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Heterologous Protein Expression by *Lactobacillus casei* Isolated from Chicken Intestine

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In this study, lactic acid bacteria (LAB) were isolated from healthy chicken intestine and determined for probiotic properties. Six isolates with potential probiotic properties were selected and screened for their feasibility of heterologous protein expression by selection of erythromycin sensitive isolates. Two erythromycin-sensitive isolates were found and further subjected to plasmid curing process. One isolate could completely eliminate its indigenous plasmids, identified as *Lactobacillus casei* by 16S rRNA sequencing and was designated as *L. casei* RCEID08. This isolate could stably maintain the *E. coli-Lactobacillus* shuttle vector, pRCEID-LC13.9. Further electrotransformation of *L. casei* RCEID08 with recombinant pLC13.9:LDH-PRO1 containing green fluorescent protein (GFP), tetanus toxin fragment C (TTFC) or VP2 of infectious bursal disease virus (IBVD) found that all recombinant *L. casei* can express these heterologous proteins. Thus, our probiotic *L. casei* isolate can be used as host for expression of various heterologous proteins and could possibly be further developed as the alternate oral delivery system of various biomolecules for biotechnological applications.

Key words: Heterologous Protein Expression, Lactic acid bacteria, Recombinant *L. casei*.

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria that can be found in diverse environments. LAB have been used in various applications, such as, in food industry and in human health. LAB are present in the intestinal tract of most animals as symbiotic organisms and confer an enhanced resistance to infection [1, 2]. Most of LAB can be used as probiotics which are defined as live microorganisms which when upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition [3]. For the probiotics in poultry industry, they can be used to improve growth performance [4, 5], increase egg production [6] and reduce gastrointestinal infection [7, 8]. Many LAB with probiotic properties have been isolated and selected from intestinal microflora of animals. A variety of *in vitro* methods have been used to evaluate for the optimal probiotic properties. The most frequent selected methods reflecting the ability to persist, adherence to cells and antagonistic activities seem to be accepted as the important factors for potential probiotic selection [9].

The most interesting application of LAB in human and veterinary medicine is to use LAB as mucosal delivery vehicles for therapeutic and prophylactic molecules. With respect to this application, besides their probiotic properties and generally regarded as safe (GRAS), one major advantage is that LAB can be engineered to express

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several heterologous proteins. Green fluorescent protein (GFP) is one of the popular heterologous proteins used for expression in several species of LAB, e.g. *L. casei*[10], *L. plantarum*[11], *Lactococcus lactis*[12] and other lactobacillus species [13, 14, 15]. Another protein, the non-toxic tetanus toxin fragment C (TTFC) has been used as a model antigen for expression in many different LAB to investigate as vaccine delivery vehicles. All the recombinant LAB expressing TTFC were able to induce protective immunity in experimental animals [16, 17, 18, 19]. Several other heterologous proteins have been successfully expressed in LAB especially various infectious agents [20, 21, 22, 23, 24]. For infectious bursal disease virus (IBDV), though several LAB were successfully expressed its viral capsid protein, the VP2, only non-LAB expression system were able to provoke protective immunity [25, 26, 27, 28, 29]. The aim of this study was to select LAB with *in vitro* probiotic properties isolated from chicken intestine and engineered it for heterologous protein expression.

MATERIALS AND METHODS

Bacteria and cultivation

The LAB isolates, two standard probiotics consist of *L. casei* TISTR 1341 (derived from Thailand Institute of Scientific and Technological Research) and *L. casei* ATCC 393 (American Type Culture Collection, USA), and all recombinant LAB constructed in this study were cultured in de Man, Rogosa and Sharpe (MRS) medium (Criterion, USA) and incubated at 37°C overnight. The indicator bacteria which commonly caused diseases in poultry industry consist of *Salmonella typhimurium* ATCC 13311, *Pasteurella multocida* (both derived from Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand) and *Escherichia coli* O157: H7 (kindly provided by Dr. Saowanit Tongpim, Faculty of Science, Khon Kaen University). These indicator bacteria were cultivated in brain heart infusion (BHI) medium (Oxoid, UK) at 37°C overnight. Agarified media were obtained by the addition of 15 g/l agar to the corresponding broth.

