# Functional properties of *Enterococcus faecalis* isolated from colostrum drawn from Thai mothers

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## Abstract

Colostrum from Thai mothers who went through vaginal delivery is an abundant source of antibacterial lactic acid bacteria (LAB). Twenty-two strains were isolated and selected according to the antibacterial activity against *Staphylococcus aureus* TISTR 1466, *Escherichia coli* TISTR 780, *Escherichia coli* O157:H7 DMST 12743, *Shigella sonnei*, *Shigella flexneri* and *Salmonella* Typhimurium SA2093. Among the isolates, *Enterococcus faecalis* was a dominant species. It also exhibited essential probiotic characteristics such as excellent adhesion to mucin and high survival under gut stresses. In addition, the functional characteristic in terms of protein digestibility of colostrum-isolated *E. faecalis* M125 was observed through the hydrolysis of the major allergenic bovine milk proteins. This strain was susceptible to all tested antibiotics, and had no enterococcal virulence factor encoding genes. These results supported that colostrum is a crucial source of beneficial bacteria for infant. *E. faecalis* M125 poses to be useful as a potential probiotic candidate for the development of functional infant formula.

## Introduction

Colostrum (breast milk) is universally known to be the best food for all newborns. Apart from its perfect nutritional value, several bioactive components were reported to protect newborns from many infectious diseases. These functional molecules include immunoglobulins, cytokines, chemokines, growth factors, hormones, oligosaccharides and glycans, antimicrobials (e.g., lysozyme, lactoferrin) and bioactive peptides (Ballard and Morrow, 2013). Moreover, it has recently been shown to contain a variety of commensal probiotic bacteria which might involve in the development of infant gut microbiota (Martín *et al*., 2003; Solís *et al*., 2010). *Staphylococcus*, *Streptococcus*, *Bifidobacterium* and *Lactobacillus* were detected as the dominant groups in all colostrum samples (Collado *et al*., 2009). However, high variation between individuals has been reported at species level (Martín *et al*., 2012). The specific shifts in the composition of colostrum microbiota were related to several factors such as lactation stage, mode of delivery, gestational age and antibiotherapy (Khodayar-Pardo *et al*., 2014; Soto *et al*., 2014). The beneficial traits and origin of colostrum bacteria were extensively reviewed by Fernández *et al*. (2013).

Probiotics are live microorganisms conveying various health benefits to the hosts when administered at an appropriate level (Holzapfel and Schillinger, 2002). Therefore, they must be able to survive gut stresses caused by gastric and bile acids and thence successfully colonise the large intestine in which their beneficial effects (e.g., prevention and/or treatment of a variety of infections) can be exerted (Olivares *et al*., 2006). To ensure the probiotic colonisation, the adhesion to mucus layer is an essential characteristic to avoid the removal by colon peristalsis (Nueno-Palop and Narbad, 2011). The antagonistic activity against intestinal pathogens is considered as one of the beneficial and functional characteristics of probiotic bacteria. Such activity can be achieved through many different mechanisms, including antimicrobial secretion, competitions for nutrients and colonising sites as well as host immune modulation and stimulation (Makras *et al*., 2006). Another probiotic function is the ability to hydrolyse bovine milk proteins and consequently improve digestibility, release bioactive peptides and reduce antigenicity of milk proteins (Muraro *et al*., 2012).

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Apart from *Lactobacillus*, *Enterococcus*, a large genus of LAB, is also frequently detected in colostrum. Many different strains of *Enterococcus faecium* and *Enterococcus faecalis* isolated from colostrum have shown great potential for probiotic characteristics (Heikkilä and Saris, 2003; Jiménez *et al*., 2008; ÖZMEN Toğay *et al*., 2014). Nevertheless, enterococcal strains are known to be opportunistic pathogens and a major cause of clinical infections (Franz *et al*., 2011). It is therefore essential to perform safety assessments on antibiotic resistance profile, virulence traits and haemolytic activity in the probiotic selection. The present work thus aimed to evaluate the impacts of lactation stages and modes of delivery on the composition of antibacterial LAB isolated from colostrum of Thai mothers. The essential probiotic mechanisms, functional characteristics in terms of proteolytic cleavage of bovine milk proteins and safety of colostrum-isolated *E. faecalis* were evaluated.

## Materials and methods

### Microorganisms and cultivation conditions

LAB strains were isolated from colostrum of healthy Thai mothers. *Lactobacillus plantarum* 299V was kindly provided by Assoc. Prof. Dr. Sunee Nitisinprasert from Kasetsart University (Bangkok, Thailand). All LAB were cultivated in de Man Rogosa Sharpe agar (MRS, Lab M, Lancashire, United Kingdom) at 37°C for 24 h. *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were obtained from the Microbiological Resources Centre (MIRCEN, Bangkok, Thailand). *Escherichia coli* O157:H7 DMST 12743 was obtained from the Department of Medical Science, Ministry of Public Health (Bangkok, Thailand). All clinical isolates of *Shigella sonnei*, *Shigella flexneri* and *Salmonella* Typhimurium SA2093 were obtained from the Microbiological Laboratory of Songklanakarind Hospital, Prince of Songkla University, Hat Yai (Songkhla, Thailand). All pathogenic strains were cultivated in Muller Hinton broth (MHB, Himedia, Mumbai, India) at 37°C for 24 h.

