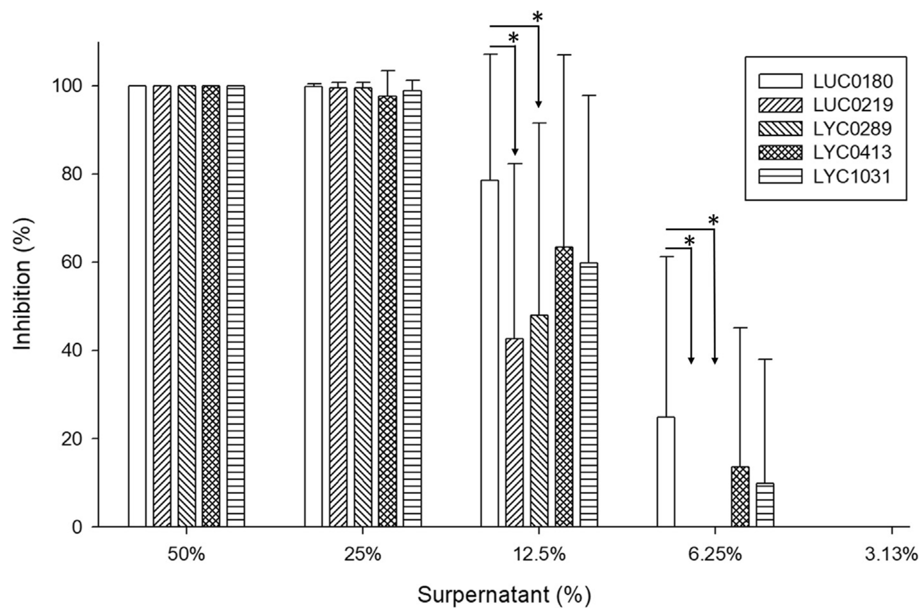

Figure 2. The inhibition percentage of serial dilution of five Lactobacilli supernatant co-cultured with eighteen CPE by broth microdilution. The CFSs were diluted with MRS broth and used at different percentages (i.e., 50, 25, 12.5, 6.25, 3.13%). The inhibition percentage was compared with original culture number (*. $P < 0.05$).

samples were diluted in normal saline and used at 50%, 25% and 12.5% cultured with 10^6 CFU/ml CPE0011 in the final volume (200 μl) 37 °C for 24 h. The inhibition percentage was calculated as above described. All tests were done in triplication.[29]

Measurement of organic acid concentration

The CSF of Lactobacilli overnight culture was prepared as descripted above. The organic acid concentrations were detected by high-performance liquid chromatography (HPLC), which was performed by the Food Industry Research and Development Institute (Hsinchu, Taiwan). The organic acids included acetic acid, citric acid, lactic acid, malic acid, and succinic acid.

Determination of antimicrobial activities of organic acids

The 1.6 mg/ml succinic, 1.6 mg/ml citric, 3.8 mg/ml acetic, and 23.2 mg/ml lactic acid (Sigma–Aldrich Corporation, USA) were prepared with MRS (mimic the organic acid concentration in LUC0180) adjusted to pH 5.0. Mix group were including all acid above in the same concentration. In addition, MRS was adjusted to pH 3.79 with 2 N HCl (mimic the pH as LUC0180). And the MRS pH 5.0 and pH 6.5 as the acidity control. All samples were diluted at 255% and 12.5% cultured with 10^6 CFU/ml CPE0011 in the final volume (200 μl) 37 °C for 24 h. The inhibition percentage was calculated as above described. All tests were done in triplication.

Statistical analysis

The paired t-test was used for statistical analysis. P-value for statistical significance for all analysis is defined as $P < 0.05$.

Results

The carbapenemase, Amp-C, and ESBL gene expression of CPE

All of CPE isolates belonged to different PFGE patterns (Fig. 1), and their resistance mechanisms were summarized in Table 1. Among six *E. coli* isolates, bla_{NDM-1} was the most common resistant gene encoding carbapenemase, followed by bla_{KPC}. In contrast, bla_{KPC} was the most common resistant gene encoding carbapenemase, followed by bla_{NDM-1} among ten *K. pneumoniae* isolates. None of all CPE isolates carried OXA-48 and VIM carbapenemase genes. In addition, Amp-C or ESBL gene was found among fourteen CPE isolates. Two *E. coli* strains carry more than one carbapenemase - one with concomitant KPC-2 and NDM-1, and another with NDM-5 and IMP 23.