Isolation of LAB from chicken intestines

Eight of 4 weeks old, male chickens were sacrificed by cervical dislocation and their intestines were removed aseptically. One gram of intestinal content was homogenized with sterile normal saline solution (NSS) using glass rod. The homogenized solution was serially diluted with NSS and the appropriate dilutions were plated onto MRS agar containing 0.5% $CaCO_3$ and incubated for 48 h at 37°C. The colonies with clear zone were selected, inoculated in MRS broth and incubated overnight at 37°C for further study. This study was approved by The Animal Ethics Committee of Khon Kaen University (permit number AEKKU 01/2553).

Bacterial identification

The identification of LAB isolates was done by partial amplification of its 16S rRNA genes. Two universal primers consist of F8 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1492 (5'-GGTTACCTTGTTACGACTT-3')[30] were used for amplification. The sequence result was compared with sequences held in the NCBI database (http://www.ncbi.nlm.nih.gov).

Gastric fluid tolerance, bile salt tolerance and hydrophobicity assays

The gastric fluid tolerance and hydrophobicity assays were done according the methods described by Mota *et al*. [14]. To determine the acid tolerance, the bacterial isolates were grown overnight in MRS medium at 37°C. The overnight culture was washed with NSS and adjusted the bacterial density to McFarland no. 0.5. One hundred microliters of adjusted bacterial suspension was mixed with 900 µl of synthetic gastric juice (2 g of NaCl and 3.2 g of pepsin in 1 liter of distilled water, the pH was adjusted to 2.5 with concentrated HCl). For the bacterial growth control tube, NSS was added instead of the gastric juice. Both the test and control tubes were incubated at 37°C for 3 h. After incubation, the numbers of viable bacteria were determined by plate count. The percentage of survival was calculated as $(1\text{-}\log cfu_{acid}/\log cfu_{control}) \times 100$

Microbial adhesion to solvents (MATS) was determined to evaluate the hydrophobicity of bacterial cell surface [14]. Briefly, the 12 h cultures were harvested by centrifugation, washed twice and adjusted with NSS to an O.D. of 0.4 - 0.5 (A0) at 600 nm. The 1.2 ml of bacterial suspension was added with 0.2 ml xylene, incubated at room temperature for 10 min and mixed by vortexing for 2 min. After mixing, the tube was stood for 30 min and the lower aqueous phase was collected to

measure the O.D. at 600 nm (A1). The percentage of MATS was calculated as (1 – A1/A0) x 100

The bile salt tolerance assay was done according to method described by Walker and Gilliland [31]. In brief, the isolate was overnight cultured in MRS medium and 1% of this inoculum was added to MRS containing 0.3% bile salt (Sigma-Aldrich, USA). The suspension was then measured for the optical density at 600 nm before and after incubation the suspension at 37°C for 24 h. The percentage of growth was calculated as (1 – $O.D._{after\ incubation}$/ $O.D._{before\ incubation}$) x 100.

Detection of antimicrobial activity

The antimicrobial activity of the isolates was done by the well diffusion assay [32]. The brain heart infusion (BHI) agar plate with a central well (diameter of 5 mm) was spread with the indicator bacteria and filled in the central well with 0.05 ml of the isolate culture. The inoculated plate was incubated at 37°C for 24 h and observed for inhibition zone.

Antibiotic susceptibility test

The antibiotic susceptibility of the isolates were performed by microdilution minimal inhibitory concentration (MIC) technique modified from National Committee for Clinical Laboratory Standards (NCCLS) [33]. The antibiotics used in this study consist of 3 groups based on mode of action. The cell wall synthesis inhibitor group consists of penicillin and vancomycin. The protein synthesis inhibitor group consists of erythromycin, tetracycline and tylosin. The nucleic acid synthesis inhibitor group consists of nalidixic acid and ciprofloxacin.

The overnight culture was washed with NSS and adjusted the bacterial density to McFarland no. 0.5. The adjusted bacterial suspension was diluted 100-fold with MRS broth and added to a 96 well plate filled with MRS containing a serial 2-fold diluted antibiotics. After incubated at 37°C for 18 h, the MIC was observed. The isolates were further selected based on the susceptible to erythromycin as the isolates would be engineered based on expression vector using erythromycin as selective marker.

