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

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\section*{ABSTRACT}

\textbf{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. \textit{Lactobacillus fermentum 47-7} was able to acquire and stable maintain the \textit{Escherichia coli-Lactobacillus} shuttle vector, pRCEID-LC13.9. Further electrottransformation of \textit{L. fermentum} 47-7 with the recombinant pLC13.9:LDH-PRO1:GFPuv containing green fluorescent protein (GFP) gene found that recombinant \textit{L. fermentum} can express GFP.

\textbf{Conclusion, significance and impact of study:} The probiotic \textit{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.

\textbf{Keywords:} Heterologous protein expression, green fluorescent protein, lactic acid bacteria, \textit{Lactobacillus fermentum}, probiotic

\section*{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 \textit{L. casei} (Pérez-Arellano and Pérez-Martinez, 2003), \textit{L. plantarum} (Geoffroy et al., 2000), \textit{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 \textit{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 \textit{Streptococcus pneumoniae} expressed in \textit{L. plantarum} and \textit{L. helveticus} (Oliveira et al., 2006), spike protein of severe acute respiratory syndrome (SARS) coronavirus expressed in \textit{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. Isolates of Gram positive, catalase negative rods and no hemolysis on sheep blood agar were selected. The colonies surrounded by a clear zone on the plate containing 0.5% CaCl₂ were selected. The bacterial suspension was added with 1 mL xylene, incubated at 37 °C for 10 min and mixed by vortexing for 2 min. The 3 mL of bacterial suspension was plated onto MRS agar. The percentage of survival was calculated as (log CFUcontrol/log CFUexperimental) × 100. For bile salt tolerance test, the numbers of viable bacteria were determined by plate count using MRS agar. The percentage of survival was calculated as (log CFUcontrol/log CFUexperimental) × 100.

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 Pethäjä (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 CFUcontrol/log CFUexperimental) × 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 | CECT² |
| L. casei ATCC393 | Bacteriocin non-producing strain | ATCC² |
| L. plantarum LL441 | Bacteriocin producing strain | CECT |
| Salmonella enterica subsp. enterica serovar Typhimurium | Pathogenic strain | ATCC |
| ATCC 13311 | Pathogenic strain | KKU³ |
| Escherichia coli O157:H7 | Pathogenic strain | CECT |
| Plasmid |                     |                  |
| pRCEID-LC13.9 | Ap', Em', E. coli-L. casei shuttle vector | RCEID², (Panya et al., 2012) |
| pLC13.9:LDH-PRO1:GFPuv | pRCEID-LC13.9-derived construct expressing the gfpuv gene | RCEID, (Panya et al., 2012) |
| Oligonucleotide primers |                     |                  |
| 27F | Sequence (5'-3') | (Eden et al., 1991) |
| 1492R | AGAGTTTGATCCTGGCAGCAG | (Eden et al., 1991) |
|                      | GGTTACCTGGTACGACTT |                  |

¹Coleción Española de Cultivos Tipo (Spanish Type Culture Collection) ²American Type Culture Collection ³Dr. Sawanit Tongpim, Faculty of Science, Khon Kaen University ⁴Research 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.
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 (A0 – A1)/A0 × 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 10^5 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 (Pany 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-PRO: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 replication 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 erythromycin-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 CaCO3 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 survive 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 marker, 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 nalidixic 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 electrot transformation. 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. fermentum47-7

pRCEID-LC13.9:LDH:PRO1:GFPuv, the plasmid containing gfpuv gene with L-lactate dehydrogenase (ldhL) promoter (Panya et al., 2012) was electrot transformed into L. fermentum 47-7 to determine its ability to express heterologous protein. After electrot transformation, 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 gfpuv gene (rL. fermentum:GFP+) can be seen as fluorescence cells under fluorescence microscopy (Figure 1A). By western blotting using antibody against GFPuv, 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.

| Strains with isolates | Cut-off values (mg/L) |
|-----------------------|------------------------|
|                       | Penicillin | Ampicillin | Erythromycin | Tetracycline | Nalidixic | Vancomycin |
| (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)
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 Reineheimer, 2003). Test for hydrophobicity has been widely accepted to reflect the ability of bacteria to adhere to host cell as the more hydrophobiect 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 electrottransformation condition for other isolates was not explored in this study. For pRCEID-LC13.9 stability in L. fermentum 47-7, its structural similarity 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 16S rRNA 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. fermentum 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 (Fingold, 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 Barbés, 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 (Hannify et al., 2007), Lactobacillus plantarum (Corthésy et al., 2005), Lactobacillus casei (Suebwongsa et al., 2013). For L. fermentum, 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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