### Subjects and breast milk sample collection

This research protocol was reviewed and approved by the Ethics Committee, Faculty of Medicine, Prince of Songkla University (EC Number: 55-243-19-2-3). A total of 20 healthy and full term (37-40 weeks) pregnancy Thai mothers who had no history of antibiotic treatment were subjected to this study. They voluntarily provided 6, 5 and 20 samples of colostrum (1st to 3rd day), transition milk (7th to 14th day) and mature milk (16th day onwards), respectively. Colostrum samples aseptically taken according to Solís *et al*. (2010) were maintained at 4°C during transportation (less than 20 min) and immediately processed upon arrival.

### Enumeration and isolation of LAB present in breast milk drawn from Thai mothers

One millilitre of each colostrum sample was diluted in a 10-fold dilution with 0.1 M phosphate buffer saline (PBS, pH 7.0) containing 0.05% (w/v) L-cysteine (Sigma, Steinheim, Germany). The appropriate dilutions were plated on MRS agar mixed with 0.05% (w/v) L-cysteine and 0.004% (w/v) bromocresol purple (Ajex Finechem, Auckland, New Zealand). The plates were incubated at 37°C for 48 h under anaerobic conditions using an anaerobic jar gassing system (Don Whitley Scientific Limited, West Yorkshire, United Kingdom). The colonies surrounded with yellow zone were enumerated and expressed as log CFU/mL of viable LAB count. They were also isolated and then selected as presumptive LAB based on their Gram-positive and catalase negative characteristics.

### Determination of antibacterial activity against foodborne pathogens

The antibacterial activity of all LAB isolates was determined by agar diffusion assay (Papamanoli *et al*., 2003). The overnight culture of LAB was centrifuged to remove the cell pellet. The obtained supernatant was then divided into two portions. One was neutralised to pH 6.5–7.0 with 1 M NaOH and the other used as the control was left unadjusted. Each aliquot (50 µL) was placed in a 5-mm-diameter agar well punched into soft MHA (1.0% agar) seeded with $10^6$ CFU/mL of the pathogenic bacteria. Following 24 h incubation, the inhibition zone around each well was measured using a Vernier calliper.

### Identification of the selected antibacterial LAB isolates

Genomic DNA was extracted from the overnight culture of each LAB isolate using a commercial kit for bacterial DNA (Omega Bio-Tek, GA, USA). The 16S rDNA was amplified through PCR reaction using primers of 27-f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-r (5'-GGTTACCTTGTTACGACTT-3'). The nucleotide sequence of the PCR product was analysed by Ward Medic Ltd. (Bangkok, Thailand; 1st Base Distributor). The 16S rDNA sequence was then compared to the GenBank nr/nt database using BLAST search program available at http://www.ncbi.nlm.nih.gov/.

*In vitro adhesion ability to mucin*

The adhesion ability to mucin of the antibacterial LAB isolates was determined using a sterilised polystyrene 96-well microtiter plate (Maxisorp Nunc, Denmark) coated with partially purified porcine gastric mucin type III (Sigma, USA) according to Uraipan *et al.* (2014). *L. plantarum* 299V, a well-known adhesive strain, was used as a positive control.

*Tolerance to upper gastrointestinal stresses*

The survival of the selected isolates under various stress conditions of the upper part of gastrointestinal tract (GIT) was tested according to Vizoso Pinto *et al.* (2006) with some modification. Overnight culture was washed twice with 0.1 M PBS and then resuspended in sterile electrolyte solution (SES; 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$), pH 6.9 in the presence of 100 mg/L lysozyme (Sigma, Steinheim, Germany). The cell suspension was incubated at 37°C for 5 min with gentle agitation and then centrifuged to discard the solution. The cell pellet was subsequently exposed to artificial gastric fluid containing 0.3% pepsin (Sigma, Steinheim, Germany) in SES (pH 2.0) at 37°C for 3 h with gentle agitation. The gastric fluid was then removed before exposing to the artificial duodenal solution (pH 7.2) consisted of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salt (Merck, Darmstadt, Germany) and 0.1% pancreatin (Sigma, St. Louis, MO, USA) for 6 h at 37°C with gentle agitation. The viable cells were counted before ($N_0$) and after each stress exposure ($N_1$) using drop plate technique according to Michida *et al.* (2006). The percentage of bacterial survival was calculated according to the equation of % survival = 100 (log $N_1$ / log $N_0$).

*Detection of enterocin structural genes present in E. faecalis*

PCR amplification of well-known structural genes of enterocin A, enterocin B, enterocin *P* and enterocin L50B was performed as Strompfová *et al.* (2008) using the primer sets specific to each type of enterocin. The genomic DNA was extracted from the overnight grown *E. faecalis* using a commercial kit for bacterial DNA (Omega Bio-Tek, GA, USA). After PCR amplification, the PCR product was detected by agarose gel electrophoresis operating for 30 min at 100 V in 2% (w/v) agarose, which was stained with SYBR® Gold (Invitrogen, USA) and then viewed under UV transillumination (Alpha Innotech Corporation, USA).