Plasmid curing in LAB isolates

For plasmid curing, novobiocin was used as curing agent in this study [34]. LAB isolates were sub-cultured every 24 h in MRS broth containing 5 µg/ml of novobiocin and examined for the presence of plasmid on day 7, 14 and 21 by random selection of at least 10% of all colonies growth on plates. The selected colonies were used for plasmid extraction by method described by Anderson and McKay [35]. The plasmid-free isolate was further used for construction of recombinant *L. casei* expressing heterologous protein.

Segregational and structural plasmid stability

Study on segregational stability of plasmid in *L. casei* isolate was done according to method described by Sorvig *et al.* [36]. The plasmid-free *L. casei* isolate derived from plasmid curing method was electrotransformed with pRCEID-LC13.9, the *E.coli*/ *L. casei* shuttle vector based on pRCEID13.9 replicon derived from *L. casei* TISTR1341 with ampicillin and erythromycin as selective markers [37]. The transformants were grown in MRS broth without erythromycin for 100 generations. An aliquot of the culture was collected every 20 generations, diluted and plated onto antibiotic-free MRS medium. Colonies were then replicated on media with and without erythromycin. The colonies on the plate were counted and calculated for the percentage of plasmid stability.

For the structural plasmid stability, the plasmids isolated from the colonies were digested with various restriction enzymes. The digestion pattern of plasmid was visualized by agarose gel electrophoresis and compared with that derived from its original plasmid.

Construction of recombinant *L. casei* RCEID08 expressing heterologous protein

Three recombinant expression plasmids each with different heterologous protein gene, i.e. GFPuv, TTFC and VP2, were used construct to three recombinant *L. casei* RCEID08 expressing GFPuv, TTFC and VP2, respectively. pLC13.9:LDH-PRO1:GFPuv, pRCEID-LC13.9-derived plasmid containing the GFPuv gene under the lactate dehydrogenase promoter of *L. casei*, was previously constructed in our laboratory [37].

To construct the recombinant plasmid expressing TTFC, the *ttfc* gene fragment was amplified from *Clostridium tetani* genomic DNA (derived from Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand) using TTFC specific primers consisted of TTFC_13.9_F with 5' *Age*I hanging (5'-ACGT*ACCGGT*AATGTCAACACC-3') and

TTFC_13.9_R with 5' *Xho*I hanging (5'-ATTA*CTCGAG*CGTCTGCAGTGT-3') primers. The *Age*I and *Xho*I-digested TTFC amplified products were replaced the GFPuv gene in the *Age*I and *Xho*I-digested pLC13.9:LDH-PRO1:GFPuv resulting in pLC13.9:LDH-PRO1:TTFC.

For construction of VP2-containing expression plasmid, IBDV RNA was extracted from the bursa of Fabricius of IBDV-infected chickens using the Trizol reagent (Invitrogen, USA) and used for amplification of *vp2* gene by RT PCR with VP2 Forward primer with 5' *Age*I hanging (5'-GTAATAC*ACCGGT*AATGACAAACCTGCAAACCC-3') and VP2 Reverse primer with 5' *Xho*I hanging (5'-TAT*CTCGAG*TCTTAGAAGCCAAATGCT CCTGCAA-3'). The *Age*I and *Xho*I-digested VP2 amplified products were replaced the GFPuv gene in the *Age*I and *Xho*I-digested pLC13.9:LDH-PRO1:GFPuv resulting in pLC13.9:LDH-PRO1:VP2.

Both *ttfc* and *vp2* gene were amplified using the following PCR conditions: 95°C for 5 min followed by 30 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 2 min.

The above three recombinant plasmids were each electrotransformed to *L. casei* RCEID08.

Electrotransformation

The competent *L. casei* RCEID08 were prepared according to Bouai protocol[38]. In brief, a single colony of *L. casei* RCEID08 from MRS plate was inoculated in 10 ml of MRS broth containing 1% glycine. After overnight incubation, the bacterial cells were collected by centrifugation at 4,500 x *g* for 5 min, transferred to 100 ml of the MRS broth containing 1% glycine and incubated until the OD600 reached 0.6. The bacterial cells were harvested by centrifugation at 4,500 x *g* for 10 min and washed three times in cold sterilized deionized water. In the final step, the washed bacterial cell pellet was resuspended in 1 ml of ice-cold 30% PEG8000 (polyethylene glycol). The competent cells can be used immediately or stored at -80°C until use.