The CPE inhibition zone sizes of five lactobacilli

All of five Lactobacilli isolates displayed the potent activity against most CPE isolates with mean zones of inhibition ranging 10.2–21.1 mm (Table 2). However, no difference was found among the inhibition zones of the five *Lactobacillus* spp. against CPE (all $p > 0.05$).

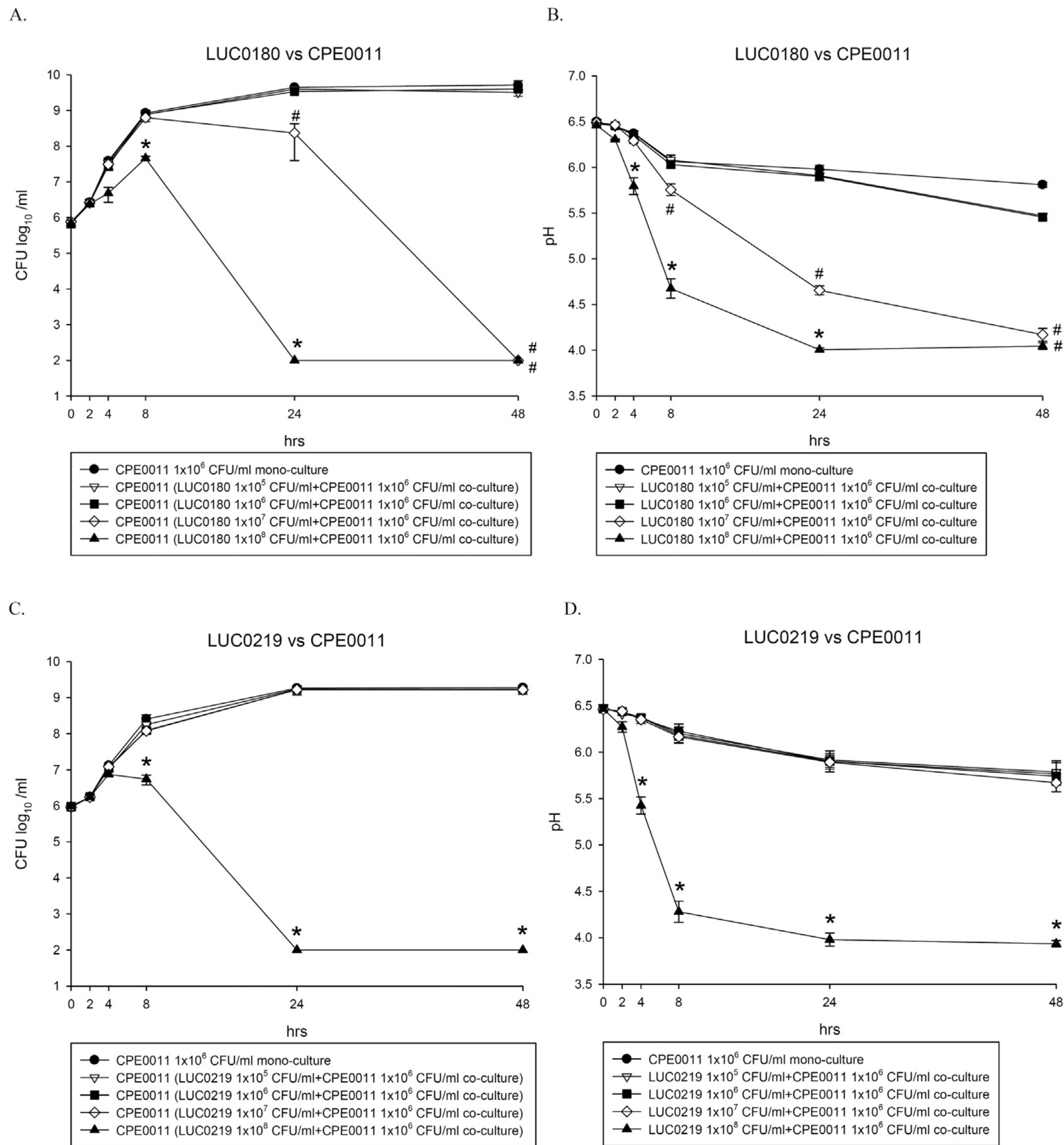

Figure 3. Time-killing curve (A, C, E, G, I) and the association between pH (B, D, F, H, J) and antibacterial effects for five different concentrations of *Lactobacillus* isolates co-cultured with CPE0011. Group A. CPE0011 1×10^6 CFU/ml mono-culture, Group B. *Lactobacillus* 1×10^5 CFU/ml + CPE0011 1×10^6 CFU/ml co-culture, Group C. *Lactobacillus* 1×10^6 CFU/ml + CPE0011 1×10^6 CFU/ml co-culture, Group D. *Lactobacillus* 1×10^7 CFU/ml + CPE0011 1×10^6 CFU/ml co-culture, Group E. *Lactobacillus* 1×10^8 CFU/ml + CPE0011 1×10^6 CFU/ml co-culture. Data are the mean $\pm$ SD in triplicate (*. $P < 0.05$, compare to Group A, B, C, D. #. $P < 0.05$, compare to Group A, B, C. &. $P < 0.05$, compare to Group A, B. $. $P < 0.05$, compare to Group A.).