*Proteolytic activity of E. faecalis using UHT skim milk as substrate*

The cell pellet from an overnight culture of *E. faecalis* was washed twice with 0.1 M sodium phosphate buffer pH 7.2 (SPB), resuspended in UHT skim milk (Agrilait, Rennes, France) and incubated at 37°C for 24 h. The equivalent UHT skim milk without adding the cells was used as control. At the end of incubation period, protein hydrolysis was determined in the supernatant by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide gels) (Bio-Rad, Hercules, CA, USA) as described by Hadji-Sfaxi *et al.* (2012). After electrophoresis, the slab gel was stained according to Ahmadova *et al.* (2011). The mixture of seven protein markers ranging from 14.4 kDa to 116 kDa was obtained from Euromedex (Souffelweyersheim, France). The stained gel was visualised by Image Scanner III (GE Healthcare, Piscataway, NJ, USA).

*Proteolytic activity of E. faecalis M125 using milk casein and whey protein as substrates*

Whey protein concentrate (WPC) and sodium caseinate (Na-caseinate) were prepared from fresh bovine milk according to El-Ghaish *et al.* (2011). The suspended WPC (5 mg/mL, heated at 85°C for 30 min) and Na-caseinate (12 mg/mL) in SPB were used as substrates. An aliquot culture of *E. faecalis* M125 was transferred onto the surface of milk citrate agar incubated at 37°C for 48 h (El-Ghaish *et al.*, 2011) to induce the expression of proteolytic enzymes. The fresh cell was collected and washed twice with SPB before the pellet was further introduced into each substrate solution to achieve final concentration of $10^{10}$ CFU/mL. The control treatment was performed along without the presence of the bacterial cells. The mixtures were incubated at 37°C and the samples were taken at 0, 9, 24 and 48 h for the determination of protein hydrolysis using SDS-PAGE (12% polyacrylamide gel). The peptide formation was determined using reversed phase-high performance liquid chromatography (RP-HPLC) performed on a Waters 2695 separation module (Waters, Milford, MA, USA). The system was equipped with a Nucleosil C18 column (5 μm, 4 mm x 100 mm; Macherey-Nagel, Hoerdt, France) and a Waters 996 photodiode array detector set and operated at 220 nm under the condition described by Hadji-Sfaxi *et al.* (2012) with some modification. Two solvents were used as mobile phases. Solvent A was 0.05% (v/v) trifluoroacetic acid (TFA) in Milli-Q water, while solvent B consisted of TFA/HPLC-grade acetonitrile/ Milli-Q water in the ratio of 0.03/90/10 (v/v/v). The gradient was generated by increasing the proportion

of solvent B from 0 to 100% within 18 min. The temperature of the column and the solvent mixture was maintained at 30°C. The flow rate was set at 1 mL/min. Data was analysed and processed by Water Millennium 32 Software.

*In vitro safety evaluation of E. faecalis M125*

*Assessment for antibiotic susceptibility*

The antibiotic susceptibility of *E. faecalis* M125 was evaluated using ampicillin, penicillin G, tetracycline (Euromedex, France), kanamycin, gentamicin (Sigma, Germany), streptomycin and vancomycin (Sigma, USA) by broth microdilution assay as described in CLSI documents M07-A8 (CLSI, 2009). The concentration ranges of antibiotics applied were 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1,024 µg/mL. The antibiotic susceptibility was expressed as minimum inhibitory concentration (MIC) value. The breakpoint values were referred to European Food Safety Authority (EFSA, 2008) to indicate susceptibility/resistance of *E. faecalis* M125 toward each antibiotic.

*Detection of virulence genes*

The presence of enterococcal virulence genes, such as *ace* (adhesin of collagen protein), *asa1* (aggregation substance), *cylA/B* (activation and expression of cytolysin), *efaAfs* (cell wall adhesin) and *gelE* (gelatinase) were determined by PCR technique according to Ahmadova *et al*. (2011). The oligonucleotide primers were purchased from Eurogentec (Belgium). Genomic DNA was extracted from overnight culture of *E. faecalis* using a commercial kit for bacterial DNA (Omega Bio-Tek, GA, USA). The amplified fragments were detected through agarose gel (1.5% w/v) electrophoresis operated at 100 V for 30 min. The gel was thereafter stained with SYBR® Gold (Invitrogen, USA) and viewed under UV transillumination (Alpha Innotech Corporation, USA). The DNA of *E. faecalis* VanB kindly provided by Assoc. Prof. Dr. Suppasil Maneerat, Prince of Songkla University was used as a positive control for the presence of *ace*, *asa1*, *efaAfs* and *gelE*. The VC 1 kb (500 – 10,000 bp) and VC 100 bp plus (100 – 3,000 bp). DNA ladder from Vivantis (Malaysia) was used as DNA marker.

*Determination for haemolytic activity*

*E. faecalis* M125 was tested for haemolytic activity by streaking on blood agar (7% v/v human blood) according to Foulquié Moreno *et al*. (2003). Haemolytic reaction was detected by the appearance of a clear zone (β-haemolysis; complete lysis of red blood cells), greenish zone (α-haemolysis; conversion of red blood cell haemoglobin to methaemoglobin) or absence of any zone (γ-haemolysis; no haemolytic activity) around the colony developing on the blood agar.

*Statistical analysis*

The data of each experiment was obtained in triplicate, and analysis of variance and Duncan's multiple range tests were performed using SPSS 16 software. Statistical significance was accepted at $p < 0.05$.