The recombinant plasmids were individually transferred to the competent cells using electrotransformation method [39]. The voltage of 2,500 V and resistance of 400 Ω was applied by using Gene PulserMXCell™(Bio-Rad, USA). After electrotransformation, the transformants were selected on MRS agar containing 2.5 µg/ml of erythromycin. The GFPuv-containing transformant was verified for the presence of GFPuv gene by PCR as previous described [37]. For TTFC- and VP2-contraning transformants, they were verified for the presence of the corresponding gene by PCR with the conditions described above.

Western blot analysis and immunodetection of heterologous protein expression

Recombinant LAB isolate was cultured overnight in MRS broth containing 2.5 µg/ml of erythromycin. The bacterial cells were harvested and resuspended in sonication buffer composed of 50 mM NaH_2PO_4, 300 mM NaCl, 1 mg/ml lysozyme (Amresco, USA), 1X proteinase inhibitor cocktail (Amresco, USA) and 10 mM dithiothreitol (Amresco, USA). Intracellular proteins were released by sonication on ice (10×30 s at full power, with a 30 s intermission between burst) using a Soniprep 150 (MSE, UK) with a fine probe. Twenty microlitters of protein samples were loaded to 12% of SDS-PAGE and run at 100 V for 2 h. The proteins were transferred from gel to PVDF membrane (Bio-Rad, USA) by using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA) with the voltage operated at 25 V for 30 min. For immunodetection, the blotted PVDF membrane was washed twice with Tris-buffered saline (TBS) buffer and then blocked with 3 % (w/v) of bovine serum albumin (BSA) in TBS buffer for 1 h. After blocking step, the membrane was washed twice with TBS containing 0.05% of Tween 20 and 0.2% of Triton X-100 (TBS Tween/Triton buffer). After washing, the membrane was incubated with primary antibody for 1 h and then washed twice with TBS Tween/Triton buffer. The primary antibodies in this study consist of the Living Color® GFP monoclonal antibody (Clontech, USA) for GFP detection, the *C. tetani* Tetanus Toxin antibody (Thermo Scientific, USA) for TTFC detection and the Infectious Bursal Disease Virus monoclonal antibody (USBiological, USA) for VP2 detection. After washing, the membrane was incubated with HRP-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology, USA) for 1 h and then washed twice with TBS Tween/Triton buffer. In the final step, the membrane was added with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) and exposed to CL-XPosure Film (Thermo Scientific, USA). The film was incubated in developing solution to generate the specific protein band and finally fixed in fixing solution.

RESULTS

Determination for *in vitro* probiotic properties of the LAB isolates

Sixty LAB isolates were isolated from healthy chicken intestine and used for determination of *in vitro* probiotic properties which included tests for hydrophobicity, acid and bile salt tolerance, and antimicrobial activity. Six isolates showed comparable performance for these *in vitro* properties with those of two standard probiotic bacteria e.g. *L. casei* TISTR 1341 [40] and *L. casei* ATCC393 [41]. Table 1 shows the results of hydrophobicity, acid and bile salt tolerance test of the six isolates. For hydrophobicity test, CH1J has the strongest hydrophobicity (99.46%) while the remaining five isolates show comparable or higher hydrophobicity (50.43% - 88.88%) than *L. casei* ATCC 393 (50.62%). For acid tolerance, all six isolates tested remained survival of more than 50% for an incubation period of 3 hours at pH 2.5. In contrast to bile salt tolerance, the results varied markedly among six isolates. CH4CB6 and CH8JS1A showed slightly growth inhibition in MRS broth containing 0.3% bile salt, while the remaining isolates showed moderated to highly susceptible to bile salts. For antimicrobial activity, none of the isolates showed antimicrobial activity

Table 1. *In vitro* colonizing properties of 6 LAB isolates. The hydrophobicity, gastric juice and bile salt tolerance results are presented as the percentage values. The *L. casei* TISTR1341 and *L. casei* ATCC 393 were used as the standard probiotic strains in this study

