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. 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. 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. 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. Park et al. reported that T3SS1 produces cytoxins in human cells, whereas T3SS2 produces endotoxins in animal infection models and cytoxins in the gut. Additionally, Zhang et al. indicated that T3SS2 is involved in the invasion of V. parahaemolyticus into human cells. Akeda et al. 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. 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; 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.

Satish Kumar et al. 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. 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. Shirazinejad et al. 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.

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 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.

Antimicrobial testing of LAB and V. parahaemolyticus co-culture

Based on the method of Varma et al., 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 × 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. and Satish Kumar et al. were adopted, albeit with some modifications, for the competitive adhesion assay. Briefly, 1 ml each of LAB (10^5 CFU/ml) and V. parahaemolyticus (10^7 CFU/ml) were mixed and subjected to the following conditions: initial centrifugation at 9500 x 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 x 10^5 cells/ml), followed by uniform mixing and incubation at 37 °C for 2 days in a 5% CO₂ incubator (Forma Direct Heat CO₂ 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 x g for 2 min at 37 °C under 5% CO₂. 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, pouring via the TCBS method, and incubating overnight at 37 °C; subsequently, the V. parahaemolyticus colonies were counted. V. parahaemolyticus grew on 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. and Zhang et al., 1 ml of the cell suspension was added into each 24-well culture plate (cell concentration adjusted to 2 x 10^5 cells/ml), mixed uniformly, and incubated at 37 °C under 5% CO₂ 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% CO₂ for 3 h. After carefully washing twice with PBS, 1 ml of kanamycin containing 100 μg/ml (cell medium without PBS) was added to each well, followed by incubation at 37 °C under 5% CO₂ 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. 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% CO₂ 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 (10^5 CFU/ml) and LAB (10^5 CFU/ml), were added. After incubating for 1 or 2 h under 5% CO₂, 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.
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 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. cholerae*.

**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† | Strain Inhibition zone† |
|-------------------------|-------------------------|
| BCRC10067, 10068, 10069, 10360, 10695, 10790, 10361, 10695, 10280, 10301, 10931, 10361, 10936, 10243, 10622, 10623, 10634, 10659, 10660, 10671, 10678, 10691, 14678 | BCRC17614, 17615, 17616, 17972, 17973, 17983, 80109 |
| 1–11, 13, 15–62, 64–70, 72–77, 79, 82, 83, 85–100, 102–113, 115, 116, 118, 121–123, 126–133 | PM1–11, 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 | PM1–11, 13, 15–62, 64–70, 72–77, 79, 82, 83, 85–100, 102–113, 115, 116, 118, 121–123, 126–133 |
| 10696, 14080, 14677, 14735, 16000, 17010 | PM124, 152, 153, 156, 158, 198, 201 |
| PM12, 14, 63, 80, 81, 84, 114, 119, 120, 157, 160, 175, 176, 178, 188, 191, 197, 204, 206, 212, 222, 229 | PM124, 152, 153, 156, 158, 198, 201 |
| 59 | PM124, 152, 153, 156, 158, 198, 201 |

† 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.

LAB adhesion to Caco-2 intestinal epithelial cells

According to Pedersen and Tannock, 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). 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; thus, Caco-2 cells are widespread and serve as an excellent model for studying the biochemical processes of bacterial adherence and invasion by other cells.

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. parahaemolyticus...
haemolyticus would damage Caco-2 cells or LAB could reduce this damage. We found that *V. para-

