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Probiotic characterization of lactic acid bacteria isolated from infants feces and its application for the expression of green fluorescent protein

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ABSTRACT

Aims: In this study, lactic acid bacteria (LAB) were isolated from 42 healthy infants and determined for probiotic properties. Twelve LAB isolates with potential probiotic properties were selected and screened for their feasibility of heterologous protein expression by selection of erythromycin sensitive isolates.
Methodology and results: One of eleven erythromycin-sensitive LAB isolates identified and designated as *Lactobacillus fermentum* 47-7 was able to acquire and stable maintain the *Escherichia coli-Lactobacillus* shuttle vector, pRCEID-LC13.9. Further electrotransformation of *L. fermentum* 47-7 with the recombinant pLC13.9:LDH-PRO1:GFPuv containing green fluorescent protein (GFP) gene found that recombinant *L. fermentum* can express GFP.
Conclusion, significance and impact of study: The probiotic *L. fermentum* isolate can be used as host for expression of heterologous proteins and could possibly be further developed as the alternate oral delivery system for various biomolecules for biotechnological application.

Keywords: Heterologous protein expression, green fluorescent protein, lactic acid bacteria, *Lactobacillus fermentum*, probiotic

INTRODUCTION

Lactic acid bacteria (LAB) consist of a heterogeneous group of Gram-positive bacteria with their common ability to produce lactic acid as the major metabolic product of carbohydrate fermentation. LAB are commonly present in the intestinal tract of both human and animal where many of LAB with probiotic properties have been isolated (Fuller, 1989). Many laboratory methods have been used to evaluate for the probiotic properties. The most common selected methods reflecting the ability of LAB to persist, to adhere to cells and to antagonize the pathogens seem to be accepted as the important factors for potential probiotic selection (Ehrmann *et al.*, 2002).

Besides their probiotic and immunomodulation properties, the feasibility to be genetically manipulated to express a wide range of heterologous proteins has drawn intense attention to explore and used LAB as mucosal delivery vehicles for therapeutic and prophylactic molecules (Well, 2011). Due to its ease to detect and quantitate, the green fluorescent protein (GFP) is a model protein used to monitor heterologous protein expression in several species of LAB, such as *L. casei* (Pérez-Arellano and Pérez-Martinez, 2003), *L. plantarum* (Geoffroy *et al.*, 2000), *Lactococcus lactis* (Scott *et al.*, 2000) and other

lactobacillus species (Gory *et al.*, 2001; Mota *et al.*, 2006; Yu *et al.*, 2007). The green fluorescent protein (GFP) is a protein isolated from the jellyfish *Aequorea victoria* that exhibit bright green fluorescence when exposed to the ultraviolet light (Shimomury *et al.*, 1962). The GFP gene is frequently used as a universal reporter of expression (Philip, 2001). To date, the GFP gene has been introduced and expressed in a broad range of organisms, such as mice (Liu *et al.*, 2003), zebrafish (Lee and Cole, 2007) and bacteria (Feilmeier *et al.*, 2000). Several other heterologous proteins have been successfully expressed in LAB especially various infectious agents such as pneumococcal surface antigen A (PspA) of *Streptococcus pneumoniae* expressed in *L. plantarum* and *L. helveticus* (Oliveira *et al.*, 2006), spike protein of severe acute respiratory syndrome (SARS) coronavirus expressed in *L. casei* (Lee *et al.*, 2006). One major advantage of LAB as mucosal delivery vehicles for prophylactic molecules is their potential to stimulate both systemic and mucosal immune responses (Well, 2011).

In this study, LAB isolated from healthy children will be screened for probiotic properties. The isolates with good probiotic properties will be selected to construct recombinant LAB expressing heterologous protein.

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MATERIALS AND METHODS

Bacterial strains, cloning vectors, primers and growth conditions

Table 1 lists the bacterial strains, oligonucleotide primers and cloning vectors used in this study. All LAB used in this study were grown in deMan, Rogosa and Sharpe (MRS) broth (Difco, UK) at 37 °C for 16 h without shaking. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311 and *Escherichia coli* O157:H7 (kindly provided by Dr. Sawanit Tongpim, Faculty of Science, Khon Kaen University) were cultured in brain heart infusion (BHI) broth (Oxoid, UK) with shaking at 37 °C for 18 h.

