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Probiotic potential of novel *Lactobacillus* strains isolated from salted-fermented shrimp as antagonists for *Vibrio parahaemolyticus*[§]

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***Lactobacillus* strains have been considered good candidates as biological control agents for prevention or treatment of plant and animal infections. One *L. plantarum* strain FB003 and three strains (FB011, FB081, and FB110) which closed to *L. sakei* were isolated from fermented and salted shrimp and their abilities in inhibiting growth of *Vibrio parahaemolyticus* were characterized. These strains were selected as potential probiotics based on their oro-gastro-intestinal resistance, gut colonization, adhesion to Caco-2 cells, antimicrobial activities, antibiotic resistance, and safety aspects. Results of this study revealed that these isolates possessed high aggregation activities against pathogens in host intestines. Strain FB011 strain showed higher coaggregation and immunomodulatory activity in the gastro-intestinal tract than *L. plantarum*. These difference effects of *Lactobacillus* strains provide valuable information about using them to prevent *Vibrio* infections in the aquaculture industry.**

Keywords: *Lactobacillus*, *Vibrio*, probiotic, salted shrimp

Introduction

Vibrio, a Gram-negative bacterium, is widely distributed in aquatic environments worldwide. It is frequently found in aquaculture pond (Baker-Austin *et al.*, 2013). Numerous infectious diseases in humans and aquatic organisms caused by *Vibrio* spp. have been reported (Gomez-Gil *et al.*, 2004; Vezzulli *et al.*, 2016). *Vibrio* is a major source of human acute gastroenteritis due to consumption of raw or undercooked seafood (Hsiao *et al.*, 2014; Wu *et al.*, 2014). It has been reported that *V. parahaemolyticus* infection can cause early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in shrimp, causing 100% losses in shrimp production (Joshi *et al.*, 2014). Antibiotics and chemotherapy have been used as traditional strategies for aquatic diseases management in the last decade. However, resistant bacteria, resistance genes, and human health risk of antibiotics via massive use and consumption of aquatic products have also become public concerns (Holmström *et al.*, 2003; Le and Munekage, 2004). The European Union and the USA have implemented bans on or restricted the use of antibiotics in aquaculture. They have set strict standards to quarantine imported aquatic products (Panel, 2012).

As effective alternatives, probiotics have received lots of attention. They have been proven to be beneficial against pathogens. Probiotics, prebiotics, and synbiotics have been useful as feed additives as immune stimulants. They can increase resistance to disease and improve the growth of host by modifying host-associated or ambient microbial community (Galdeano and Perdigon, 2004; Qi *et al.*, 2009; Gareau *et al.*, 2010).

Lactic acid bacteria (LAB) have been isolated from various environments and fermented foods for use as probiotics (Naidu *et al.*, 1999; Saarela *et al.*, 2000). It has been found that *L. salivarius* is able to directly inhibit non-staphylococcal intestinal bacterial strains through a peptide bacteriocin (Corr *et al.*, 2007). LAB isolated from salted fish have been found to be able to inhibit *V. parahaemolyticus* growth (Satish Kumar *et al.*, 2011). However, studies focusing on LAB isolated from salted and fermented shrimp have not been reported. Therefore, the objective of this study was to determine the probiotics potential of LAB isolated from salted and fermented shrimp and evaluate their antimicrobial activities again pathogenic *V. parahaemolyticus*.

Materials and Methods

Bacterial strains and growth conditions

Ten pathogenic bacteria were used in the study: *Bacillus cereus* KACC 11240, *Shigella boydii* KACC 10792, *Listeria monocytogenes* KACC 10764, *Yersinia enterocolitica* subsp. *enterocolitica* KACC 15320, *Escherichia coli* K99 KCTC 261, *Salmonella enterica* serotype Choleraesuis KCTC 2932, *Salmonella enterica* serovar Typhi KCTC 2514, *Salmonella enterica* subsp. *enterica* serovars Gallinarum KCTC 2931, *S. aureus* KCCM 11335, and *V. parahaemolyticus* ATCC 43996. They were obtained from Korean Agricultural Culture Collection (KACC), Korean Collection for Type Cultures (KCTC), Korean Culture Collection of Microorganisms (KCCM), or American Type Culture Collection (ATCC). Commercial probiotic strain *Lactobacillus rhamnosus* GG obtained from ATCC was used as reference strain. All strains were grown routinely on either brain heart infusion (BHI; Difco) or deMan, Rogosa and Sharpe (MRS; Difco) broth at 37°C.

