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Lactic acid bacteria inhibit the growth of *Vibrio parahaemolyticus* and the invasion of Caco-2 cells

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ABSTRACT: *Vibrio parahaemolyticus* is a major cause of foodborne illnesses worldwide resulting from the consumption of raw seafood. Lactic acid bacteria (LAB) influence the health of human intestine by enhancing immune system function and acting as antibacterial agents. In this study, 7 LAB (PM12, PM14, PM63, PM212, PM222, BCRC14735, and BCRC17010) were found to adhere to the intestinal epithelial Caco-2 cells as well as to inhibit the growth of *V. parahaemolyticus*. The viability of Caco-2 cells infected with *V. parahaemolyticus* was tested by the lactate dehydrogenase (LDH) assay. Our results indicated that PM12, PM14, PM63, PM212, BCRC14735 and BCRC17010 inhibited *V. parahaemolyticus* BCRC10806 and BCRC12865. While, PM222 could exhibit the maximum comparative adherence ability to Caco-2 cells. The LDH assay results revealed that 5 LAB (PM12, PM14, PM63, PM212, and PM222) significantly decreased the cytotoxicity induced by the *V. parahaemolyticus* BCRC10806 and BCRC12865 after 2 h of incubation. In the competition assay, the prevention group demonstrated inhibitory effects against two *V. parahaemolyticus*. Besides, we noted that these 7 LAB inhibited the invasion of *V. parahaemolyticus* into the Caco-2 cells.

KEYWORDS: *Vibrio parahaemolyticus*, lactic acid bacteria, adherence, invasion

INTRODUCTION

Vibrio parahaemolyticus, a gram-negative bacterium, produces more than three types of hemolysin virulence factors[1]. Its prevalence is widespread across oceans, coasts, estuaries, and swamps worldwide, and it coexists with plankton and large marine animals as well as with other living beings and inanimate objects[1]. The major regions where poisoning by *V. parahaemolyticus* is observed are Japan and East Asian countries, although the frequency is lower in some of the Asian countries possibly due to differences in the form of aquatic product consumption[2]. *V. parahaemolyticus* is mainly detected in raw fish and shellfish. The common sources of infection are clams, oysters, crabs, shrimps, and lobsters. *V. parahaemolyticus* occurrence in fresh fish and shellfish during the summer is more than 90%, and the consumption of these fishes causes food poisoning when the number of bacteria counts on the fish exceed 10^5 CFU/g.

In recent years, *V. parahaemolyticus* possessing a type III secretion system (T3SS) has been found. *In vitro* studies have confirmed that T3SS induces cytotoxicity in Caco-2 cells[3]. Park et al[4] reported that T3SS1 produces cytotoxins in human cells, whereas T3SS2 produces endotoxins in animal infection models and cytotoxins in the gut. Additionally, Zhang et al[5] indicated that T3SS2 is involved in the invasion of *V. parahaemolyticus* into human cells. Akeda et al[6] demonstrated that some isolated *V. parahaemolyticus* not only produces toxins but also invades intestinal cells. *V. parahaemolyticus* was revealed to invade Caco-2 cells associated with Rho, Rac, and Cdc42 proteins[7]. Typical clinical symptoms of *V. parahaemolyticus*-induced food poisoning are acute dysentery and abdominal pain accompanied by diarrhea, nausea, vomiting, fever, shivering, and watery stools[8,9]; also, some patients complain of mucus or blood in their stools, which lowers their blood pressure and may eventually result in unconsciousness, convulsions, paleness, and even death[10,11].

Satish Kumar et al[12] reported that *Lactobacillus plantarum* AS1 can inhibit the adsorption of *Vibrio* spp. by HT-29 intestinal cells via its antimicrobial activity by producing bacteriocins, lactic acid, and

exopolysaccharides. Yang et al[13] also demonstrated that LAB can delay the intestinal fluid accumulation and villus damage caused by *V. parahaemolyticus* in mice. The authors further reported that strains with stronger adsorption ability are more protective than those with stronger antibacterial ability, indicating that the adsorption capacity is more important[14]. Shirazinejad et al[15] soaked fresh shrimp in 3.0% lactic acid for 10 min and observed that it effectively inhibited the growth of pathogenic bacteria. The cell-free supernatant (LBP102) of *L. plantarum* NTU 102 was found to be effective against *V. parahaemolyticus*[16].

The present study aimed to screen for LAB from pickled vegetables and from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and evaluate their effects on the growth inhibition and invasion of *V. parahaemolyticus* into Caco-2 epithelial cells.