Isolation of LAB from infant feces

LAB were isolated from fecal specimens of 42 healthy infants at Srinagarind Hospital, Khon Kaen, Thailand. One gram of fecal sample was suspended and serially diluted with sterile normal saline solution (NSS). The appropriate dilutions of fecal suspension were plated onto MRS agar containing 0.5% $CaCO_3$ and incubated for 48 h at 37 °C. The colonies surrounded by a clear zone on the plate were selected. Isolates of Gram positive, catalase negative rods and no hemolysis on sheep blood agar (Ghrairi *et al.*, 2008) were further subjected to determine for *in vitro* probiotic properties which consist of acid and bile salt tolerance test, hydrophobicity test and antimicrobial activity assay and for antibiotic susceptibility test. This study was approved by Khon Kaen Ethics Committee for Human Research, Ref. No. HE521269.

Acid and bile salt tolerance test

LAB isolates were incubated in MRS broth at 37 °C for 16 h. After incubation, the culture was washed with phosphate saline buffered (PBS) and adjusted the bacterial density to McFarland no. 0.5 and used for acid and bile salt tolerance test. Acid tolerance test was modified from method described by Erkkilä and Petäjä (2000), in brief one-tenth dilution of the bacterial cell suspension was incubated in PBS pH 2, 3 and 6.2 (as control) for 3 h at 37 °C. After incubation, the numbers of viable bacteria were determined by plate count using MRS agar. The percentage of survival was calculated as (log CFU_{acid}/log $CFU_{control}$) × 100. For bile salt tolerance test, method described by Wang *et al.* (2010) with some modifications was used. The bacterial suspension was adjusted to 1% and inoculated in MRS broth (as control) and MRS broth with 0.3% and 0.5% Oxgall (Sigma, USA). After incubation at 37 °C for 12 h, the numbers of viable bacteria were determined by plate count using MRS agar. The percentage of survival was calculated as (log CFU_{bile}/log $CFU_{control}$) × 100.

Table 1: Bacterial strains, cloning vectors and oligonucleotide primers used in this study.

Materials	Relevant properties	Source/reference
Bacteria		
Lactobacillus fermentum 47-7	Human isolate	This study
L. sakei CECT906	Bacteriocin-sensitive strain	CECT[a]
L. casei ATCC393	Bacteriocin non-producing strain	ATCC[b]
L. plantarum LL441	Bacteriocin producing strain	CECT
Salmonella enterrica subsp. enterica serovar Typhimurium ATCC 13311	Pathogenic strain	ATCC
Escherichia coli O157:H7	Pathogenic strain	KKU[c]
Plasmid		
pRCEID-LC13.9	Ap[r], Em[r], *E. coli-L. casei* shuttle vector based on pRCEID13.9	RCEID[d], (Panya *et al.*, 2012)
pLC13.9:LDH-PRO1:GFPuv	pRCEID-LC13.9-derived construct expressing the *gfp*UV gene	RCEID, (Panya *et al.*, 2012)
Oligonucleotide primers	Sequence (5' - 3')	
27F	AGAGTTTGATCCTGGCTCAG	(Eden *et al.*, 1991)
1492R	GGTTACCTTGTTACGACTT	(Eden *et al.*, 1991)

[a]Colección Española de Cultivos Tipo (Spanish Type Culture Collection)
[b]American Type Culture Collection
[c]Dr. Sawanit Tongpim, Faculty of Science, Khon Kaen University
[d]Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University

Hydrophobicity test

Microbial adhesion to solvents (MATS) was determined to evaluate the hydrophobicity of bacterial cell surface. After incubation at 37 °C for 16 h, the bacterial cells were washed twice in PBS by centrifugation at 10,000 ×*g*. for 5 min at 4 °C and adjusted to an optical density (O.D.) of 0.4 -0.5 at 600 nm with PBS (A0). The 3 mL of bacterial suspension was added with 1 mL xylene, incubated at room temperature for 10 min and mixed by vortexing for 2

min. After mixing, the tube was left in the upright position for 20 min for phase separation and the lower aqueous phase was collected to measure the O.D. at 600 nm (A1). The percentage of hydrophobicity was calculated as $(A_0 - A_1)/A_0 \times 100$ (Pérez-Sánchez et al., 2011).