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Isolation of LAB

Lactic acid bacteria were isolated from salted and fermented shrimp collected from a household in Yeosu, Korea. Immediately after sampling, they were spread onto MRS agar plates. These plates were incubated at 37°C for 24 to 48 h under anaerobic conditions. Colonies with different morphologies were identified through procedures consisting of morphology, Gram-staining, catalase test, and motility test. All lactobacilli were frozen at -80°C in MRS broth supplemented with 30% sterile glycerol.

Screening of LAB against pathogenic *V. parahaemolyticus*

Antagonistic activities of potential LAB isolates against four *V. parahaemolyticus* strains were tested by using well diffusion agar assay (WDAA). Briefly, pathogenic bacteria were prepared in 10 ml tryptic soy broth (TSB; Difco) supplement with 2.5% NaCl and cultured for 24 h at 37°C. After incubation, 1 ml was spread over TSA plates. These isolates were also cultured in 10 ml MRS broth for 24 h. Then 100 µl of supernatant was introduced into wells and incubated at 37°C for 24 h. Antibacterial activity was defined as clear inhibitory diameter (mm) zone formed around the well.

Identification of isolated *Lactobacillus* strains

To identify bacteria, biochemical test, API 50 CHL kit, and API ZYM kit (bioMérieux S.A.) were used according to the manufacturer's instructions. For molecular identification of isolated strains, 16S rDNA sequence analysis was performed using universal primers 27F (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were sequenced by Bioneer Co. Sequences of related strains were retrieved from GenBank database. They were used to construct a phylogenetic tree by neighbor-joining method using MEGA7 program.

Safety assessment

All isolates were subjected to safety assessments, including biogenic amine production, hemolytic activity, and degradation of type III mucin from porcine stomach (Sigma). Møller decarboxylase medium supplement with 0.5% w/v of individually amino acids (L-phenylalanine, L-lysine, L-tryptophan, L-tyrosine, L-arginine, L-ornithine, or L-histidine) was used to determine biogenic amine production as described, by (Bover-Cid and Holzapfel, 1999). Hemolytic activity of isolates was investigated as described previously with minor modifications (Eaton and Gasson, 2001). Mucin degradation activity was determined by petri dish-based methods as described by (Zhou *et al.*, 2001).

Oro-gastro-intestinal transit assay

To evaluate survival potential of LAB in human GI tract, *in vitro* oro-gastro-intestinal transit assay was performed using published method (Bove *et al.*, 2012) with slight modifications. For oral stress, cultured LAB cells were resuspended into 10 ml of gastric electrolyte solution (GES) (Marteau *et al.*, 1997) supplemented with 150 mg/L lysozyme to reach a final concentration of 10^9 CFU/ml for 10 min. After each incubation period, cells were collected by centrifugation at 10,000 × *g* for 5 min and washed twice with PBS. Then 1 ml aliquot was plated onto MRS agar for viability analysis. For gastric stress, cells were resuspended in 10 ml of GES adjusted to pH 3 and pH 2 in the presence of 0.3% w/v pepsin for 30 min. For intestinal stress, cells were treated with GES containing 0.3% bile oxgall and 0.1% pancreatin for 120 min. Incubations were performed at 37°C with shaking. Survival rate was determined by comparing the number of viable cells to that of unstressed control after incubation.

Autoaggregation and coaggregation assays

Autoaggregation and coaggregation assays were performed according to published methods (Damodharan *et al.*, 2015). Briefly, cells were cultured for 18 h and harvested by centrifugation at 10,000 × *g* for 5 min at 4°C, washed twice with PBS, and then re-suspended in PBS to reach final concentration of 10^8 CFU/ml. The suspension was incubated at 37°C in 4 ml aliquots for 5 h without shaking. Autoaggregation percentage was determined every hour by comparing the absorbance at wavelength of 600 nm. For coaggregation analysis, LAB and pathogens suspensions were prepared as described earlier for autoaggregation. They were then mixed at equal volume followed by incubation at 37°C without agitation for 5 h. Coaggregation was calculated as follows:
where and were absorbance values (600 nm) of pathogen and LAB suspension in control tube and was absorbance value of mixed bacterial suspension at 5 h after incubation.