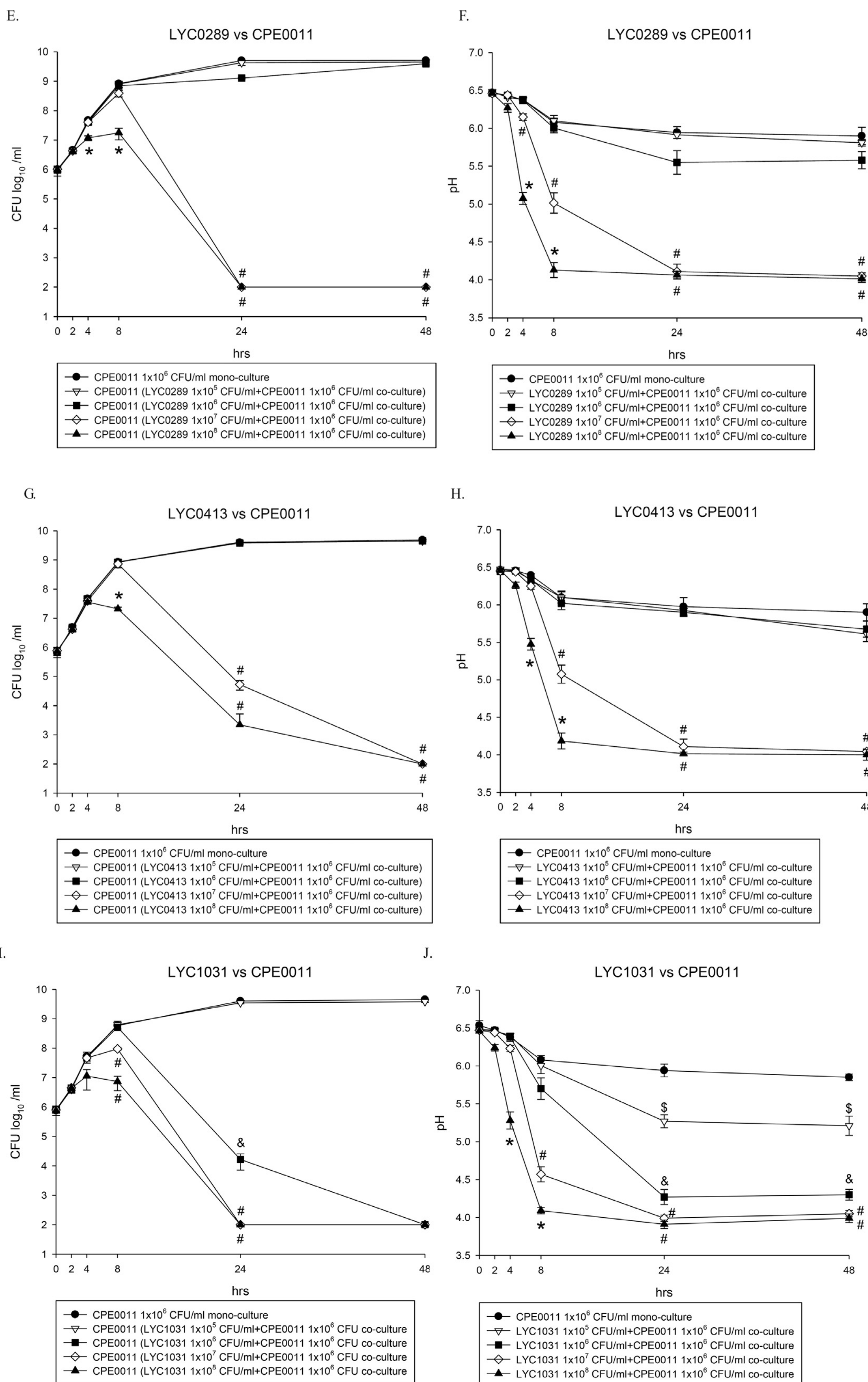

Figure 3. (*continued*).

Table 3 Organic acid concentration (mg/ml) analysis of five lactobacilli cell free supernatant.

	pH	Total organic acid	Succinic acid	Citric acid	Malic acid	Acetic acid	Lactic acid
LUC0180	3.79	30.2	1.6	1.6	ND	3.8	23.2
LUC0219	3.84	28.2	ND	1.0	ND	3.9	23.3
LYC0289	3.75	32.3	ND	1.4	ND	3.9	27
LYC0413	3.85	27.2	1.3	1.5	ND	3.5	20.9
LYC1031	3.75	30.6	ND	1.3	ND	3.8	25.5

ND. Non-detectable.

The inhibition percentage against CPE of five lactobacilli

Initial, 100% of CPE were inhibited using 50% of all five lactobacilli supernatant, and 97.8%–99.9% were inhibited under 25% supernatant. While the concentration of supernatant was decreased to 12.5%, only 45.0%–78.8% could be inhibited. Under the concentration of 6.25%, no inhibition was noted for LUC0219 and LUC0289, and only 23.6%, 12.8%, and 9.38% could be inhibited by LUC0180, LYC0413, and LYC1031, respectively. Finally, none of CPE could be inhibited by 3.13% of any lactobacilli supernatant (Fig. 2).

The antibacterial effect and the association with pH value in time killing test

Fig. 3 showed the results from time-killing test and the association between the antibacterial effect and pH. Overall, we can find the growth of CPE0011 was significantly inhibited by high concentration of lactobacilli (10^7 or 10^8 CFU/ml) (Fig. 3A, C, E, G and I). Moreover, the decrease in pH was observed in the co-culture with 10^7 or 10^8 CFU/ml lactobacilli and CPE (Fig. 3B, D, F, G and H).

The organic acids analysis of lactobacilli cell free supernatant

Using HPLC, lactic acid was the highest amount of organic acid in the five lactobacilli supernatant with the concentration ranged from 20.9 to 27.0 mg/ml. In addition, the concentration of acetic acid and citric acid ranged from 3.5 to 3.9 mg/ml and 1.0–1.6 mg/ml among five lactobacilli supernatant, respectively. Succinic acid was only detected in LUC0180 and LYC0413, but not in three other lactobacilli supernatant. Finally, malic acid was not detected in any of lactobacilli supernatant (Table 3).