MATERIALS AND METHODS

Bacteria and cell culture condition

A total of 386 strains, comprising 86 LAB purchased from BCRC, and 300 LAB isolated from pickled vegetables were used in this study. LAB were cultured in *Lactobacillus* MRS broth (Difco; Detroit, Michigan, USA) supplemented with 0.05% (w/v) L-cysteine and incubated at 37 °C for 20 h. The cultured cells were then transferred into MRS broth containing 25% (v/v) glycerol and frozen at −80 °C. *V. parahaemolyticus* purchased from BCRC was cultured in Tryptic soy broth (TSB) and Tryptic soy agar (TSA) supplemented with 2.5% (w/v) NaCl at 37 °C for 18 h. *V. parahaemolyticus* was then transferred into TSB supplemented with 2.5% NaCl and 25% glycerol and then frozen at −80 °C.

Human rectal cancer cells (Caco-2) purchased from BCRC were subsequently cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) nonessential amino acid transferrin. All media and supplement were obtained from GIBCO BRL Laboratories (NY, USA).

The inhibition zone of LAB

LAB (1 ml) were cultured for 20 h and centrifuged at 8000 rpm for 10 min, then filtered through a 0.22-μm filter to obtain the supernatant, which was stored at −20 °C until further use. In this study, *V. parahaemolyticus* incubated overnight in a 2.5% NaCl-supplemented TSB were diluted to approximately 10^7 CFU/ml[17,18] and spread on the agar. A sterile 1-ml tip was used to make a 9-mm-diameter well in the agar plate. LAB supernatant (100 μl) was transferred into the well, and the plate was incubated at 4 °C for 2 h, followed by incubation at 37 °C for 12 h and a measurement of the inhibition zone diameter.

The bacteriostasis ability of LAB was categorized into 4 grades depending on the size of the zone of inhibition. The zone of inhibition was defined according to the zone diameter as: −, ⩽ 11 mm; +, 12–16 mm; ++, 17–22 mm; and +++, ⩾ 23 mm[17]. Also, the LAB and its supernatant were adjusted to pH 7.2 and heated at 100 °C for 15 min. They were then diluted twice, and the inhibition zone test was performed to assess the ability of the LAB to inhibit *V. parahaemolyticus*.

Antimicrobial testing of LAB and *V. parahaemolyticus* co-culture

Based on the method of Varma et al[18], the antibacterial activity was performed by culturing 1 ml of *V. parahaemolyticus* (10^5 CFU/ml), 1 ml LAB (10^7 CFU/ml), 4 ml TSB, and 4 ml of 2.5% NaCl-containing MRS broth. Above mediums were mixed (total 10 ml) afterwards then culture for 4 h at 37 °C. After 4 h, the cells were centrifuged at $1000 \times g$, washed twice with phosphate-buffered saline (PBS), serially diluted, and then plated on a Petri dish with thiosulfate-citrate-bile salts-sucrose (TCBS) agar. The bacteria were then incubated overnight at 37 °C; then, the number of *V. parahaemolyticus* colonies was counted. *V. parahaemolyticus* colonies appeared blue-green on differential TCBS agar medium. The survival of *V. parahaemolyticus* was calculated using the following equation:

$$\text{Survival}\,(\%) = \frac{\text{No. after co-culture with LAB}}{\text{No. after co-culture with MRS}} \times 100$$

Antibiotic susceptibility of *V. parahaemolyticus*

V. parahaemolyticus was diluted to 10^7 CFU/ml. The cells were stained with a sterile cotton swab and spread uniformly on an approximately 4-mm-thick Mueller-Hinton agar plate. Once the bacterial suspension dried, sterile antimicrobial disc (Disc; Oxoid) were placed on the surface of the agar plate using sterile forceps and gently pressed to ensure that it completely contacted on the agar plate surface and to ensure error-free measurement of the inhibition zone. The antimicrobial discs were placed at ⩾1.4 cm away from the edge of the dish and at intervals of ⩾2.2 cm between the discs. After incubation at 37 °C for 14–16 h, the clear zone

around the discs was measured. The following 9 antimicrobial discs were used in the experiment: ampicillin (10 μg), kanamycin (30 μg), tetracycline (30 μg), penicillin G (10 units), neomycin (15 μg), streptomycin (10 μg), gentamicin (30 μg), chloramphenicol (30 μg), and spiramycin (100 μg).

Competitive adhesion assay of LAB and *V. parahaemolyticus* on Caco-2 cells

The methods of Gueimonde et al[19] and Satish Kumar et al[12] were adopted, albeit with some modifications, for the competitive adhesion assay. Briefly, 1 ml each of LAB (10^9 CFU/ml) and *V. parahaemolyticus* (10^7 CFU/ml) were mixed and subjected to the following conditions: initial centrifugation at $9500 \times g$ for 10 min and discarding of the supernatant, addition of 1 ml PBS and vortex, final centrifugation and removal of supernatant, washing of cells, and addition of a 1 ml cell culture medium.