Caco-2 cell culture and adhesion assay

For immune stimulation experiments, human colonic cell line Caco-2 was obtained from KCTC (Korea) and cultured in Dulbecco's Modified Eagle Medium culture medium which was routinely changed every 2 days until a confluent monolayer was obtained. Caco-2 monolayer was washed twice with PBS (pH 7.2) and resuspended in serum- and antibiotic-free medium for 12 h before adhesion assay. One milliliter of bacterial suspension (10^8 CFU/ml) was added to each well and incubated at 37°C with 5% CO_2 for 1 h. After incubation, each well was washed twice with PBS to remove non-attached bacterial cells followed by treatment with 1 ml of 1% (v/v) Triton X-100. The number of adhered bacterial cells was determined after plating serial dilutions of the suspension onto MRS plates followed by incubation at 37°C for 24 h.

Antibacterial activity

Antibacterial activity of isolates against foodborne pathogens was assessed using agar well diffusion method described above.

Minimum inhibition concentration of antibiotics

Antibiotic susceptibility of potential probiotics was determined by minimal inhibitory concentration (MIC) using broth microdilution method (Wiegand *et al.*, 2008). MIC value was defined as the lowest concentration at which visible bacterial growth was inhibited after overnight incubation.

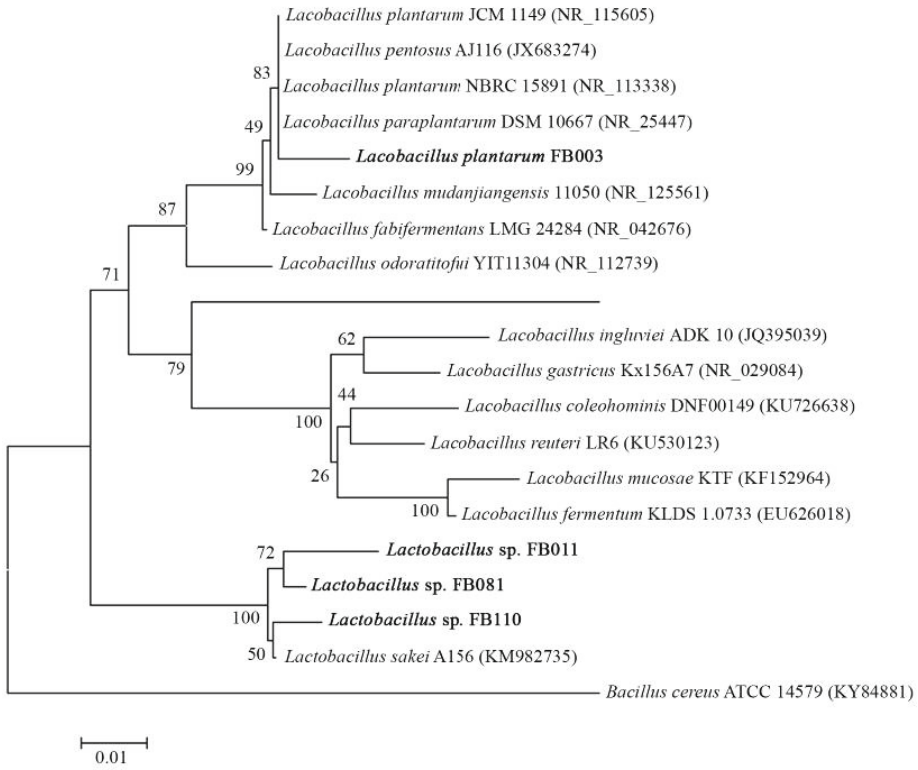

Fig. 1. Phylogenetic tree constructed based on 16S rRNA gene sequence of strains FB003, FB011, FB081, FB110 isolated from salted and fermented shrimp.