**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              |                |         |     |
| BCRC10806             | PM14| 13                    | 12      | 9              |                |         |     |
| BCRC10806             | PM63| 13                    | 11      | 12             |                |         |     |
| BCRC10806             | PM80| 13                    | 11      | 10             |                |         |     |
| BCRC10806             | PM81| 12                    | 12      | 11             |                |         |     |
| BCRC10806             | PM84| 12                    | 12      | 11             |                |         |     |
| BCRC10806             | PM114| 12                | 12      | 9              |                |         |     |
| BCRC10806             | PM119| 11                | 12      | 9 ⩽ 11         | ⩽ 11           |         |     |
| BCRC10806             | PM120| 12                | 12      | 9              |                |         |     |
| BCRC10806             | PM206| 12                | 12      | 9              |                |         |     |
| BCRC10806             | PM212| 12                | 15      | 9              |                |         |     |
| BCRC10806             | PM222| 10                | 10      | 10             |                |         |     |
| BCRC10806             | 14677| 9                 | 9       | 9              |                |         |     |
| BCRC10806             | 14735| 10                | 9       | 9              |                |         |     |
| BCRC10806             | 17010| 10                | 12      | 10             |                |         |     |
| BCRC12865             | PM12| 12                    | 10      | 9              |                |         |     |
| BCRC12865             | PM14| 13                    | 11      | 9              |                |         |     |
| BCRC12865             | PM63| 12                    | 11      | 11             |                |         |     |
| BCRC12865             | PM80| 12                    | 11      | 11             |                |         |     |
| BCRC12865             | PM81| 11                    | 11      | 10             |                |         |     |
| BCRC12865             | PM114| 12                | 11      | 9              |                |         |     |
| BCRC12865             | PM119| 11                | 11      | 11             | ⩽ 11           | ⩽ 11    |     |
| BCRC12865             | PM206| 13                | 12      | 9              |                |         |     |
| BCRC12865             | PM212| 12                | 11      | 9              |                |         |     |
| BCRC12865             | PM222| 10                | 10      | 10             |                |         |     |
| BCRC12865             | 14677| 9                 | 9       | 9              |                |         |     |
| BCRC12865             | 14735| 10                | 9       | 9              |                |         |     |
| BCRC12865             | 17010| 10                | 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† | 0.57† | <0.01† | <0.01† |
| BCRC10806    | 14735| 22.45† | 6.55† | <0.01† | <0.01† |
| BCRC10806    | 17010| 60.27† | 0.19† | <0.01† | <0.01† |
| BCRC10806    | PM14| 48.56† | 0.65† | <0.01† | <0.01† |
| BCRC10806    | PM63| 35.50† | 0.85† | <0.01† | <0.01† |
| BCRC10806    | PM80| 142.37† | 1.66† | <0.01† | <0.01† |
| BCRC10806    | PM81| 159.96† | 2.26† | <0.01† | <0.01† |
| BCRC10806    | PM84| 20.64† | 0.03† | <0.01† | <0.01† |
| BCRC10806    | PM114| 45.21† | 0.37† | <0.01† | <0.01† |
| BCRC10806    | PM119| 119.92† | 1.71† | <0.01† | <0.01† |
| BCRC10806    | PM120| 192.38† | 1.16† | <0.01† | <0.01† |
| BCRC10806    | PM206| 166.73† | 0.34† | <0.01† | <0.01† |
| BCRC10806    | PM212| 27.15† | 1.18† | 0.01† | <0.01† |
| BCRC10806    | PM222| 181.14† | 43.11† | 0.55† | <0.01† |
| BCRC12865    | 14677| 15.07† | 0.55† | <0.01† | <0.01† |
| BCRC12865    | 14735| 74.71† | 1.98† | 0.49† | <0.01† |
| BCRC12865    | 17010| 12.64† | 0.30† | <0.01† | <0.01† |
| BCRC12865    | PM12| 31.62† | 1.63† | <0.01† | <0.01† |
| BCRC12865    | PM14| 12.24† | 2.09† | <0.01† | <0.01† |
| BCRC12865    | PM63| 59.74† | 0.39† | <0.01† | <0.01† |
| BCRC12865    | PM80| 66.77† | 4.01† | <0.01† | <0.01† |
| BCRC12865    | PM81| 71.78† | 1.63† | <0.01† | <0.01† |
| BCRC12865    | PM84| 46.72† | 0.63† | <0.01† | <0.01† |
| BCRC12865    | PM114| 60.79† | 2.74† | <0.01† | <0.01† |
| BCRC12865    | PM119| 14.27† | 0.37† | <0.01† | <0.01† |
| BCRC12865    | PM120| 32.71† | 0.10† | <0.01† | <0.01† |
| BCRC12865    | PM206| 19.48† | 0.17† | <0.01† | <0.01† |
| BCRC12865    | PM212| 49.80† | 1.80† | 0.01† | <0.01† |
| BCRC12865    | PM222| 178.95† | 28.83† | 0.05† | <0.01† |

† A,B Values in the same column with different letters indicate significant difference (p < 0.05). A,B,C Values in the same row with different letters indicate significant difference (p < 0.05).
respectively (Fig. 1), indicating that the two Vibrio strains grew with time and gradually induced cell damage.