Subsequently, 1 ml of the Caco-2 cell suspension was added to each 24-well culture plate (at a cell concentration of 2×10^5 cells/ml), followed by uniform mixing and incubation at 37 °C for 2 days in a 5% CO_2 incubator (Forma Direct Heat CO_2 Incubator, Thermo). After confirmation of complete cell attachment, the old medium was aspirated and the cells were washed twice with PBS. Next, 800 μl of the fresh medium, 100 μl each of LAB (for the control group, 100 μl of cell culture medium), and *V. parahaemolyticus* were added, followed by centrifugation of the 24-well culture plate at $90 \times g$ for 2 min at 37 °C under 5% CO_2. The culture fluid was aspirated again and the cells were carefully washed twice with PBS. Cells were subsequently diluted by adding 1 ml of 0.25% (w/v) trypsin/EDTA and then scraped using a sterile tip. To these cells, 9 ml of PBS was added, followed by serial dilution, pour plating via the TCBS method, and incubating overnight at 37 °C; subsequently, the *V. parahaemolyticus* colonies were counted. *V. parahaemolyticus* grew on the differential medium TCBS agar as blue-green colonies. The survival of *V. parahaemolyticus* was calculated using the following equation:

$$\text{Survival}\,(\%) = \frac{\text{No. in the presence of LAB}}{\text{No. in the control group}} \times 100$$

The competitive adhesion assay was conducted in three experimental modes: (1) the exclusion group, the LAB was added for 1 h, after addition of *V. parahaemolyticus* for 1 h; (2) the competition group, LAB and *V. parahaemolyticus* were added simultaneously for adhesion and incubated for 1 h; and (3) the displacement group, *V. parahaemolyticus* was added before the addition of LAB (for adhesion for 1 h) and incubated for 1 h.

Assay of the inhibition of the invasion of *V. parahaemolyticus* into Caco-2 cells by LAB

Based on the method of Akeda et al[6,7] and Zhang et al[5], 1 ml of the cell suspension was added into each 24-well culture plate (cell concentration adjusted to 2×10^5 cells/ ml), mixed uniformly, and incubated at 37 °C under 5% CO_2 for 2 days. After confirming that the cells were completely attached, the old medium was aspirated, added 200 μl of glutaraldehyde for 10 min, and washed twice with PBS. Next, 800 μl of fresh medium, 100 μl each of LAB (10^9 CFU/ml) (or control group: 100 μl of fresh medium), and 100 μl *V. parahaemolyticus* (10^7 CFU/ml) were added. Then centrifuged the resulting mixture at 1000 rpm for 2 min and incubated at 37 °C under 5% CO_2 for 3 h. After carefully washing twice with PBS, 1 ml of kanamycin containing 100 μg/ml (cell medium without FBS) was added to each well, followed by incubation at 37 °C under 5% CO_2 for 1 h and washing twice with PBS. After 10 min of 0.1% (v/v) Triton X-100 treatment, the lysate was transferred into 9 ml PBS and serially diluted. Suitable dilutions were then plated on a Petri dish with TCBS agar. Following overnight incubation, the number of *V. parahaemolyticus* colonies was counted. *V. parahaemolyticus* grew on differential TCBS agar medium as blue-green colonies. The *V. parahaemolyticus* inhibition was calculated as follows:

$$\text{Inhibition}\,(\%) = \left[1 - \frac{\text{No. in the presence of LAB}}{\text{No. in control group}}\right] \times 100$$

Lactate dehydrogenase (LDH) analysis

LDH exists in the cytoplasm. When cell damage occurs, LDH will release. Quantification of the amount of LDH can allow the determination of the degree of cell injury.

LDH was measured according to the method of Matlawska-Wasowska et al[20] using the CytoScanTM LDH Cytotoxicity Assay Kit (G-Biosciences, USA). In brief, 100 μl of a Caco-2 cell suspension was added to each 96-well culture plate (cell concentration was adjusted to 10^4 cells/100 μl), uniformly mixed, and then incubated at 37 °C under 5% CO_2 for 24 h. After confirmation of complete cell attachment, the medium was aspirated and washed twice with PBS, and 100 μl each of *V. parahaemolyticus* (10^6 CFU/ml) and LAB (10^8 CFU/ml), were added. After incubating for 1 or 2 h under 5% CO_2, the cell suspension was centrifuged at 1100 rpm for 5 min. The supernatant (50 μl) was pipetted into a fresh 96-well plate, and 50 μl of the substrate mix was

added to each well, followed by incubation in the dark at 37 °C for 20 min. Then, 50 µl of the stop solution was added to each well and the absorbance was read at 490 nm. Cytotoxicity was calculated as follows:

$$\text{Cytotoxicity}\,(\%) = \left[\frac{\text{Experimental} - \text{Control}}{\text{Lysis}}\right] \times 100$$

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (version 20.0) software package. The experimental results were analyzed by one-way analysis of variance and expressed as mean ± SD. The differences between the mean values of each experimental group were tested using the Duncan's Multiple Range Test, $p < 0.05$ was considered as statistical significance.