Antimicrobial activity assay

The antimicrobial activity of the isolates was done by the well diffusion assay (Mishra and Prasad, 2005). The brain heart infusion (BHI) agar was used for culture indicator pathogenic bacteria, S.Typhimurium ATCC 13311 and E. coli O157:H7. The MRS agar were used for culture indicator LAB, Lactobacillus sakei CECT906 which is bacteriocin-sensitive strain, L. casei ATCC393 (bacteriocin non-producing strain) and L. plantarum LL441 (bacteriocin producing strain). The agar plate with a central well with the diameter of 5 mm. was spread with the indicator bacteria (L. sakei CECT906, S. Typhimurium ATCC13311 and E. coli O157:H7) and filled the well with 0.05 mL of the culture supernatant neutralized to pH 6.5. The plate was incubated at 37 °C for 48 h and observed for inhibition zone.

Antibiotic susceptibility test

The antibiotic susceptibility of the isolates was determined by the minimal inhibitory concentration (MIC) technique (Santini et al., 2010) for penicillin, ampicillin, vancomycin, erythromycin, tetracycline and nalidixic acid. Each antibiotic was serially diluted with concentration ranging 0.5 to 256 μg/mL in 96 well plate. Fifty microliter of the bacterial isolates at 10^6 CFU/mL were inoculated into the plate filled with 50 μL of the serial diluted antibiotics. The MIC was defined as the lowest antibiotic concentrations that showed growth inhibition.

Electrotransformation of LAB isolates

The competent LAB isolates were prepared according to method of Bruce and Jeannette (1987). In brief, a single colony of LAB isolate from MRS plate was grown in MRS broth with 1% glycine and incubated at 37 °C for 16 h. The bacterial pellet harvested from overnight culture was transferred into a new MRS broth with 1% glycine and incubated until the OD 600 reached 0.6. The bacterial cells were harvested, washed three times in cold sterilized deionized water and resuspended in ice-cold 30% polyethylene glycol 8000. The competent cells can be used immediately or stored at –80 °C until use. For electrotransformation (Panya et al., 2012), the frozen competent cells were thawed on ice, mixed with 1 μL (100 ng/ μL) pRCEID-LC13.9 or pLC13.9:LDH-PRO1:GFPuv and filled in ice-chilled electroporation cuvette. The electric pulse with the voltage of 2.5 kV and resistance of 400Ω was applied by using Gene PulserMXCell™ (BioRad, USA). Following the pulse, the electroporated cells were incubated in MRS broth at 37 °C for 3 h, and plated onto MRS agar plates containing 2.5 μg/mL of erythromycin and incubated at 37 °C for 48 h.

Segregational and structural stability of the plasmid in LAB isolate No. 47-7

Segregational and structural stability of the plasmid in LAB isolate 47-7 was done by method described by Sorvig et al. (2005). The plasmid-free LAB isolate 47-7 prepared by plasmid curing method (McHugh and Swartz, 1977) was electrotransformed with pRCEID-LC13.9. This plasmid is an E. coli / L. casei shuttle vector using the replicon of pRCEID13.9 derived from L. casei TISTR1341 and has ampicillin and erythromycin resistant gene as selective markers. The transformants carrying pRCEID-LC13.9 were grown in MRS broth without erythromycin for 100 generations. Every 20 generations (around 42 min/generation), an aliquot of the culture was taken, diluted and plated onto erythromycine-free MRS medium. Colonies were then replicated on media with and without erythromycin (2.5 μg/mL). The colonies on the plate were counted and calculated for the percentage of plasmid stability. For plasmid structural stability, the transformants carrying pRCEID-LC13.9 were grown in MRS broth without erythromycin for 100 generations. Every 20 generations, an aliquot of the culture was taken, diluted and plated onto erythromycin-free MRS medium. The plasmids isolated from the colonies derived at 20, 40, 60, 80 and 100 generations by method of O'Sullivan and Klaenhammer (1993) were digested with EcoRI. The digested pattern of the plasmid was visualized by agarose gel electrophoresis and compared with that derived from its original plasmid.