Effect of LAB against *V. parahaemolyticus*

To determine the protective effect of four potential LAB strains against pathogenic *V. parahaemolyticus* cells, pathogen was preincubated with 10 ml tryptic soy broth (TSB; Difco) supplement with 2.5% NaCl at 30°C for one day with shaking at 200 rpm. These tested LAB strains were incubated with MRS broth at 37°C for one day. Both pathogen and LAB cells were centrifuged, washed, and suspended with 10 ml tryptic soy broth (TSB; Difco) supplement with 2.5% NaCl and adjusted to about 8 log CFU/ml. The same volume of pathogen and LAB cells was mixed and incubated at 30°C for 48 h with shaking. After every 4 h of incubation, 0.1 ml of the suspension was immediately collected to analyze the number of surviving pathogen cells on thiosulphate citrate bile salts sucrose agar plates (TCBS; Difco).

Statistical analysis

All experiments were performed in at least three times and the results represented by mean ± standard deviation. Test of significant difference were carried out by one-way ANOVA and Duncan's Multiple Range Test at ($P < 0.05$) using SPSS program (ver. 22, SPSS Inc.).

Results and Discussion

Isolation and identification of two *Lactobacillus* isolates

In this study, we screened lactic acid bacteria isolated from fermented and salted shrimp for their anti-*Vibrio* activities. All isolates were Gram-positive and catalase negative LAB strains. Among 215 bacteria tested for anti-Vibrio activity, five lactic acid bacteria showed clear inhibition zone diameters ranging from 4 to 10 mm. These potential strains were selected for further identification. Carbohydrate catalase and enzyme production profiles were identical among these five LAB strains based on tests using API 50CHL (Supplementary data Table S1) and API 20 Strep system (bioM'erieux) (Supplementary data Table S2). FB011, FB081, and FB110 shared

Table 1. Oro-gastro-intestinal transit tolerance of the LAB strains

Candidate strains	Oro-gastrointestinal transit tolerance [Log_{10} (CFU/ml)]					Viability after OGT assay (Log reduction)
	Initial	Oral stress (10 min)	Gastric stress pH 3 (30 min)	Gastric stress pH 2 (30 min)	Intestinal stress (120 min)	
Lactobacillus plantarum FB003	9.10 ± 0.04	8.94 ± 0.03	8.89 ± 0.07	7.88 ± 0.02	7.53 ± 0.04	1.57 ± 0.06^{ab}
Lactobacillus sp. FB011	9.10 ± 0.06	8.84 ± 0.06	8.75 ± 0.03	7.90 ± 0.03	7.54 ± 0.04	1.56 ± 0.07^{ab}
Lactobacillus sp. FB081	9.11 ± 0.02	8.82 ± 0.08	8.73 ± 0.06	7.70 ± 0.02	7.53 ± 0.05	1.58 ± 0.05^{a}
Lactobacillus sp. FB110	9.06 ± 0.03	8.52 ± 0.05	8.70 ± 0.07	7.91 ± 0.03	7.80 ± 0.07	1.26 ± 0.08^{b}
Lactobacillus rhamnosus GG	9.01 ± 0.02	8.44 ± 0.02	8.39 ± 0.02	7.28 ± 0.04	7.29 ± 0.01	1.72 ± 0.02^{a}

^{a,b} Values within a column without a common superscript letter are significant different ($P < 0.05$).

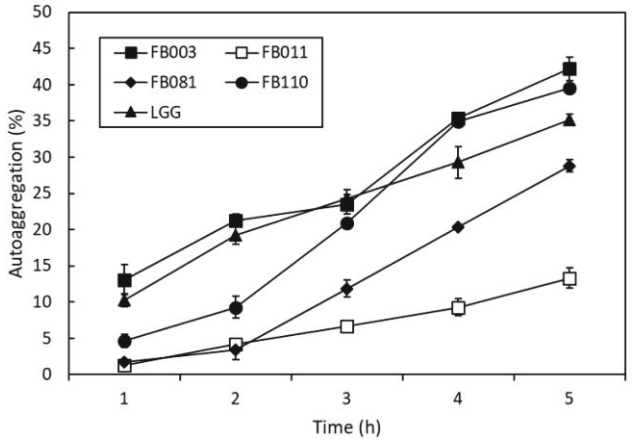

Fig. 2. Autoaggregation percentage of selected LAB strains and reference *L. rhamnosus* GG strain. Values are expressed as mean ± SD (n = 3).

quite similar sugar fermentation patterns while FB003 was found to share 99.7% similarities with published reference strain L. plantarum JCM 1149. None of these four isolates produced enzymes such as trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, or α-fucosidase. LAB FB003 produced the highest number of enzymes.