RESULTS AND DISCUSSION

LAB inhibited the growth of *V. parahaemolyticus*

In this study, the zone of inhibition was calculated by the diffusion method to analyze 386 LAB sourced from BCRC and plant pickles. Table 1 shows that 25 strains of LAB supernatant inhibited *V. parahaemolyticus* BCRC10806 and BCRC12865 with + inhibitory capacity, and the maximum inhibition zone observed was 15 mm. In our previous study, we indicated that LAB can inhibit *V. parahaemolyticus*-mediated inflammatory responses and can also effectively inhibit the growth of *V. parahaemolyticus* in seafood products[21].

Effect of LAB cultured medium and different treatments of the supernatant on the inhibitory capacity of *V. parahaemolyticus*

From this study, 10 LAB (PM12, PM14, PM63, PM80, PM81, PM114, PM120, PM206, PM212, and BCRC17010) showed first-class inhibitory effect on *V. parahaemolyticus* BCRC10806 and BCRC12865, with a maximum inhibition zone of 13 mm (Table 2).

Nine strains of LAB (PM14, PM81, PM84, PM114, PM119, PM120, PM206, PM212, and BCRC17010) showed a first-class inhibitory effect on *V. parahaemolyticus* BCRC10806 after heating at 100 °C for 15 min. The inhibition zone of PM212 reached 15 mm (Table 2). Four strains of LAB (PM81, PM120, PM206, and BCRC17010) showed a first-class inhibitory effect against *V. parahaemolyticus* BCRC12865, with an inhibition zone of 12 mm (Table 2). Only one strain (PM63) showed a first-class inhibitory effect on *V. parahaemolyticus* BCRC10806 after the LAB supernatant was heated at 100 °C for 15 min (Table 2). The above results demonstrated a decrease in the bacteriostatic capacity of heated LAB or even the loss of bacteriostatic effect. It was found that the antibacterial capacity of *L. rhamnosus* supernatant heated at 90 °C for 20 min decreased, suggesting that the proteins of the antibacterial ingredients may be denatured by heating[22].

Gopal et al[23] revealed that the lactic acid secreted by LAB and the small peptides possess a synergistic effect on bacteriostasis. Therefore, the small peptide molecules of LAB showed effective bacteriostasis under acidic condition. Here, when the LAB and supernatant were adjusted to pH 7.2, the inhibitory activity for *V. parahaemolyticus* was lost (Table 2). Also, two dilutions of LAB and supernatant revealed no inhibition for the two strains of *V. parahaemolyticus* (Table 2). Kaur et al[24] reported that the culture supernatant of lactobacilli inhibited biofilm formation in *Vibrio cholerae* by more than 90%, but neutralization of the pH of culture supernatant also abrogated their antimicrobial ability against *V. cholera*.

LAB and *V. parahaemolyticus* co-culture for antibacterial test

LAB (10^8 CFU/ml) and *V. parahaemolyticus* (10^6 CFU/ml) were diluted to a 100 : 1 ratio in a culture tube for the inhibition test. Table 3 shows that the residual rates of *V. parahaemolyticus* decreased for the 15 strains of LAB co-cultured with the two strains of *V. parahaemolyticus* (BCRC10806 and BCRC12865) for 4 h. Additionally, after 2 h of co-culture, the survival of *V. parahaemolyticus* decreased significantly; after 3 h, the survival was approximately zero, indicating that the growth of *V. parahaemolyticus* was effectively inhibited by the cell culture supplementation with LAB. According to literature, Hwanhlem et al[25] showed that LAB can completely inhibit the growth of *V. parahaemolyticus* within 24 h.

Antimicrobial disc sensitivity test

The 10 antimicrobial discs used in this test revealed that two *V. parahaemolyticus* strains were susceptible to ampicillin and penicillin G (Table 4). The screened LAB supernatants repressed the inhibitory activity of *V. parahaemolyticus* BCRC10806 in a manner similar to that of the neomycin and streptomycin antimicrobial discs, with an inhibition zone diameter of 12–16 mm; also, LAB supernatants inhibited the invasion of *V. parahaemolyticus* BCRC12865

Table 1 Agar diffusion test showing the antagonistic activity of the spent culture supernatants (SCS) of LAB against *V. parahaemolyticus*.