Strains/Isolates	Hydrophobicity (%)	Gastric Juice Tolerance (%)	Bile Salt Tolerance (%)
*L. casei*TISTR1341	68.88	62.80	14.29
L. casei ATCC 393	50.62	42.88	33.33
CH1J	99.46	87.22	15.00
CH1CB1	88.87	91.18	6.11
CH4CB6	52.58	70.89	99.25
CH6CS1A	76.15	59.66	13.23
CH7CS1	50.43	83.23	30.57
CH8JS1A	58.88	52.18	91.43

Table 2. Antibiotic susceptibility profiles of the six LAB isolates. The antibiotics in this study consisted of erythromycin (Ery), ciprofloxacin (Cip), nalidixic acid (Nal), penicillin (Pen), tetracycline (Tet), tylosin (Tyl) and vancomycin (Van)

Isolates	Antibiotics (µg/ml)						
	Ery	Cip	Nal	Pen	Tet	Tyl	Van
CH1J	S (< 0.25)	R (16)	R (>128)	R (>128)	R (>128)	R (>128)	R (>128)
CH1CB1	R (>128)	R (16)	R (>128)	S (4)	R (32)	R (>128)	R (>128)
CH4CB6	R (>128)	R (64)	R (>128)	R (>128)	R (>128)	R (>128)	R (>128)
CH6CS1A	S (< 0.25)	R (16)	R (>128)	S (2)	R (64)	S (< 0.25)	R (>128)
CH7CS1	R (>128)	R (16)	R (>128)	R (>128)	R (>128))	R (>128)	R (>128)
CH8JS1A	R (>128)	R (32)	R (>128)	S (<0.25)	R (16)	R (>128)	R (>128)

Interpretation of susceptibility to antibiotics (µg/ml):- Erythromycin (Ery): sensitive ≤ 0.5, resist ≥8; Ciprofloxacin (Cip): sensitive ≤ 1, resist ≥ 4; Nalidixic acid (Nal): sensitive ≤ 8, resist ≤ 32; Penicillin (Pen): sensitive ≤ 8, resist ≥16; Tetracycline (Tet): sensitive ≤ 4, resist ≥ 16; Tylosin (Tyl): sensitive ≤ 0.5, resist ≥ 8; Vancomycin (Van): sensitive ≤ 4, resist ≥ 32.

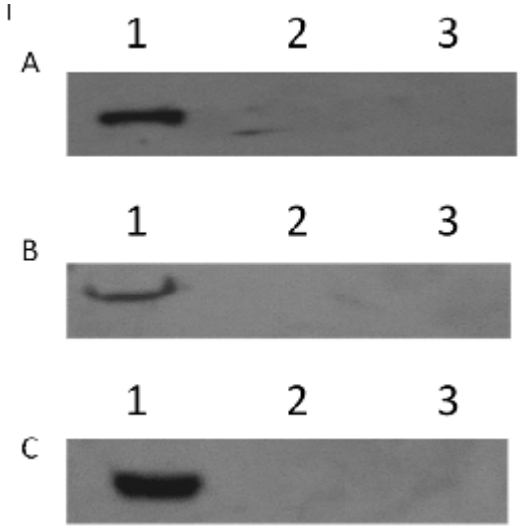

Fig. 1. Western blot analysis of the protein expression in *L. casei* RCEID08 consisting of GFP (A), TTFC (B) and VP2 (C). Lanes 1A, 1B and 1C= recombinant *L. casei* RCEID08 containing pLC13.9:LDH-PRO1:GFPuv, pLC13.9:LDH-PRO1:TTFC and pLC13.9:LDH-PRO1:VP2. Lanes 2A, 2B and 2C = recombinant *L. casei* RCEID08 containing pRCEID-LC13.9. Lanes 3A, 3B and 3C = plasmid-free *L. casei* RCEID08

against all indicator bacteria, i.e., *S. typhimurium* ATCC 13311, *P. multocida* and *E. coli* O157:H7.

Selection of the suitable isolate for heterologous protein expression.