Identification of LAB isolates

The LAB isolates with good in vitro probiotic properties, erythromycin sensitive and can acquire and maintain the heterologous plasmid will be identified by 16S rRNA sequencing using method described by Eden et al. (1991). In brief, the genomic DNA of LAB isolate was purified from overnight culture using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) and subjected to polymerase chain reaction (PCR) for the partial amplification of its 16S rRNA gene with the universal primer pairs 27F and 1492R (Table 1). PCR condition consists of pre-denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 48 °C for 30 sec, 72 °C for 2 min each and final extension at 72 °C for 10 min. PCR products with the length of 1516 base pairs were sequenced based on cycle sequencing at 1st BASE Company, Malaysia. The sequencing results were compared with sequences held in NCBI database (http://www.ncbi.nlm.nih.gov).

Detection of GFP expression in recombinant L. fermentum

Determination of GFP expression in recombinant L. fermentum carrying pLC13.9:LDH-PRO:GFPuv was done by culturing the bacteria in MRS broth with 2.5 μg/mL erythromycin at 37 °C for 16 h. After incubation, the bacterial cells were washed, resuspended in PBS and

examined the cells under fluorescence microscope for GFP expression. Further confirmation of GFP expression was done by western blotting analysis (Lizier, 2010). The bacterial cells were resuspended in lysis buffer, disrupted by sonication on ice (10×30 sec at full power, with a 30 sec intermission between burst) using a Soniprep 150 (MSE, UK) with a fine probe. The bacterial lysate were loaded to 12% of SDS-PAGE and run at 100V for 2 h. The proteins were blotted to PVDF membrane (Bio-Rad, USA) by using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA) with the voltage setting at 25 V for 30 min. For immunodetection of GFP, mouse monoclonal anti-GFP (Abcam, UK) and goat anti-mouse monoclonal IgG conjugated-HRP (Santa Cruz Biotechnology, USA) were used as primary and secondary antibodies. For signal detection, the membrane was applied with SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific, USA) and exposed to CL-Xposure Film (Thermoscientific, USA). After brief exposure, the film was incubated in developing solution for generating the specific protein band and finally fixed in fixative solution.

RESULTS

Isolation of LAB from infants

The fecal samples from 42 children aged under 12 months were used for LAB isolation. Two hundred and thirty-seven isolates with clear zone on MRS medium containing $CaCO_3$ were found. Only 119 LAB isolates identified as Gram positive, catalase-negative rods and no hemolysis on sheep blood agar were selected for further studies.

Determination of LAB isolates for *in vitro* probiotic properties

One hundred and nineteen LAB isolates were determined for *in vitro* probiotic properties which included tests for hydrophobicity, acid and bile salt tolerance and antimicrobial activity. Based on hydrophobicity values (Ehrmann *et al.*, 2002), 28 isolates (24.37%) were strongly hydrophobic, 38 isolates (31.09%) showed hydrophobic and the remaining 53 isolates (44.54%) were hydrophilic. The LAB isolates with hydrophobic property (66 isolates) were further screened for acid and bile salt tolerance. For acid tolerance, thirty-five isolates with the survival rate more than 80% after 3 h incubation at pH 3 were found. In addition, 3 isolates (LAB No. 23-2, 23-5 and 28-3) showed high acid tolerance with more than 80% survival after 3 h incubation at pH 2. For the bile salt tolerance test, seventeen LAB isolates were tolerance at 0.3% bile salt medium and 6 isolates (LAB No. 21-4, 23-2, 23-5, 28-3, 28-5, and 28-6) can survival in culture with 0.5% bile concentration. From acid and bile salt tolerance results, 12 LAB isolates were selected for determination for antimicrobial activity. It was found that eight isolates showed growth inhibition activities against bacteriocin-sensitive *L. sakei* CECT906 and six isolates showed inhibition against *S.* Typhimurium. Among these isolates,

LAB No. 28-3 and 47-7 showed growth inhibition against both *L. sakei* CECT906 and *S.* Typhimurium. None shows antimicrobial activity against *E. coli* O157:H7. Table 2 summarizes the acid and bile salt tolerance and antimicrobial activities of the selected 12 LAB isolates.

Determination of antibiotic susceptibility for selection of suitable isolate for heterologous protein expression

Since the shuttle and expression vectors used in this study has erythromycin resistance gene as selective maker, thus the suitable host for heterologous protein expression should be erythromycin sensitive isolate. Table 3 showed the antibiotic susceptibility pattern of the selected 12 LAB isolates. All LAB isolates, except LAB 18-5, are erythromycin-sensitive isolates. In addition, all isolates were susceptible to β-lactam antibiotics, i.e. penicillin and ampicillin but resist to nalixidic acid.