	Strain	Inhibition zone†	
		10806	12865
BCRC	10067, 10068, 10069, 10360, 10361, 10695, 10790, 11051, 11652, 11662, 11846, 11847, 12187, 12188, 12190, 12191, 12193, 12194, 12248, 12250, 12251, 12256, 12260, 12301, 12580, 12931, 12936, 12943, 12944, 13869, 14002, 14008, 14011, 14019, 14024, 14060, 14064, 14065, 14069, 14602, 14606, 14618, 14619, 14622, 14625, 14630, 14634, 14659, 14660, 14662, 14663, 14665, 14667, 14668, 14671, 14678, 14691, 14728, 14741, 14759, 15416, 15477, 15971, 16061, 16092, 17002, 17004, 17009, 17012, 17394, 17474, 17615, 17616, 17972, 17973, 17983, 80109	−	−
PM	1–11, 13, 15–62, 64–70, 72–77, 79, 82, 83, 85–100, 102–113, 115, 116, 118, 121–123, 126–133, 135–151, 155, 163, 165, 167, 170–174, 177, 180–187, 190, 192–196, 199, 200, 202, 203, 207–211, 213–218, 220, 221, 223, 225–228	−	−
FM	1–58, 60–66, 68–71	−	−
BCRC	17614	−	+
PM	71, 78, 101, 117, 125, 134, 164, 166, 205	−	+
BCRC	10696, 14080, 14677, 14735, 16000, 17010	+	−
PM	124, 152, 153, 156, 158, 198, 201	+	−
FM	67	+	−
BCRC	12574, 14084	+	+
PM	12, 14, 63, 80, 81, 84, 114, 119, 120, 157, 160, 175, 176, 178, 188, 191, 197, 204, 206, 212, 222, 229	+	+
FM	59	+	+

† The inhibition zone: −, ⩽ 11 mm; +, 12–16 mm; ++, 17–22 mm; and +++, ⩾ 23 mm.

in a manner similar to that of the erythromycin, streptomycin, and spiramycin discs, with an inhibition zone diameter of 12–13 mm (Table 4). Increasing numbers of antibiotic-resistant pathogens is an important issue in the world. However, the application of probiotics to inhibit the growth of *Vibrio* spp. might be a good method for preventing the pathogen and may also reduce its chances of antibiotic resistance[26].

LAB adhesion to Caco-2 intestinal epithelial cells

According to Pedersen and Tannock[27], LAB can attach more than 15 bacterial cells to pig porcine squamous epithelial cells, evidencing the adhesion properties of this strain. As shown in Fig. 1, a total of 14 LAB (BCRC14677, BCRC14735, BCRC17010, PM12, PM14, PM63, PM80, PM81, PM84, PM114, PM119, PM120, PM206, and PM222) could adhere to Caco-2 cells. Our results showed that PM222 possessed the highest adhesion ability, averaging 34 cells/Caco-2 cell, while the other 7 strains (PM80, PM84, PM114, PM119, PM120, PM206, and BCRC17010) were found to adhere at a rate of more than 15 cells/Caco-2 cell (Fig. 1).

Several *in vitro* assays employ lactobacilli adsorption assays on intestinal cell lines such as human rectal cancer cells (Caco-2 and HT-29)[23,28,29]. Caco-2 cells isolated from human with rectal cancer reportedly exhibited *in vitro* morphology, functional differentiation, and mature gut characteristics such as functional brushlike microvilli[30]; thus, Caco-2 cells are widespread[31] and serve as an excellent model for studying the biochemical processes of bacterial adherence and invasion by other cells[29].

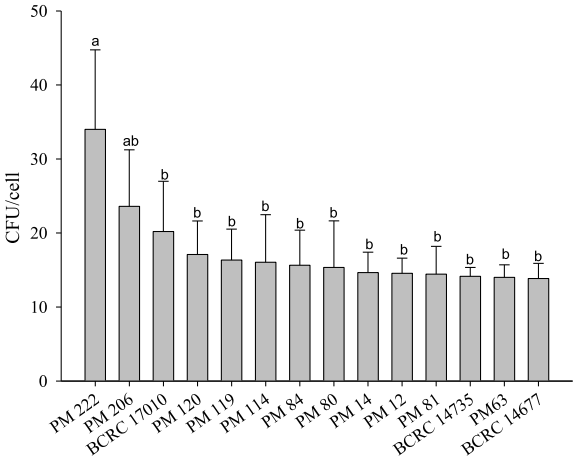

Fig. 1 Adhesion assays of 14 LAB on the Caco-2 cells. Adhesion assays were monitored after 2 h of incubation. Ten of the Caco-2 cells were used to calculate the average number of adhering LAB cells per epithelial cell. [a,b] Values in the same column with different letters indicate significant difference ($p < 0.05$).

LDH analysis

Here, LAB and *V. parahaemolyticus* were co-cultured with Caco-2 intestinal epithelial cells to determine the activity of LDH and to observe whether *V. para-*