As our expression vectors have erythromycin as selective marker, further selection for the suitable host for heterologous protein expression was done by using the antibiotic susceptibility test to select for erythromycin sensitive isolate. As shown in Table 2, CH1J and CH6CS1A are two isolates with erythromycin sensitive character. These two strains were selected and determined for the presence of cryptic plasmid. It was found that CH1J and CH6CS1A contained 2 and 3 plasmids, respectively (data not shown). To eliminate the possible incompatibility with our introduced expression vector, both isolates were subjected to plasmid curing using novobiocin as curing agent in order to eliminate their cryptic plasmids. It was found that only CH6CS1A could completely eliminate its cryptic plasmids. This plasmid-free CH6CS1A showed the same probiotic properties and antibiotic susceptibility profile as its parent strain was selected as host for heterologous protein expression. This LAB isolate was identified by 16S rRNA as *Lactobacillus casei* and designated as *L. casei* RCEID08.

Segregational and structural stability of pRCEID-LC13.9 in *L. casei* RCEID08

To determine the segregational and structural stability of the exogenous plasmid in *L. casei* RCEID08, the *E.coli-L.casei* shuttle vector, pRCEID-LC13.9, was electrotransformed to *L. casei* RCEID08. The vector was maintained at 64% after 100 generations in the absence of selective pressure (data not shown). The structural stability of the vector was determined by isolation of the vector from the transformants every 20 generations under non-selective conditions and digested with *Pst*I and *Sal*I. It was found that the plasmid had the same molecular size compared with that obtained from its original vector up to 80 generations (data not shown).

Expression of heterologous proteins in *L. casei* RCEID08

The above segregational and structural stability of pRCEID-LC13.9 in *L. casei* RCEID08 reflect the ability to use the pRCEID-LC13.9-based expression vector for heterologous protein expression. Three heterologous proteins consist of GFP, TTFC, and VP2 of IBVD virus were selected for expression in *L. casei* RCEID08.

For GFP expression, pLC13.9:LDH-PRO1:GFPuv was electrotransformed to *L. casei* RCEID08, the transformants containing gfp_{UV} gene were validated by PCR amplification of GFPuv gene and Western blot analysis. Figure 1A showed the Western blot analysis for GFPuv protein using specific antibody against GFPuv protein. In addition, the expression of GFPuv could be visualized under fluorescent microscope (data not shown).

For TTFC and VP2 expression, pLC13.9:LDH-PRO1:TTFC and pLC13.9:LDH-PRO1:VP2, respectively, were each introduced to *L. casei* RCEID08. Both TTFC –containing and VP2-containing transformants were validated using PCR and Western blot analysis. Figure 1B and 1C showed the Western blot analysis for TTFC and VP2, respectively, using specific antibodies against the corresponding proteins.

DISCUSSION

From this study, the LAB were isolated from healthy chicken intestine and determined for the *in vitro* probiotic properties, which consisted of tests for the resistance to gastric juice and bile salt, hydrophobicity assay and detection of antimicrobial activity. There are several *in vitro* tests used for evaluation of probiotic potential and diverse criteria for determination of an appropriate LAB isolate for probiotic usage [9]. Among numerous existing tests, the basis methods for evaluation are those that determined for certain functional characteristics involving tolerance to acid and bile salt condition, and adherence to the epithelial cells. Tolerance to acid pH and bile salt is the important factor in determination of survival and growth of bacteria in the intestinal tract and hence, is a critical requirement for probiotics [42]. In this study, hydrophobicity test had been used to reflect the adhesion properties as a correlation of the hydrophobic nature of the outermost surface of bacteria has been implicated in the attachment of bacteria to epithelial and intestinal mucosal cells [9, 43, 44, 45, 46]. In comparison with the standard probiotic strains, *L. casei* TISTR1341 [40] and *L. casei* ATCC 393 [41], our six isolates showed comparable *in vitro* probiotic properties for acid and bile salt tolerance and hydrophobicity with those of the standard probiotic strains and were sufficient to select for further study, although none of the six isolates had antimicrobial activity against three indicator bacteria, i.e. *S. typhymurium* ATCC 13311, *P. multocida* and *E. coli* O157:H7.