**Results**

*Abundance, isolation and identification of antibacterial LAB present in colostrum drawn from Thai mothers*

LAB were detected and enumerated in all colostrum samples from Thai mothers with the mean value of $3.58 \pm 0.59$ log CFU/mL (Table 1). The highest and lowest numbers of total LAB were counted from the mature milk from mothers who went through caesarean section (log 2.30-4.87 CFU/mL) (Table 1). A total of 189 LAB strains were isolated and selected according to antibacterial activity against many different foodborne pathogens. The highest percentage of antibacterial LAB isolates was obtained from colostrum (15.6%) whereas the lowest prevalence was observed in the transition milk (9.7%) as shown in Table 1. Among these, 22 isolates accounting for 11.6% (Table 1) exhibited the inhibitory activity against all pathogens with the clear zone diameter greater than 10 mm. The complete loss of the inhibition was observed in the neutralised supernatant of all selected antibacterial isolates suggesting that acids played a major role in the inhibition. However, the enterococcal enterocin had no influence on the antibacterial activity of these isolates as the genes encoding enterocin A, B, P and L50B were absent (Table 2).

According to 16S rDNA nucleotide sequence analysis, all 22 isolates were identified as *E. faecalis* (10 isolates), *E. faecium* (6 isolates), *Lactobacillus plantarum* (4 isolates) and *L. rhamnosus* (2 isolates) as shown in Table 2. Interestingly, *E. faecalis* and *L. plantarum* were isolated from mothers who went through vaginal delivery. Only antibacterial-producing *E. faecalis* was selected from colostrum obtained from such mothers. In the meantime, *E. faecium*, *E. faecalis* and *L. rhamnosus* were obtained from mothers who went through caesarean procedure. *E. faecium* was the only species obtained from colostrum of such group of mothers.

Table 1 Total LAB counts, isolate numbers and prevalence of antibacterial LAB present in breast milk drawn at various lactation stages from Thai mothers who went through different modes of delivery.

| Lactation stages | Cesarean section | | | Vaginal delivery | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | Average LAB counts (Min-Max) | Isolate Numbers | Antibacterial Isolates (%) | Average LAB counts (Min-Max) | Isolate Numbers | Antibacterial Isolates (%) | Average LAB counts (Min-Max) | Isolate Numbers | Antibacterial Isolates (%) |
| Colostrum | 3.26 ± 0.12 (3.13-3.38) | 15 | 2 (13.3%) | 3.70 ± 0.58 (3.29-4.36) | 17 | 3 (17.6%) | 3.48 ± 0.45 (3.13-4.36) | 32 | 5 (15.6%) |
| Transition | 3.66 ± 0.28 (3.41-3.96) | 19 | 1 (5.3%) | 3.69 ± 0.21 (3.54-3.84) | 12 | 2 (16.7%) | 3.67 ± 0.22 (3.41-3.96) | 31 | 3 (9.7%) |
| Mature | 3.59 ± 0.78 (2.30-4.87) | 79 | 7 (8.9%) | 3.57 ± 0.57 (2.75-4.25) | 47 | 7 (14.9%) | 3.58 ± 0.70 (2.30-4.87) | 126 | 14 (11.1%) |
| All stages | 3.55 ± 0.66 (2.30-4.87) | 113 | 10 (8.8%) | 3.62 ± 0.50 (2.75-4.36) | 76 | 12 (15.8%) | 3.58 ± 0.59 (2.30-4.87) | 189 | 22 (11.6%) |

Total LAB counts performed in MRS agar cultivated anaerobically at 37°C for 48 h are mean values of three replicates ± standard deviation (log CFU/mL).

Table 2 DNA homology of 16S rDNA, detection of enterocin A, B, P and L50B encoding genes and % survival after sequential exposure to gut stresses in mouth (0.01% lysozyme for 5 min), stomach (pH 2 with 0.3% pepsin for 3 min), stomach (pH 2 with 0.3% pepsin for 3 h) and duodenum (0.5% bile salts and 0.1% pancreatin for 6 h) among the selected antibacterial LAB isolated from breast milk drawn at different stages of lactation from Thai mothers who went through different modes of delivery.