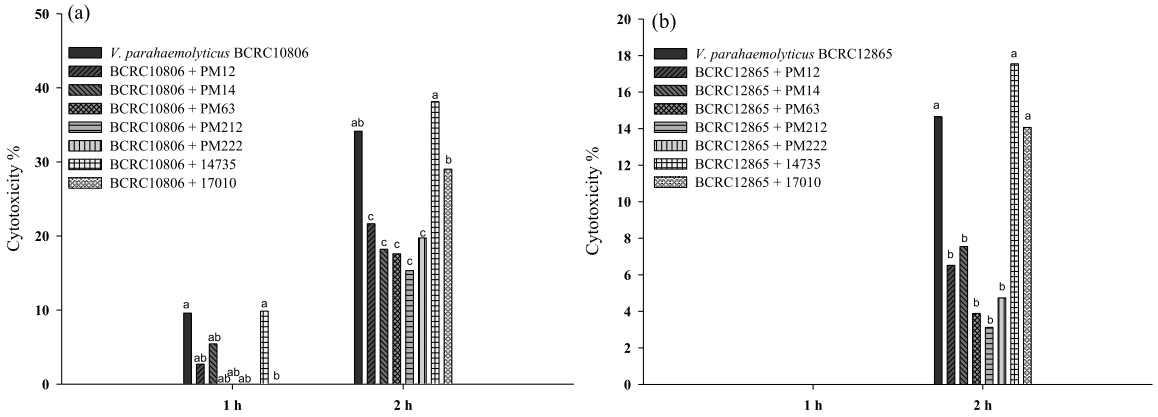

Fig. 2 Effect of *V. parahaemolyticus* (a) BCRC10806 and (b) BCRC12865 and LAB on Caco-2 cell cytotoxicity, assayed by LDH release. Each value is expressed as mean ± SD of two replicate analyses. [a,b] Means with different superscript letters in the same hour are significantly different ($p < 0.05$).

Table 2 Effect of heat treatment (100 °C, 15 min), dilution, and pH 7.2 on the activity of cell free-spent culture supernatants (SCS) and viable cells of LAB against *V. parahaemolyticus* BCRC10806 or BCRC12865.

V. parahaemolyticus	LAB	Inhibition zone (mm)[†]				
		Culture	100 °C, 15 min		Dilution pH 7.2	
			Culture	SCS		
BCRC10806	PM12	13	11	9		
	PM14	13	12	9		
	PM63	13	11	12		
	PM80	13	11	10		
	PM81	12	12	11		
	PM84	12	11	11		
	PM114	12	12	9		
	PM119	11	12	9	⩽ 11	⩽ 11
	PM120	12	12	9		
	PM206	12	12	9		
	PM212	12	15	9		
	PM222	10	10	10		
	14677	9	9	9		
	14735	10	9	9		
	17010	13	12	10		
BCRC12865	PM12	12	10	9		
	PM14	13	11	9		
	PM63	12	11	11		
	PM80	12	11	11		
	PM81	12	12	11		
	PM84	11	11	10		
	PM114	12	11	9		
	PM119	11	11	9	⩽ 11	⩽ 11
	PM120	12	12	9		
	PM206	13	12	9		
	PM212	12	11	9		
	PM222	10	10	10		
	14677	9	9	9		
	14735	10	9	10		
	17010	13	12	11		

[†] The inhibition zone: −, ⩽ 11 mm; +, 12–16 mm; ++, 17–22 mm; and +++, ⩾ 23 mm.

Table 3 The survival of *V. parahaemolyticus* BCRC10806 or BCRC12865 in co-culture with LAB[†].

Survival (%)	LAB	1 h	2 h	3 h	4 h
BCRC10806	14677	34.80^{Aa}	0.57^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	14735	22.45^{Aa}	6.55^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	17010	60.27^{Aa}	0.19^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM14	48.56^{Aa}	0.65^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM63	35.50^{Aa}	0.85^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM80	142.37^{Ba}	1.66^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM81	159.96^{Ba}	2.26^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM84	20.64^{Aa}	0.03^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM114	45.21^{Aa}	0.37^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM119	119.92^{Ba}	1.71^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM120	192.38^{Ba}	1.16^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM206	166.73^{Ba}	0.34^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM212	27.15^{Aa}	1.18^{Ab}	0.01^{Ac}	$<0.01^{c}$
	PM222	181.14^{Ba}	43.11^{Bb}	0.55^{Bc}	$<0.01^{d}$
BCRC12865	14677	15.07^{Aa}	0.55^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	14735	74.71^{Aa}	1.98^{Ab}	0.49^{Bc}	$<0.01^{c}$
	17010	12.64^{Aa}	0.30^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM12	31.62^{Aa}	1.63^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM14	12.24^{Aa}	2.09^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM63	59.74^{Aa}	0.39^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM80	66.77^{Aa}	4.01^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM81	71.78^{Aa}	1.63^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM84	46.72^{Aa}	0.63^{Ab}	$<0.01^{Ac}$	$<0.01^{c}$
	PM114	60.79^{Aa}	2.74^{Ab}	$<0.01^{Ab}$	$<0.01^{c}$
	PM119	14.27^{Aa}	0.37^{Ab}	$<0.01^{Ac}$	$<0.01^{b}$
	PM120	32.71^{Aa}	0.10^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM206	19.48^{Aa}	0.17^{Ab}	$<0.01^{Ab}$	$<0.01^{b}$
	PM212	49.80^{Aa}	1.80^{Ab}	0.01^{Ac}	$<0.01^{c}$
	PM222	178.95^{Ba}	28.83^{Bb}	0.03^{Ac}	$<0.01^{c}$