Matlawska-Wasowska et al.\textsuperscript{20} tested the cytotoxicity of \textit{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.\textsuperscript{32} also reported that \textit{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 \textit{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 \textit{V. parahaemolyticus}**

As shown in Table 5, seven LAB competitively adhered to the Caco-2 intestinal epithelial cells with \textit{V. parahaemolyticus} BCRC10806. The prevention group and the simultaneous action revealed a significant decrease in the residual rate of \textit{V. parahaemolyticus} as compared with that of the control group, and the prevention of \textit{V. parahaemolyticus} survival rate was less than 10%. Seven LAB strains competitively adhered to the Caco-2 intestinal epithelial cells with \textit{V. parahaemolyticus} BCRC12865 (Table 5), and the residual ratio of \textit{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 \textit{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\textsuperscript{33}. Therefore, inhibiting pathogen adherence can prevent the infection and colonization of the intestine\textsuperscript{34}. Probiotics can inhibit pathogenic bacteria through competitive repulsion and antagonism to maintain a healthy gut flora\textsuperscript{35}. Numerous \textit{in vitro} tests have demonstrated that LAB can inhibit pathogen adherence or invasion into intestinal cells\textsuperscript{36–38}. According to literature, \textit{L. plantarum} AS1 was inhibitory to \textit{V. parahaemolyticus} during adhesion of the HT-29 cell line\textsuperscript{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\textsuperscript{39}.

**LAB inhibition on the invasion of \textit{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\textsuperscript{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\textsuperscript{6}. Therefore, we examined whether LAB could inhibit the invasion of Caco-2 cells by \textit{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 \textit{V. parahaemolyticus} BCRC12865 than for that by \textit{V. parahaemolyticus} BCRC10806 (Table 6). In literature, the \textit{L. acidophilus} had the potential to bind to epithelial cell brush borders by calcium cations acting as an ion bridge\textsuperscript{12}. LAB can inhibit the pathogen attachment via the steric hindrance mechanism\textsuperscript{12}. The invasion and adherence of \textit{V. cholerae} to Caco-2 cells was reduced by \textit{L. acidophilus}\textsuperscript{39}.

**CONCLUSION**

In conclusion, LAB from this study can inhibit the growth of \textit{V. parahaemolyticus}. The LDH assay revealed that certain LAB could significantly decrease the cytotoxicity induced by both \textit{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 (log CFU/ml) | Treatment (log CFU/ml) | Competition (log CFU/ml) |
|-----------------------|--------------|-------------------------|------------------------|--------------------------|
| BCRC10806             | Control†     | 4.77 ± 0.70             | 4.41 ± 0.40            | 4.01 ± 0.62              |
|                       | PM12         | 3.51 ± 1.19             | 3.87 ± 0.74            | 2.32 ± 0.21              |
|                       | PM14         | 3.40 ± 0.84             | 4.37 ± 0.70            | 2.14 ± 0.08              |
|                       | PM63         | 3.38 ± 1.01             | 4.21 ± 2.15            | 2.35 ± 0.17              |
|                       | PM212        | 3.68 ± 0.03             | 4.03 ± 1.50            | 1.98 ± 0.13              |
|                       | PM222        | 3.09 ± 0.08             | 4.01 ± 1.28            | 2.62 ± 0.00              |
|                       | 14735        | 2.51 ± 0.38             | 3.95 ± 1.21            | 3.48 ± 0.85              |
|                       | 17010        | 2.88 ± 0.07             | 4.10 ± 1.49            | 4.96 ± 0.67              |
| BCRC12865             | Control†     | 4.87 ± 0.32             | 5.55 ± 0.44            | 4.29 ± 1.62              |
|                       | PM12         | 4.12 ± 0.76             | 4.98 ± 0.03            | 3.60 ± 0.62              |
|                       | PM14         | 3.94 ± 0.50             | 5.20 ± 0.22            | 3.97 ± 1.14              |
|                       | PM63         | 4.29 ± 0.87             | 4.98 ± 0.10            | 3.96 ± 1.86              |
|                       | PM212        | 4.02 ± 0.36             | 4.96 ± 0.03            | 2.95 ± 2.12              |
|                       | PM222        | 3.38 ± 0.09             | 5.13 ± 0.22            | 4.25 ± 2.26              |
|                       | 14735        | 3.60 ± 0.09             | 5.52 ± 0.13            | 3.24 ± 1.25              |
|                       | 17010        | 2.95 ± 0.26             | 5.28 ± 0.03            | 3.14 ± 1.64              |

† Control: 900 μl of fresh medium and 100 μl *V. parahaemolyticus* (10⁷ 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* (10⁷ 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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