| Mode of delivery | Lactation stage | Strains | Closest species in NCBI | Similarity (%) | Sequence accession number | Enterocin structural genes | Survival rate (percentage) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Mouth | Stomach | Duodenum |
| Cesarean section | Colostrum | MC316 | Enterococcus faecium T110 | 98 | CP006030.1 | None | 98.00 ± 0.01 A/bcd | 71.99 ± 1.15 B/bcdef | 63.39 ± 6.78 B/defg |
| | Colostrum | MC318 | Enterococcus faecium T110 | 99 | CP006030.1 | None | 99.26 ± 0.57 A/ab | 66.56 ± 2.84 B/g | 62.14 ± 4.04 B/defg |
| | Transition | MC321 | Enterococcus faecium T110 | 99 | CP006030.1 | None | 98.57 ± 0.74 A/abc | 75.38 ± 2.24 B/abcd | 62.09 ± 0.32 C/defg |
| | | MC192 | Enterococcus faecium T110 | 99 | CP006030.1 | None | 99.10 ± 0.73 A/abc | 68.28 ± 0.18 B/fg | 61.80 ± 2.14 C/efg |
| | | MC195 | Enterococcus faecium T110 | 99 | CP006030.1 | None | 98.71 ± 1.58 A/abc | 71.72 ± 1.49 B/bcdef | 65.48 ± 0.63 C/cdef |
| | | MC13104 | Enterococcus faecium T110 | 99 | CP006030.1 | None | 99.03 ± 0.03 A/abc | 72.54 ± 0.68 B/bcdef | 65.74 ± 1.36 C/cdef |
| | Mature | MC1273 | Enterococcus faecalis ATCC 29212 | 99 | CP008816.1 | None | 99.25 ± 0.62 A/ab | 72.49 ± 2.62 B/bcdef | 59.64 ± 2.32 C/fg |
| | | MC237 | Enterococcus faecalis ATCC 29212 | 98 | CP008816.1 | None | 99.09 ± 0.66 A/abc | 62.05 ± 0.14 B/h | 58.22 ± 0.47 C/g |
| | | MC5123 | Lactobacillus rhamnosus LOCK908 | 99 | CP005485.1 | None | 99.80 ± 0.17 A/a | 58.73 ± 0.75 B/h | 46.59 ± 1.10 C/h |
| | | MC5124 | Lactobacillus rhamnosus LOCK908 | 99 | CP005485.1 | None | 99.68 ± 0.08 A/ab | 49.82 ± 3.13 B/i | 40.82 ± 1.54 C/h |
| Vaginal delivery | Colostrum | M114 | Enterococcus faecalis ATCC 29212 | 100 | CP008816.1 | None | 98.66 ± 0.77 A/abc | 75.32 ± 2.02 B/abcd | 68.87 ± 0.73 C/abcd |
| | Colostrum | M317 | Enterococcus faecalis ATCC 29212 | 99 | CP008816.1 | None | 99.56 ± 0.02 A/ab | 75.03 ± 1.63 B/abcde | 63.36 ± 1.91 C/defg |
| | | M318 | Enterococcus faecalis ATCC 29212 | 100 | CP008816.1 | None | 98.02 ± 0.59 A/bcd | 70.89 ± 2.15 B/defg | 60.03 ± 2.42 C/fg |
| | Transition | M125 | Enterococcus faecalis ATCC 29212 | 99 | CP008816.1 | None | 99.87 ± 0.07 A/a | 79.53 ± 0.07 B/a | 75.40 ± 0.22 C/a |
| | | M126 | Enterococcus faecalis ATCC 29212 | 100 | CP008816.1 | None | 99.51 ± 0.08 A/ab | 77.86 ± 3.59 B/a | 70.38 ± 0.40 C/abc |
| | Mature | M145 | Enterococcus faecalis ATCC 29212 | 100 | CP008816.1 | None | 99.75 ± 0.03 A/a | 76.92 ± 2.34 B/ab | 65.67 ± 0.93 C/cdef |
| | | M534 | Enterococcus faecalis ATCC 29212 | 99 | CP008816.1 | None | 98.54 ± 0.03 A/abc | 70.08 ± 2.48 B/efg | 67.60 ± 4.92 B/bcde |
| | | M535 | Enterococcus faecalis ATCC 29212 | 98 | CP008816.1 | None | 99.57 ± 0.34 A/ab | 71.38 ± 2.72 B/cdefg | 65.33 ± 2.76 B/cdef |
| | | M495 | Lactobacillus plantarum B21 | 99 | CP010528.1 | Not tested | 96.72 ± 1.31 A/de | 68.44 ± 3.22 B/fg | 58.93 ± 5.92 B/fg |
| | | M496 | Lactobacillus plantarum B21 | 99 | CP010528.1 | Not tested | 95.40 ± 0.25 A/e | 68.78 ± 0.9 B/fg | 58.03 ± 4.08 C/g |

Results are mean values of three replicates ± standard deviation (SD).
Different superscripts letters within the same row/column indicate significant differences ($p < 0.05$)
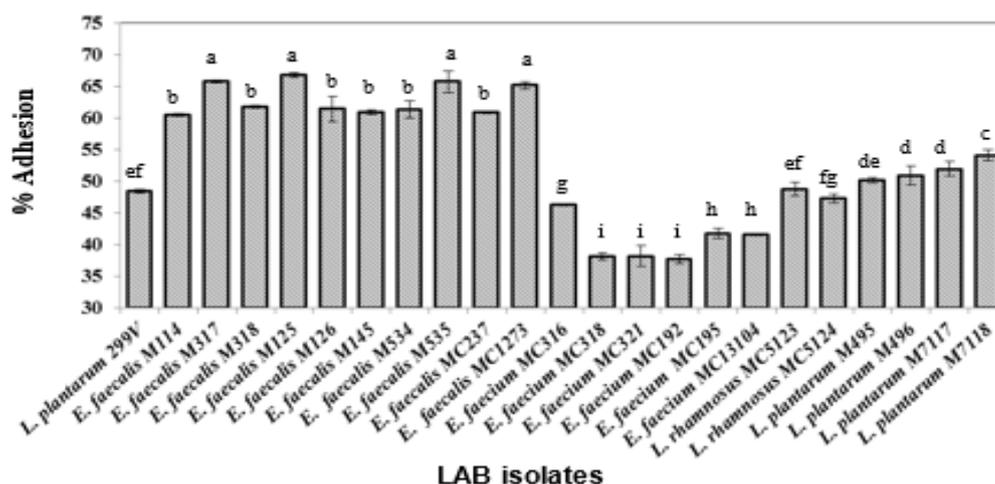
Figure 1 Adhesion ability to porcine mucin of antibacterial LAB isolated from breast milk compared to the mucin adhesive *L. plantarum* 299V used as positive control. Data are mean values ± SD from triplicate analyses. Different superscripts indicate significant differences (*p* < 0.05).