FB011, FB081, and FB110 isolates were of isolates clustered with 16S rRNA sequences of *Lactobacillus sakei* A156 (sharing 98%, 97%, and 98% similarities with *L. sakei* A156, respectively). A phylogenetic tree was constructed based on their 16S rRNA sequences compared to related species obtained from NCBI database (Fig. 1). *Lactobacillus* strains are good candidates as biological control agents for prevention or treatment of plant and animal infections for several reasons. For example, they can produce organic acids, fatty acids, hydrogen peroxide, and bacteriocins (Gerez *et al.*, 2013; Roselló *et al.*, 2013). Interestingly, antimicrobial activity and probiotics properties of *L. plantarum* strains have been reported (Anyogu *et al.*, 2014; Li *et al.*, 2016). However, such properties of *L. sakei* have not been well reported.

Oro-gastro-intestinal transit

Survival capabilities of these LAB under oro-gastro-intestinal conditions were investigated using an *in vitro* model. Results are shown in Table 1. Overall, bacterial viabilities were not influenced by oral stress. However, they were significantly affected by gastric conditions in a pH-dependent manner (pH 2 and 3). These strains showed no significant differences in survival capabilities compared to the reference strain *Lactobacillus rhamnosus* GG except that FB110 had stronger survival ability than the reference strain. Tolerance for extreme GI tract condition is an essential requisite for probiotics (Panel, 2012). *L. plantarum* species have been commonly isolated from human gastric system (De Vries *et al.*, 2006) while *L. sakei* is detected only occasionally. Based on our data, these five *Lactobacillus* strains differed considerably in their resistance to gastric acid under simulated gastric condition, even between closed species (FB011, FB081, and FB110). Moreover, FB011, FB081, and FB110 strains show to be highly resistant to oro-gastro-intestinal parameters. Therefore, they are more acceptable than reference LGG strain, similar to some previous findings for other LAB strains (Bove *et al.*, 2013; Angmo *et al.*, 2016).

Autoaggregation and coaggregation

Adherence to intestinal epithelial cell is one of various mechanisms involved in aggregation (Collado *et al.*, 2008). Results of autoaggregation rates of isolates are shown in Fig. 2. Only two strains, FB003 and FB0110, exhibited relatively higher (34.3%) autoaggregation rates compared to the reference strain LGG. *L. plantarum* FB003 strain exhibited the lowest autoaggregation phenotype at 5 h of test period. High autoaggregation is a very important criterion to enable the presence of probiotics in host intestines for a long time (Xu *et al.*, 2009). Based on aggregation, FB011, FB081, and FB110 isolates might have higher immunomodulatory activity in the GI tract than *L. plantarum*.

Coaggregation potential of each isolate against pathogen was estimated and compared based on absorbance values (Fig. 3). Overall, all three FB011, FB081, and FB110 isolates showed higher percentages of against pathogens compared to *L. plan-*