The ability of LAB isolates to acquire and maintain heterologous plasmid

To determine the ability of erythromycin-sensitive LAB isolates to acquire heterologous plasmid, the *E. coli* / *L. casei* shuttle vectors-pRCEID-LC13.9 with the length of 5,280 bp and with erythromycin resistance gene as selection marker were used for electrotransformation. It was found that among eleven erythromycin-sensitive isolates only LAB isolate No. 47-7 can acquire pRCEID-LC13.9. For segregational and structural stability study in this isolate, it was found that in the absence of erythromycin in the medium, pRCEID-LC13.9 was maintained at 84% after 100 generations while the structural stability was intact after 100 generations. This result indicated that LAB isolate No. 47-7 can acquire, maintain the plasmid, pRCEID-LC13.9 and is suitable for use as bacterial host for heterologous protein expression. Based on 16S rDNA sequencing, the LAB isolate 47-7 was identified as *L. fermentum* and was then designated as *L. fermentum* 47-7.

Expression of GFP in *L. fermentum* 47-7

pRCEID-LC13.9:LDH:PRO1:GFPuv, the plasmid containing *gfp*uv gene with L-lactate dehydrogenase (*ldh*L) promoter (Panya *et al.*, 2012) was electrotransformed into *L. fermentum* 47-7 to determine its ability to express heterologous protein. After electrotransformation, the transformant *L. fermentum* 47-7was determined for GFP expression by visualization under fluorescence microscopy and by western blotting. It was found that recombinant *L. fermentum* 47-7 carrying *gfp*uv gene (r*L. fermentum*:GFP+) can be seen as fluorescence cells under fluorescence microscopy (Figure 1A). By western blotting using antibody against GFP_UV, the specific GFP band with the molecular weight of 29.53 kDa was found in *L. fermentum*:GFP+ (Figure 1B, lane 2) but not in *L. fermentum* carrying the empty plasmid, pRCEID-LC13.9. (Figure 1B, lane 1).

Table 2: Acid and bile salt tolerance and antimicrobial activities of 12 selected LAB isolates.

Isolate name	Acid tolerance (% survival)		Bile salt tolerance (% survival)		Antimicrobial activity		
	pH2	pH3	0.3%	0.5%	*L. sakei*	*S.* Typhimurium	*E. coli*
LAB 16-5	0.00	90.72	81.85	0	+	-	-
LAB 18-5	0.00	96.02	76.7	0	-	-	-
LAB 21-4	59.49	98.58	98.67	83.08	-	-	-
LAB 23-2	95.34	96.93	87.32	81.23	-	-	-
LAB 23-5	92.79	97.98	70.91	70.07	-	-	-
LAB 28-3	81.75	96.93	86.02	76.74	+	+	-
LAB 28-5	48.69	91.87	82.52	64.47	-	-	-
LAB 28-6	52.86	91.02	0	0	-	-	-
LAB 47-5	0.00	87.75	0	0	-	-	-
LAB 47-7	56.12	93.95	94.83	93.45	+	+	-
LAB 48-5	0.00	74.77	0	0	-	-	-
LAB 48-6	0.00	88.19	0	0	-	-	-

+, The diameter of clear inhibition zone extending laterally around the well ≥ 2.0 mm; –, No inhibition zone or the diameter of clear inhibition zone extending laterally around the well < 2.0 mm.

Table 3: Antibiotic susceptibility profile of 12 LAB isolates and the microbiological cut-off values (mg/L). Strains with MICs higher than the breakpoints below are considered as resistant.

	Penicillin	Ampicillin	Erythromycin	Tetracycline	Nalidixic	Vancomycin
Cut-off values (mg/L). (*Lactobacillus* obligate heterofermentative [a])	4	2	1	8	n.r.	n.r.
(*Lactobacillus* obligate homofermentative [a])	4	1	1	4	n.r.	2
LAB 16-5	S	S	S	S	R	R
LAB 18-5	S	S	R	R	R	S
LAB 21-4	S	S	S	S	R	S
LAB 23-2	S	S	S	S	R	R
LAB 23-5	S	S	S	S	R	R
LAB 28-3	S	S	S	S	R	S
LAB 28-5	S	S	S	S	R	S
LAB 28-6	S	S	S	S	R	S
LAB 47-5	S	S	S	R	R	S
LAB 47-7	S	S	S	S	R	R
LAB 48-5	S	S	S	R	R	R
LAB 48-6	S	S	S	S	R	S