*Tolerance of E. faecalis isolates to GIT stresses*

All selected isolates showed viability after sequential exposure to simulated GIT with survival rates ranging from 40.82.22 to 75.40% (Table 2). They exhibited high viability (95.40-99.87%) after exposure to lysozyme for 5 min (Table 2). However, significant reduction was observed in all isolates after sequential exposure to simulated gastric acid and bile for 3 and 6 h, respectively. Among all, *L. rhamnosus* was most sensitive to gut stresses, while *E. faecalis* M125 was the most tolerant.

*Mucin adhesion ability of antibacterial producing LAB isolated from colostrum*

The adhesion ability of the antibacterial LAB isolates to porcine gastric mucin type III is shown in Fig. 1. *E. faecalis* exhibited distinctive adhesion capacity with extremely higher level than all tested LAB isolates and the mucin adhesive *L. plantarum* 299V. *L. plantarum* displayed moderately high adhesion with slightly greater than *L. plantarum* 299V and *L. rhamnosus*. The extremely low adhesion was clearly observed among all isolates of *E. faecium*. The highest adhesion percentage was particularly shown in *E. faecalis* M125 (67.29%). According to the results obtained, 10 isolates of *E. faecalis* were chosen for further study.

*Proteolytic activity of the selected isolates of high mucin adhesive E. faecalis*

The proteolytic activity of *E. faecalis* grown in UHT skim milk was observed using SDS-PAGE (Fig. 2A). Following 24 h incubation, only *E. faecalis* M125 was able to hydrolyse all casein proteins ($\alpha$S$_{1^-}$, $\alpha$S$_{2^-}$ and $\beta$-caseins). However, $\beta$-lactoglobulin (BLG)

and $\alpha$-lactalbumin (ALA) abundantly present in whey protein remained intact. Both BLG and ALA were not degraded even after 48 h incubation. This was confirmed by performing protein hydrolysis of partial purified whey proteins (Fig. 2B) and Na-caseinate (Fig. 2C). Although many degrading bands with much smaller size than ALA were observed after 24 h incubation, these represented short peptides released from hydrolysis of residual casein proteins present in whey proteins. Likewise, all casein proteins present in Na-caseinate were digested after 24 h and the complete digestion was observed at 48 h. Majority of peptide bands were generated with molecular weight smaller than 14 kDa corresponding to RP-HPLC profile of Na-caseinate degradation as shown in Fig. 2D. After 24 h hydrolysis, HPLC chromatogram confirmed similar pattern of hydrolytic products, which were mainly medium-sized peptides with moderate hydrophilic property. Nevertheless, the unhydrolysed casein was still present albeit of little peak area, corresponding to the faint bands observed from SDS-PAGE as shown in Fig. 2C. However, the complete hydrolysis of Na-caseinate was observed after 48 h hydrolysis confirming proteolytic activity of *E. faecalis* M125 specifically targeted to milk casein.

*In vitro safety evaluation of E. faecalis M125*

*E. faecalis* M125 exhibited MIC values for tetracycline, ampicillin, penicillin G, vancomycin, gentamicin, streptomycin and kanamycin at the level of 1, 0.5, 0.5, 2, 16, 16 and 256 $\mu$g/mL, respectively. These results were lower level than the break points recommended by EFSA (2008) for all the tested antibiotics (2, 4, 4, 4, 32, 128 and 512 $\mu$g/
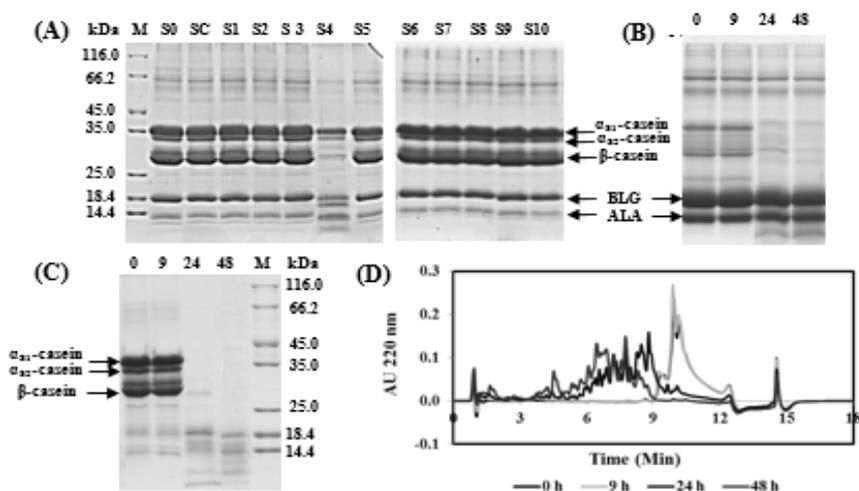
Figure 2 SDS-PAGE profiles showing protein hydrolysis of (A) UHT skim milk proteins at 0 h (S0) and 24 h by various strains of *E. faecalis* (S1–S10 representing *E. faecalis* M114, M317, M318, M125, M126, M145, M534, M535, MC237 and MC1273, respectively) and the control without the bacterial cells (SC). The *E. faecalis* M125 hydrolysis of (B) whey proteins containing β-lactoglobulin (BLG) and α-lactalbumin (ALA) and (C) sodium caseinate at 0, 9, 24 and 48 h of hydrolysis. Lanes M, protein molecular weight marker (Euromedex, Souffelweyersheim, France). (D) The RP-HPLC chromatogram confirms casein hydrolysis of *E. faecalis* M125
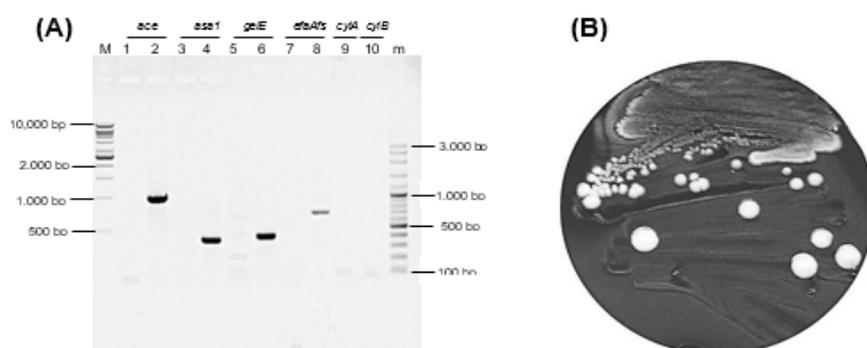