The antimicrobial mechanisms of lactobacilli cell free supernatant

When the LUC0180 CSF were treated with heating at 121 °C for 15 min, catalase breaks down hydrogen peroxide or proteinase K digest protein, the inhibitory effects were not be changed when comparing with untreated group at 25% CSF (Fig. 4). When we adjust the pH concentration of MRS with HCl to 3.79, the same pH level of LUC0180 CSF, only 60.6% inhibitory effect was noted. However, the inhibitory effect can be totally disappeared when we neutralized LUC0180 CSF with NaOH to pH 6.5. On the other hand, when the CSF concentration was diluted to 12.5%, the inhibitory effect still could be 100% preserved when heating at 121 °C for 15 min and treated with proteinase K. Besides the inhibitory effect still could be partially 93.4% preserved when treated with catalase. However, at such a low CSF concentration, the inhibitory effect was totally lost at pH 3.79 MRS (Fig. 4). In addition, the inhibitory effects of neutralized CFS and MRS with pH of 6.5 were lower than those of other conditions, including post treatment of autoclave, catalase, and proteinase K irrespective of CSF concentration (all $p < 0.05$). Moreover, the inhibitory effects of MRS with pH of 3.79 were lower than those undergoing autoclave, catalase and proteinase K management in the CSF concentration of 25% and 12.5%.

The antimicrobial activities of organic acids

Under the concentration of 50% CSF, lactic acid, and mix acid could totally inhibit the growth of CPE0011, and acetic acid could inhibit 67.8%. In contrast, succinic acid and citric acid could not inhibit the growth of CPE0011 (Fig. 5). While we decreased the concentration to 25%, only lactic acid and mix acid displayed 100% inhibition. In contrast, succinic acid, citric acid and acetic acid did not show any inhibitory effect (Fig. 5).

Discussion

This study investigating the antibacterial effect of Lactobacilli against CPE had several significant findings. First, five selected Lactobacilli showed potent *in vitro* activity against CPE, which was demonstrated by three different methods including agar well diffusion method, broth microdilution method and time-killing method. These findings are consistent with our previous study,[1] and suggests that the potential usefulness of *Lactobacillus* strains for inhibiting CPE. In addition to limited antibiotic options for treating multidrug-resistant organisms (MDROs), *Lactobacillus* spp. as a probiotic may play a promising role in this era of rapidly growing burden of MDROs. However, further *in vivo* study and large-scale study are warranted to clarify this issue before it can be safely applied in this field.

Second, this study also tried to elucidate the antimicrobial mechanism of *Lactobacillus* spp. Initially, we found the significant association between pH and antibacterial effect of *Lactobacillus* against CPE and pH neutralization could eliminate the antimicrobial activity of CFS. However, the low pH was not absolutely the only mechanism according to our result. The presence of organic acid especially lactic and acetic acid is the major inhibitory