n.r. not required.
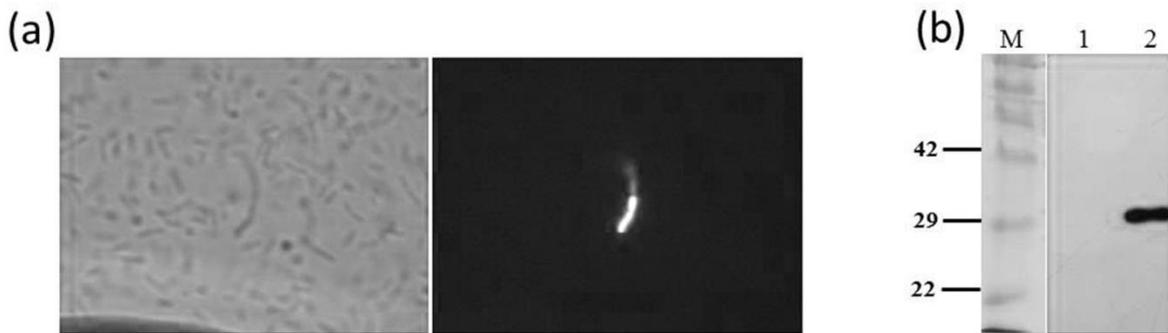[a] European Food Safety Authority (EFSA)

Figure 1: The expression of GFP in *L. fermentum*:GFP+. a, as seen under the brightfield (left) and fluorescent (right) microscope with the magnification of 1000X; b, as detected by immunodetection using mouse anti-GFP monoclonal antibody in whole cell lysate of *L. fermentum*:GFP+.   Lane 1, *L. fermentum*:pRCEID-LC-13.9; Lane 2, *L. fermentum*:GFP+; Lane M, Protein molecular weight marker (Chromatein Prestained Protein Ladder, Vivantis, Malaysia).

DISCUSSION

One important property of probiotic bacteria is their ability to survive in the gastrointestinal tract of the host. *In vitro* experiment to reflect this property is the determination of the hydrophobicity, acid and bile salt tolerance (Vinderola and Reinheimer, 2003). Test for hydrophobicity has been widely accepted to reflect the ability of bacteria to adhere to host cell as the more hydrophobicity the bacteria has, the greater adhesion the bacteria would be acquired (Rosenberg *et al.*, 1983; Li, 2008). The resistance to both gastric and intestinal environment is another important factor that the probiotic bacteria must have in order for survival before they can exert their beneficial effect (Ehrmann *et al.*, 2002). In this study, twelve LAB isolates with acceptable good resist to acid and bile salt were selected. Besides the above properties, the antimicrobial activity is another important characteristic that probiotic bacteria should have. Six of twelve LAB isolates have antimicrobial activity against *S.* Typhimurium.

The erythromycin-sensitive isolates with potential *in vitro* probiotic properties were selected to determine their ability to acquire and maintain the exogenous plasmid. It was found that only the LAB47-7 isolate which had been identified and designated as *L. fermentum* 47-7, is able to acquire and permissible for the pRCEID-LC13.9. Several factors besides the bacterial host have the influence on the transformation efficiency, such as the ingredients and concentration of cell wall weakening reagent used, the strength and time of electric pulse, electroporation buffer and recovery medium (Luchansky *et al.*, 1988; Mason *et al.*, 2005) however, such extensive optimization of electrotransformation condition for other isolates was not explored in this study. For pRCEID-LC13.9 stability in *L. fermentum* 47-7, its structural stability is similar to that of *L. casei* RCEID02 (Panya *et al.*, 2012), the native host of this cloning vector, but its segregation is slightly lower than that of *L. casei* RCEID02 (84% versus 90%), but the plasmid stability at this level is sufficient for use in the construction of recombinant LAB expressing heterologous protein in most applications, especially those for delivery of the desired protein to stimulate the host immune response. There are several factors that can influence both the segregation and structural stability, such as plasmid structure and copy number, type of host especially its genetic background (Ponciano *et al.*, 2007).