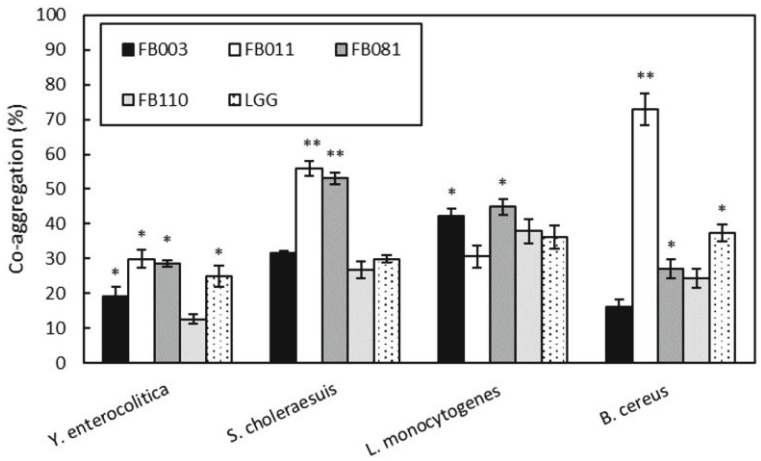

Fig. 3. Co-aggregation values recorded for lactic acid bacteria strains and *L. rhamnosus* GG (LGG) with *Yersinia enterocolitica* ssp. *enterocolitica* KACC 15320, *Salmonella* Choleraesuis KCTC 2932, *Listeria monocytogenes* KACC 10764, and *Bacillus cereus* KACC 11240 after 5 h incubation at 37°C in PBS. Error bars represent standard deviations of the mean values (n = 3). *$P < 0.05$; **$P < 0.01$, significantly different from each other against same pathogen.

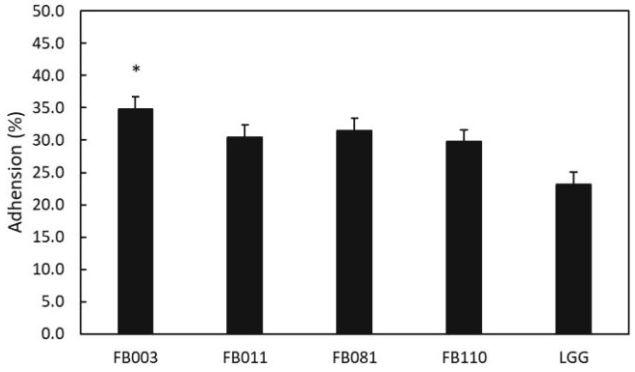

Fig. 4. Adherence ability of isolates to Caco-2 cell monolayer. Error bars represent standard deviations of mean values (n = 3). *$P < 0.05$, significantly different from each other.

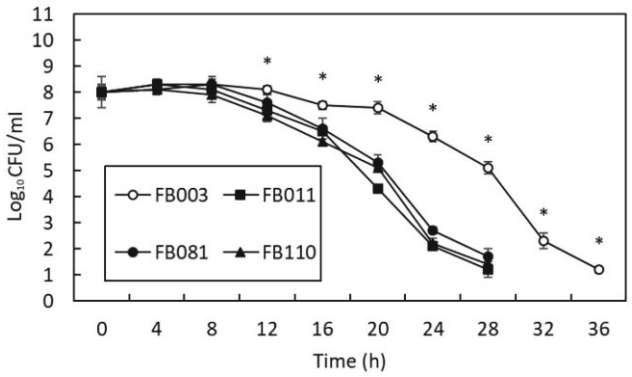

Fig. 5. Reducing population densities of *V. parahaemolyticus* CM1 by LAB. *$P < 0.05$, significantly different from each other at same time.

tarum FB003. *Lactobacillus* spp. FB011 and FB081 isolates exhibited significantly higher percentage of coaggregation against all tested pathogenic strains compared to the reference LGG strain except *B. cereus*. In this study, all four LAB strains showed strong coaggregation activities against tested pathogen strains. Among these LAB strains, *Lactobacillus* sp. FB011 was found to be the superior strain in terms of forming coaggregates.

Adhesion to Caco-2 cells

In order to select potential probiotics, *in vitro* adhesion of these LAB strains to human epithelial cells derived from colon adenocarcinoma (Caco-2 line) for 1 h was assayed. Figure 4 shows the efficiency of these four LAB isolates in adhering to Caco-2 cells, in which *L. plantarum*, about 34.5%. These results are similar to earlier reports about adhesion capabilities of *Lactobacillus* spp. (García-Cayuela *et al.*, 2014; García-Ruiz *et al.*, 2014; Saxami *et al.*, 2016).