[†] [A,B] Values in the same column with different letters indicate significant difference ($p < 0.05$). [a,b,c,d] Values in the same row with different letters indicate significant difference ($p < 0.05$).

haemolyticus would damage Caco-2 cells or LAB could reduce this damage. We found that *V. parahaemolyticus* BCRC10806 induced only a slight injury in cells, with cytotoxic activity of approximately 9.59% when incubated for 1 h (Fig. 2); after 2 h, the cytotoxicity of *V. parahaemolyticus* BCRC10806 and BCRC12865 was found to be 34.15% and 14.64%,

Table 4 Effect of inhibitory activity against *V. parahaemolyticus* by antimicrobial disc.

Antimicrobial disc	Inhibition zone (mm)	
	BCRC10806	BCRC12865
Ampicillin (10 μg)	7.5 ± 0.7	9.0 ± 0.0
Kanamycin (30 μg)	17.0 ± 1.4	18.5 ± 0.7
Tetracycline (30 μg)	23.0 ± 2.8	21.5 ± 0.7
Penicillin G (10 μg)	6.0 ± 0.0	7.0 ± 1.4
Neomycin (30 μg)	16.0 ± 1.4	16.0 ± 1.4
Erythromycin (15 μg)	18.0 ± 0.0	13.0 ± 1.4
Streptomycin (10 μg)	12.5 ± 0.7	12.5 ± 0.7
Gentamicin (30 μg)	19.5 ± 0.7	19.0 ± 1.4
Chloramphenicol (30 μg)	28.0 ± 1.4	26.0 ± 1.4
Spiramycin (100 μg)	16.5 ± 0.7	12.0 ± 2.8

respectively (Fig. 1), indicating that the two *Vibrio* strains grew with time and gradually induced cell damage.

Matlawska-Wasowska et al[20] tested the cytotoxicity of *V. parahaemolyticus* on epithelial cells. They reported no cell lysis before 2 h; however, cell lysis increased with time (from 3–4 h). Fernández et al[32] also reported that *V. parahaemolyticus* was cytotoxic to Caco-2 epithelial cells in a time-dependent manner; cell lysis increased in 3 or 4 h later. Similarly, the amount of cell lysis was found to be time-dependent in this study: when LAB and *V. parahaemolyticus* were co-cultured with the Caco-2 intestinal epithelial cell line (Fig. 2), 5 LAB (PM12, PM14, PM63, PM212, and PM222) reduced the damage caused by *Vibrio* spp. to the Caco-2 cells.

Competitive adherence between LAB and *V. parahaemolyticus*

As shown in Table 5, seven LAB competitively adhered to the Caco-2 intestinal epithelial cells with *V. parahaemolyticus* BCRC10806. The prevention group and the simultaneous action revealed a significant decrease in the residual rate of *V. parahaemolyticus* as compared with that of the control group, and the prevention of *V. parahaemolyticus* survival rate was less than 10%. Seven LAB strains competitively adhered to the Caco-2 intestinal epithelial cells with *V. parahaemolyticus* BCRC12865 (Table 5), and the residual ratio of *V. intestinalis* was significantly reduced (1–25%) in the prevention group as compared with that in the control group. Based on the above results, we concluded that LAB preferentially inhibited the competitive adherence of both strains of *V. parahaemolyticus*, suggesting that LAB has a preventive effect. Here, strain BCRC17010 was found to be the best for inhibition.

The adherence of pathogenic bacteria onto the intestinal mucosal surface is considered the first step in intestinal tract infection[33]. Therefore, inhibiting pathogen adherence can prevent the infection and colonization of the intestine[34]. Probiotics can inhibit pathogenic bacteria through competitive repulsion and antagonism to maintain a healthy gut flora[35]. Numerous *in vitro* tests have demonstrated that LAB can inhibit pathogen adherence or invasion into intestinal cells[36–38]. According to literature, *L. plantarum* AS1 was inhibitory to *V. parahaemolyticus* during adhesion of the HT-29 cell line[12]. Lactobacilli were implicated as a physical barrier to inhibit direct contact with epithelial cells via competitive exclusion, and bacteriocin of lactobacilli also was suggested to exhibit a direct inhibitory effect on the pathogen's adherence to epithelial cells[39].