The selected *L. fermentum* isolate was identified through its 16SrRNA gene sequence as *Lactobacillus fermentum*. This bacterial species, though not a predominant species, is commonly found in the human gut and vagina (Kaewnopparat *et al.*, 2013). Around 11.8% of *L. fermemtum* were isolated from faecal samples derived from 50 breast-feeding infants with the age of 3-30 days (Ozgun and Vura, 2011). In addition, it has been demonstrated that this bacterial species can colonize the mammary gland after administered them in the capsule form to lactating mothers (Slomiany *et al.*, 1985; Handley *et al.*, 1987; Kang and Conway, 2006). Apart from its safety (Finegold, 1977), the health benefit of *L. fermentum* has been reported by several studies. Maldonado *et al.* (2012) reported that infants receiving a follow-on formula enriched with *L. fermentum* demonstrated significant decrease in the number of community-acquired gastrointestinal and respiratory infections. Olivares *et al.* (2007) demonstrated that oral administration of *L. fermentum* CECT5716 potentiates the immune response against influenza in subjects receiving influenza vaccine and may provide enhanced systemic protection from infection by increasing both the T-helper type 1 (Th1) response and virus-neutralizing antibodies. In addition, *L. fermentum* CECT5716 has been reported to enhance tumor necrosis factor (TNF) response of murine bone-marrow derived macrophages, Th1 cytokine production by mice spleen cells and to increase the intestinal IgA concentration but not serum IgA in mice through oral administration (Díaz-Ropero *et al.*, 2007). For our *L. fermentum* 47-7 shows potential *in vitro* probiotic properties, with antimicrobial activity against both

bacteriocin-sensitive strain, *L. sakei* CECT906 and food borne pathogen, *S.* Typhimurium. The precise mechanism of *in vitro* antimicrobial activity of this isolate was not explored in this study. The antimicrobial activity of probiotic bacteria both *in vivo* and *in vitro* can be mediated through several mechanisms such as the production of antimicrobial compounds such as lactic acid, short chain fatty acids, hydrogen peroxide, bacteriocin-like substances and biosurfactants (Boris and Barbes, 2000), bacteriocin, including the competition for nutrient (Reid and Burton, 2002) the ability to adhere to the surface of the intestinal epithelial to inhibit the adhesion of pathogens (Stöber *et al.*, 2010) and modulate the immune response (Lebeer *et al.*, 2008). In case of *L. fermentum*, it has been demonstrated that this bacteria can produce hydrogen peroxide, bacteriocin and biosurfactants to inhibit the growth of intestinal and urogenital pathogens (Anukam and Reid, 2007; Kaur *et al.*, 2013).

There is now a growing interest in the use of LAB as delivery vehicles for mucosal immunization and treatment (Well, 2011). The major advantage of LAB is LAB-based mucosal vaccine is able to elicit both secretory Ig A and serum antibody (Lee *et al.*, 2006). The other advantages include their safety as they are generally recognized as safe or GRAS (Yuki *et al.*, 1999) and the ability to be engineered to express various heterologous proteins especially those from various pathogenic microorganisms (Well, 2011). The LAB species commonly used to engineer for heterologous protein expression are *Lactococcus lactis* (Hanniffy *et al.*, 2007), *Lactobacillus plantarum* (Corthésy *et al.*, 2005), *Lactobacillus casei* (Suebwongsa *et al.*, 2013). For *L. fermemtum,* isolate of *L. fermentum* from guinea pig vaginal tract has been used to express and secrete the VD4 region of chlamydial major outer-membrane protein (Rush *et al.*, 1997) and also used for expression of *Chlamydia psittaci* and human immunodeficiency-derived antigens as anchorage form (Turner and Giffard, 1999). As the *L. fermentum* isolate in this study can be successfully engineered to express GFP protein by using *L. casei* replicon-based expression vector (pRCEID-LC13.9) (Panya *et al.*, 2012), this indicated that this *L. fermentum* isolate has the flexibility to be engineered with heterologous replicon-based expression vector for expression other interesting proteins.

In conclusion, we had screened and isolated probiotic LAB derived from healthy infants. One isolate identified as *L. fermentum* was selected based on erythromycin sensitivity for expressing heterologous proteins. The recombinant *L. fermentum* isolate was successfully expressed GFP by using expression vector with *ldh* promoter. Thus, this *L. fermentum* isolate could be further exploited as protein delivery vehicle for various biotechnological applications.

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