From antibiotic susceptibility result, the six isolates showed resistance to several antibiotics and were similar to previous studies [47, 48]. Two of six selected isolates, CH1J and CH6CS1A, were further selected based on the susceptible to erythromycin as these isolates will be engineered based on the expression vector used erythromycin as selective maker. Only isolate CH6CS1A could eliminate all of its plasmids and was used as host to express heterologous proteins. From 16S rRNA sequencing result, this selected isolate was identified as *Lactobacillus casei* and designated as *L. casei* RCEID08. The *Lactobacillus* sp. can normally be found in milk product, environments and avian intestinal tract [49]. From previous studies, the predominant species of lactobacilli commonly found in the chicken gastrointestinal tract consist of *L. johnsonii*, *L. agilis*, *L. crispatus*, *L. vaginalis*, *L. reuteri*, *L. salivarius* and *L. aviarius*[50, 51, 52, 53] and *L. casei*[54]. Lactobacilli have several advantages as mucosal delivery system [19], including GRAS (Gererally Recognize as Safe) status, survival within the gut, stimulate mucosal immune response and potential to be engineered to express therapeutic proteins. For *L. casei*, they have been used in chicken as probiotics [55] for several purposes, e.g. to improve feed conversion [56, 57, 58, 59], increase egg production [6] and also considered as immunoadjuvant[60].

With the adequate segregational (64% maintenance after 100 generation) and structural stability (intact vector up to 80 generation) of pRCEID-LC13.9 in *L. casei* RCEID08, it indicated that this isolate can be used as host for expression of heterologous protein with the pRCEID-LC13.9-based expression vector. Moreover, the resulting transformant and its parent strain were shown to be similar in probiotic properties (data not shown). For GFP expression using pLC13.9:LDH-PRO1:GFPuv, it was found that *L. casei* RCEID08 can express the GFP as determined by both visualization under the UV microscope and Western blot analysis. Due to its ease to detect and quantitate, the GFP gene has been used to monitor heterologous protein expression in many lactobacillus species, such as *L. casei*[10], *L. sakei*[13], *L. delbrueckii* and *L. fructosus*[15] and recently *L. agilis* and *L. vaginalis*[61]. For TTFC, this non-toxic tetanus toxin fragment has been widely used as a model antigen to express in some genera of LAB. For example, mucosal delivery of TTFC-expressing *L. plantarum* and *L. lactis* promoted both T helper 1 and 2 responses and conferred protective immunity in mice [62, 16, 63]. In this study, the recombinant *L. casei* RCEID08 containing pLC13.9:LDH-PRO1:TTFC were able to express the TTFC as determined by Western blot assay. Another protein that we selected for expression in our isolate is the VP2 of IBDV. IBDV is the causative agent of a highly contagious disease in chicken known as Gumboro[64] and is a major concern in the poultry industry. VP2 is the viral capsid which elicit neutralizing antibodies [65]. Current IBDV vaccine used in poultry industry is inactivated IBDV vaccines given through subcutaneous or intramuscular injection. Several alternative IBDV

vaccines are under investigation, such as recombinant and DNA vaccines [66, 67]. With respect to lactobacillus, Dieye *et al.*, [68] had developed *L. lactis* expressing VP2 and VP3, it was found that VP2 could express in cytoplasm and cell wall-anchorage but not extracellular compartment whereas VP3 could express in all compartments. However, none of these recombinant *L. lactis* could elicit antibody against both VP2 and VP3. To our knowledge, VP2-expressing lactobacillus inducing protective immunity against IBDV has not been reported. Thus, in this study, we had constructed recombinant *L. casei* RCEID008 expressing VP2 by using pLC13.9:LDH-PRO1:VP2. This construct might be useful as the alternative VP2 delivery system to further study and/or improvement the immune response against IBDV in chicken.

In summary, we had screened and isolated LAB derived from chicken intestine with *in vitro* probiotic properties. One isolate identified and designated as *L. casei* RCEID08, was selected based on erythromycin sensitivity for expressing heterologous proteins. The recombinant *L. casei* RCEID08 was successfully expressed GFP, TTFC or VP2 of IBDV by using expression vector with *ldhL* promoter. Thus, this *L. casei* isolate could be further used as protein delivery vehicle for a variety of applications.

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