Figure 3 (A): Agarose gel electrophoresis of enterococcal virulence factors (*ace, asa1, gelE, efaAfs, cylA and cylB*) absent in *E. faecalis* M125. Lanes M and m: VC 1 kb (500 – 10,000 bp) and VC 100 bp plus (100 – 3,000 bp) DNA ladder, respectively. Lanes# 1, 3, 5, 7, 9, 10 belong to *E. faecalis* M125 genes, whereas lanes# 2, 4, 6, 8 are *E. faecalis* VanB (positive control). (B): Haemolytic activity of *E. faecalis* M125 (γ-haemolysis) on blood agar (7% v/v human blood) after 48 h of incubation at 37°C.

mL, respectively). This reflected its susceptibility to all antibiotics tested and therefore no chance in the occurrence of transferable antibiotic resistance genes. None of the genes associated with virulence factors were detected (Fig. 3). *E. faecalis* M125 showed no haemolytic activity (γ-haemolysis reaction) on blood agar (Fig. 3) corresponding to the absence of cytolysin genes (cylA and cylB).

## Discussion

Colostrum has been recognised to contain a variety of commensal probiotic bacteria. In particular, many probiotic LAB were isolated and reported previously (Martín *et al*., 2005; Mehanna *et al*., 2013). In the present work, LAB were found and counted in all colostrum samples drawn from

Thai mothers. The result is in good agreement with Marín *et al*. (2009) reporting the similar bacterial counts of log 3.29 and 3.30 CFU/mL present in fresh and frozen-stored colostrum samples, respectively. The fact that colostrum contained viable LAB was duly emphasized. No statistically significant differences were observed in LAB numbers among Thai colostrum samples drawn at various lactation stages and delivery modes in the present work. On the contrary, lactation stage, mode of delivery, gestational age and antibiotherapy were all influential factors relating to the number of LAB in breast milk samples of European mothers (Cabrera-Rubio *et al*., 2012; Soto *et al*., 2014).

The present work demonstrated that *Enterococcus* was the major LAB isolates obtained from colostrum drawn from Thai mothers, when the strain selection

was performed based on antibacterial activity. *Enterococcus* was screened from colostrum drawn at all lactation period, whereas *Lactobacillus* isolates were obtained only in mature milk. These results agree with the abundance of *Enterococcus* in the faeces of breast-fed newborn reported by Solís *et al*. (2010). Moreover, the highest level of strong antibacterial activity was detected among all *E. faecalis* strains isolated from colostrum of vaginal delivery mothers (17.6%). Jiménez *et al*. (2008) also noted that *E. faecalis* was the dominant species in colostrum of healthy mothers. In addition, *E. faecalis* and *L. plantarum* were the main species isolated from colostrum of mothers who went through vaginal delivery. In the meantime, *E. faecalis*, *E. faecium* and *L. rhamnosus* were abundant in caesarean-sectioned mothers. These results are in agreement with Cabrera-Rubio *et al*. (2012) describing the differences of colostrum microbiome between mothers who gave birth through caesarean section and vaginal delivery. The distinction might be due to the physiologic changes of vaginal delivery mothers during labour. These results evinced that the lactation stage and mode of delivery seemed to be the most important influence on the diversity and richness of antibacterial LAB present in colostrum of Thai mothers.

Thai mothers' colostrum-isolated LAB species showing strong antibacterial activity against all pathogens appeared to be *E. faecalis*. Heikkilä and Saris (2003) found that *E. faecalis* isolated from colostrum was able to inhibit and suppress the growth of S. aureus. The presence of *E. faecalis* in colostrum was also reported previously together with their probiotic characterisation (Heikkilä and Saris, 2003; Jiménez *et al*., 2008; Özmen Toğay *et al*., 2014). However, the probiotic mechanism in terms of adhesion capability, which is the major probiotic criterion to ensure the ability to establish and multiply in highly competitive habitat of colon (Servin and Coconnier, 2003), has not yet been assessed. In the present work, colostrum-isolated *E. faecalis* revealed distinctively high adhesion ability as compared to the other LAB isolates. The adhesion property was a specific characteristic depending on the individual LAB species as previously reported (Uraipan and Hongpattarakere, 2015). The excellent adhesion ability of *E. faecalis* strains from colostrum might support the persistency of this species in maternal breast milk and faeces of her infant apart from their tolerances to gut stresses.