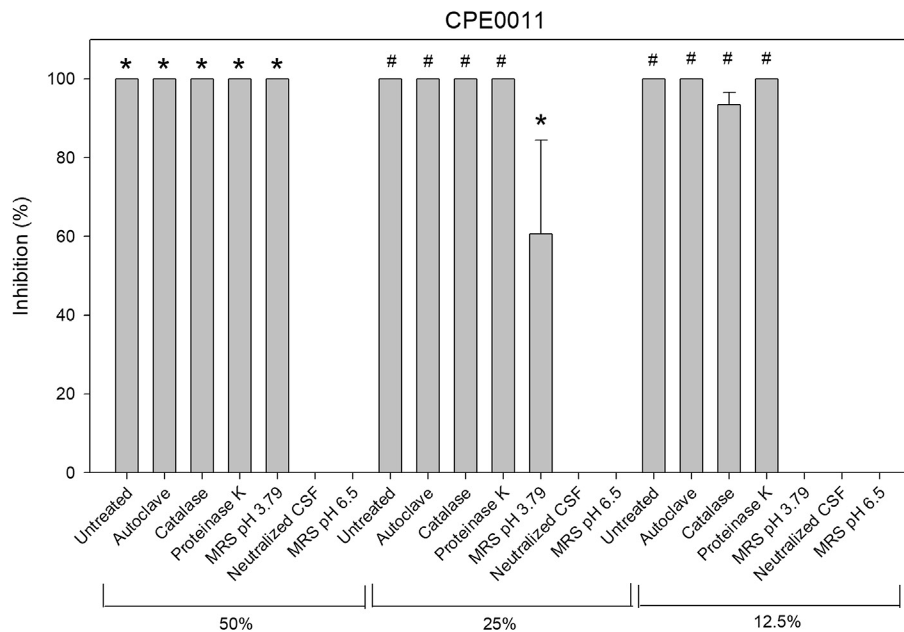

Figure 4. The inhibition percentage of 10^6 CFU/ml CPE0011 co-cultured with different concentrations (50, 25 and 12.5%) of LUC0180 CSF were treated with heating (autoclave), catalase or proteinase K, pH 3.79 MRS and NaOH neutralized (*. $p < 0.05$ compare to neutralized CFS and MRS pH 6.5. #. $p < 0.05$ compare to MRS pH 3,79, neutralized CFS and MRS pH 6.5).

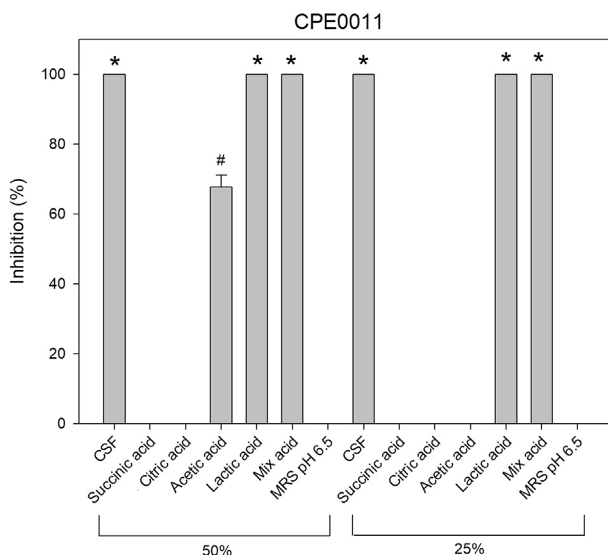

Figure 5. Succinic, citric, acetic and lactic acid alone or all mixed with MRS (mimic the organic acid concentration in LUC0180) adjusted to pH 3.79 and diluted at 50% and 25% co-cultured with 10^6 CFU/ml CPE0011. The inhibition percentage was calculated (*. $p < 0.005$ compare to succinic acid, citric acid, acetic acid, and MRS pH 6.5. #. $p < 0.05$ compare to succinic acid, citric acid, and MRS pH 6.5).

mechanism. Besides, the antimicrobial activity was not affected by heating, catalase, and proteinase treatment. It means the inhibitory effect of H_2O_2 and bacteriocins was not obvious. This finding was consistent with previous study that *L. plantarum* cultures to inhibit pathogens grow depends on a pH-lowering effect of supernatant and/or on the presence of organic acid.[29] Furthermore, we found that lactic acid was the major component of organic acid, and lactic acid was the most active product of *Lactobacillus* spp. against CPE. In contrast, the amount and anti-CPE activity of succinic acid, and citric acid were much less than lactic acid. The similar findings have been observed in previous studies. Tejero-Sariñena et al.[30] showed that lactic and acetic acid were the principal end product of probiotic metabolism and the production of organic acid from glucose fermentation and consequent lowering of culture pH may be the antimicrobial mechanism of probiotics. De Keersmaecker et al.[31] reported that the strong antimicrobial activity of *L. rhamnosus* GG against *Salmonella* was mediated by lactic acid. In summary, lactic acid is the principle organic acid, produced by *Lactobacillus* spp. and exert the antimicrobial effect through lowering pH.

To our knowledge, the present work is the first study about the inhibitory mechanism of *Lactobacillus* spp. against CPE. Our findings emphasized the critical role of lactic acid production by *Lactobacillus* in the antimicrobial mechanism. In the era of high prevalence CPE, especially colonization in gut, extensive use of *Lactobacillus* spp. to control and inhibit the colonization may be considered in future. Further *in vivo* study should be carried to document such effect someday.

Declaration of Competing Interest

The authors declare no conflict of interests.

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