Antimicrobial activity, antibiotic resistance, and safety assessments

Antimicrobial activities of these LAB strains showed similar patterns (Table 2). Among 10 tested pathogens, cell free supernatant of FB011, FB081, and FB110 showed high levels of antibacterial activity against six pathogenic strains: *E. coli* K99, *S.* Gallinarum, *S.* Choleraesuis, *S.* Typhi, *S. boydii*, and *V. parahaemolyticus*. *L. plantarum* strongly inhibited *L. monocytogenes* and *S. aureus* than other isolates. It also possessed high level of antimicrobial activity against *S.* Choleraesuis and *V. parahaemolyticus*. For safety reason, susceptibility of these strains to antibiotics was determined. Results are summarized in Table 2. All strains were moderately susceptible to penicillin, ampicillin, streptomycin, kanamycin, and tetracycline. No multiple drug resistance was detected based on suggested MIC values of EFSA (European Food Safety Authority) cut-off (Panel, 2012). These strains did not possess bioamine activity, hemolytic activity, or mucin degrading activity. Absence of these biosynthesis enzymes is a criterion to used them as starter cultures or probiotics (Lee *et al.*, 2011; Angmo *et al.*, 2016). Thus, these strains might potential as new probiotics for starter culture in food or aquaculture.

Effect of LAB against *V. parahaemolyticus*

Growth inhibition of *V. parahaemolyticus* was detected in co-culture assay. Results are shown in Fig. 5. Survival rate of pathogen was significantly reduced during the test period after co-culture. At 32 h after co-culture with three FB011, FB081, and FB110 strains, survival rates of pathogen were reduced 100% ($P < 0.001$). The same was found for the survival rate of pathogen after co-culture with *L. plantarum* FB003

Table 2. Antimicrobial activity of cell free supernatant broth of isolates against common pathogens, antibiotic resistances, and safety assessment

		FB003	FB011	FB081	FB110			FB003	FB011	FB081	FB110
Pathogenic strains	*B. cereus*	+[1]	+	+	+	(MIC)(μg/ml)	Penicillin	4[2]	4	4	4
	E. coli K99	+	++	++	++		Ampicillin	1	0.5	1	0.5
	L. monocytogenes	+++	+	+	+		Vancomycin	64	128	128	64
	S. Gallinarum	+	++	++	++		Streptomycin	4	16	4	16
	S. aureus	+++	+	+	++		Kanamycin	8	8	8	8
	S. Choleraesuis	++	++	++	+		Tetracycline	2	1	2	2
	S. Typhi	+	++	++	++	Bioamine production		ND	ND	ND	ND
	S. boydii	+	++	++	++	Hemolytic activity		ND	ND	ND	ND
	Y. enterocolitica	+	+	+	+	Mucin degradation		ND	ND	ND	ND
	V. parahaemolyticus	+++	+++	+++	+++						

[1] +, zone ≤ 4 mm; ++, ≤ 8 mm; +++, > 8 mm
[2] ND, non-detected

for 36 h. These results indicated that the inhibitory effect of *L. plantarum* against *V. parahaemolyticus* was lower than that of *Latobacillus* spp. FB011, FB081, and FB110. In this study, four isolates effectively inhibited *V. parahaemolyticus* strains after 2 days of co-culture. We also confirmed that *L. plantarum* also had strong bactericidal effect against *Vibrio*. It has been previously demonstrated that *L. plantarum* can kill different pathogenic bacteria such as *Salmonella*, *Clostridia*, and *E. coli* (Murry Jr *et al.*, 2004; De Vries *et al.*, 2006). Moreover, three FB011, FB081, and FB110 isolates which closed to *Lactobacillus sakei* showed stronger inhibition effect on *Vibrio* than *L. plantarum*, suggesting that mechanisms of action of these two *Lactobacillus* species might differ.

In summary, we found that one *L. plantarum* strain FB003 and three strains FB011, FB081, and FB110 isolated from fermented and salted shrimp showed high potential as probiotics. They were also found to be safe as starter culture in food industry or cultivation. *L. sakei* showed stronger killing effect on pathogenic *V. parahaemolyticu* than *L. plantarum*, making it a promising candidate for treating *Vibrio* infections. Although further studies are needed to identify the inhibitory mechanism, results of this study might provide novel anti-*Vibrio* strategies through the use of *Lactobacillus*.

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