In the present work, *E. faecalis* M125 exhibited highest viability after sequential exposure to simulated GIT. More than 99% survival of this strain was observed after passing through the mimicking condition in human mouth. However, the survival rate significantly decreased ($p < 0.05$) after sequential condition of stomach. Though gastric juice was the strongest inhibiting factor against *E. faecalis* M125 in vitro, such conditions might not happen in vivo in human gut when the pH value in stomach after food intake was increased (Huang and Adams, 2004). Moreover, Charteris *et al*. (1998) reported that milk proteins and gastric mucin were able to protect the tested probiotic bacteria during the simulated gastric transit.

Antagonistic activity is the important probiotic property beneficial to human health. Several low molecular weight compounds, secondary metabolites and many types of bacteriocins were previously reported as the antibacterial substances produced by LAB (Makras *et al*., 2006). In the present work, the production of organic acids was the major attribute of the antibacterial activity due to loss of the activity after neutralisation. Moreover, the absence of enterocin structural genes in all *Enterococcus* isolates confirmed no involvement of bacteriocin in such antibacterial activity. The major end-products generated from glucose fermentation of *Enterococcus* was lactic acid (Cebrián *et al*., 2012). This particular acid displayed the antibacterial effect through the collapse of proton motive force and the destruction of cell membrane permeability resulting to the inhibition of substrate transport systems (Ammor *et al*., 2006; Kanjan and Hongpattarakere, 2016). According to this antibacterial activity of probiotics, several strains showed the potential to be applied for adjunctive therapy to treat children suffering from diarrhoea. For example, *L. rhamnosus* strain GG was effective on the reduction of severity and duration of the disease (Canani *et al*., 2007).

Another positive outcome of probiotic is the anti-allergenic property, especially in dairy products. Certain strains of probiotics LAB were able to degrade cow milk proteins into smaller peptides. These strains were supplemented into infant formula recommended for infants who suffered from cow milk allergy (Muraro *et al*., 2012). The large protein allergens are generally more immuno-reactive than the smaller peptides (Mouécoucou *et al*., 2004). The major allergens have been characterised as proteins or glycoproteins that have molecular weights (MW) ranging from 15 to 40 kDa or 10 to 70 kDa, respectively (Żukiewicz-Sobczak *et al*., 2013). In the present work, *E. faecalis* M125 showed the proteolytic activity generating peptides smaller than 14 kDa after hydrolysis of the casein fractions (MW within the range 19 to 25 kDa).

Casein, accounting for 80% of total cow milk

protein, is considered the major milk allergen (Hochwallner *et al.*, 2014). Thus, *E. faecalis* M125 possessed beneficial property by specifically hydrolysing the major allergenic proteins present in cow milk. Further study is therefore necessary in order to demonstrate the main epitopes, which can be hydrolysed by this particular strain.

The safety assessment of colostrum-isolated enterococci regarding antibiotic resistance, virulence traits and haemolysis were essential before being applied as a probiotic supplement. Antibiotic resistance was the major concern since antibiotic resistance genes of enterococci were often harboured in plasmid or transposon, presenting a risk of horizontal gene transfer to pathogenic bacteria (Nueno-Palop and Narbad, 2011). According to the results obtained in the present work, *E. faecalis* M125 was susceptible to tetracycline, ampicillin, penicillin G, gentamicin, streptomycin, and kanamycin. More importantly, it was sensitive to vancomycin. The vancomycin-resistant enterococci were a frequent cause of nosocomial infections in the last decade (Ahmadova *et al.*, 2013). A similar profile of susceptibility to ampicillin, penicillin and vancomycin was also observed in other *E. faecalis* strains isolated from colostrum (Jiménez *et al.*, 2008; Özmen Toğay *et al.*, 2014).

Additionally, the non-pathogenicity of *E. faecalis* M125 was confirmed according to its negative haemolytic reaction on blood agar as well as the absence of six major genes encoding enterococcal virulence factors. However, these virulence genes were found in other strains of *E. faecalis* isolated from colostrum and breast milk samples (Jiménez *et al.*, 2008; Özmen Toğay *et al.*, 2014). Virulence factors of *E. faecalis* were considered to facilitate the adherence of bacteria to the host cell membranes and to environmental surfaces where they could assess to nutrients and escape host immune response (Medeiros *et al.*, 2014). Overall, *E. faecalis* M125 was neither resistant to any antibiotics tested nor carrying transferable virulence genes. It was therefore proposed as potential probiotic candidate that can be safely applied in food products without spreading antibiotic resistant and virulence trait encoding genes to other gut microflora present in the gut ecosystem.

## Conclusion

Antibacterial producing enterococci were abundantly selected from colostrum drawn from Thai mothers. *E. faecalis* was the major LAB species dominantly obtained from breast milk secreted at all stages of lactation. This was observed in correlation to their extremely high mucin adhesion as well as gut stress tolerances. *E. faecalis* M125 displayed an interesting functional characteristic in terms of proteolytic cleavage of the major allergenic components present in bovine casein, apart from its strong antibacterial activity against foodborne pathogenic bacteria. Additionally, antibiotic resistances, virulence factors and haemolytic activity were absent in this strain. Further studies should be done to elucidate its potential health benefit and application as promising probiotic candidate for developing novel functional food products to serve dairy and infant formula industry.

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