LAB inhibition on the invasion of *V. parahaemolyticus* in Caco-2 intestinal epithelial cells

Invasive pathogens can break through the host mucosal barrier, thereby invading and colonizing it to cause intestinal diseases[40,41]. Some studies have shown that diseases are caused by *Vibrio* spp. not only because of the toxins they produce but also because of their invasion into the gut epithelium[6]. Therefore, we examined whether LAB could inhibit the invasion of Caco-2 cells by *V. parahaemolyticus*. We found that 7 LAB (PM12, PM14, PM63, PM212, PM222, BCRC14735, and BCRC17010) inhibited this invasion. The inhibitory effect was greater for the invasion by *V. parahaemolyticus* BCRC12865 than for that by *V. parahaemolyticus* BCRC10806 (Table 6). In literature, the *L. acidophilus* had the potential to bind to epithelial cell brush borders by calcium cations acting as an ion bridge[39]. LAB can inhibit the pathogen attachment via the steric hindrance mechanism[12]. The invasion and adherence of *V. cholerae* to Caco-2 cells was reduced by *L. acidophilus*[39].

CONCLUSION

In conclusion, LAB from this study can inhibit the growth of *V. parahaemolyticus*. The LDH assay revealed that certain LAB could significantly decrease the cytotoxicity induced by both *V. parahaemolyticus* BCRC10806 and BCRC12865 strains after a 2 h-incubation. In the competition adherence assay, the prevention group revealed inhibitory effects against

Table 5 Effect of LAB on the survival of *V. parahaemolyticus* BCRC10806 or BCRC12865 from colonizing Caco-2 cells.

V. parahaemolyticus	LAB	Prevention		Treatment		Competition	
		log CFU/ml	%	log CFU/ml	%	log CFU/ml	%
BCRC10806	Control*	4.77 ± 0.70	100	4.41 ± 0.40	100	4.01 ± 0.62	100
	PM12	3.51 ± 1.19	5.40	3.87 ± 0.74	28.74	2.32 ± 0.21	2.01
	PM14	3.40 ± 0.84	4.19	4.37 ± 0.70	91.46	2.14 ± 0.08	1.33
	PM63	3.38 ± 1.01	4.02	4.21 ± 2.15	62.97	2.35 ± 0.17	2.18
	PM212	3.68 ± 0.03	8.02	4.03 ± 1.50	42.36	1.98 ± 0.13	0.92
	PM222	3.09 ± 0.08	2.09	4.01 ± 1.28	39.70	2.62 ± 0.00	4.00
	14735	2.51 ± 0.38	0.54	3.95 ± 1.21	34.88	3.50 ± 0.85	30.52
	17010	2.88 ± 0.07	1.27	4.10 ± 1.49	49.06	2.66 ± 0.77	4.46
BCRC12865	Control*	4.87 ± 0.32	100	5.55 ± 0.44	100	4.29 ± 1.62	100
	PM12	4.12 ± 0.76	17.83	4.98 ± 0.03	26.91	3.60 ± 0.62	20.54
	PM14	3.94 ± 0.50	11.64	5.20 ± 0.22	45.04	3.97 ± 1.14	48.09
	PM63	4.29 ± 0.87	25.92	4.98 ± 0.10	27.20	3.96 ± 1.86	46.28
	PM212	4.02 ± 0.36	13.97	4.96 ± 0.03	25.92	4.21 ± 2.12	83.25
	PM222	3.38 ± 0.09	3.19	5.13 ± 0.22	38.10	4.25 ± 2.26	90.62
	14735	3.60 ± 0.09	5.31	5.52 ± 0.13	93.06	3.24 ± 1.25	8.82
	17010	2.95 ± 0.26	1.20	5.28 ± 0.03	54.25	3.41 ± 1.64	13.20

* Control: 900 µl of fresh medium and 100 µl *V. parahaemolyticus* (10^7 CFU/ml)

Table 6 Effect of LAB on the invasion of the Caco-2 cell line by *V. parahaemolyticus* BCRC12865†.

V. parahaemolyticus	LAB	Invasion (log CFU/ml)	Inhibition (%)
BCRC10806	Control*	2.24 ± 0.18	0
	PM12	1.77 ± 0.95	34.99
	PM14	1.53 ± 0.72	72.31
	PM63	1.86 ± 0.75	39.25
	PM212	1.71 ± 1.06	34.72
	PM222	1.73 ± 0.93	43.88
	14735	1.62 ± 0.36	74.95
	17010	1.81 ± 0.02	61.32
BCRC12865	Control*	3.57 ± 0.94	0
	PM12	2.20 ± 0.28	93.10
	PM14	2.24 ± 0.41	93.46
	PM63	2.13 ± 0.07	92.15
	PM212	2.02 ± 0.55	83.98
	PM222	2.01 ± 0.62	82.61
	14735	1.80 ± 0.32	93.25
	17010	1.56 ± 0.45	95.32

† The number of *V. parahaemolyticus* BCRC12865 before the invasion is 7.64 log CFU/ml.

* Control: 900 µl of fresh medium and 100 µl *V. parahaemolyticus* (10^7 CFU/ml)

the two *V. parahaemolyticus* strains. Besides, we revealed that the LAB inhibited *V. parahaemolyticus* invasion of Caco-2 cells. The mechanism of LAB against *V. parahaemolyticus* will be explored in near future.

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