Isolation, Identification, and Evaluation of Novel Probiotic Strains Isolated from Feces of Breast-Fed Infants

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Objective: *To isolate, identify, and evaluate the probiotic properties of lactic acid bacteria (LAB) isolated from the feces of breast-fed infants.*
Material and Method: *The probiotic tests included investigation of hemolysis activity, survival in simulated gastrointestinal tract conditions (acid and bile salt tolerance), susceptibility to antibiotics, and ability to inhibit selected bacterial pathogens (Escherichia coli O157:H7, Vibrio cholerae and Salmonella enterica subsp enterica serovar Typhimurium). The bacterial species identification was performed by both carbohydrate utilization and partial 16S ribosomal RNA sequencing.*
Results: *Five of fifty LAB isolates (UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37) showed good probiotic properties. These five isolates showed non-hemolysis type (gamma-hemolysis), susceptibility to all antibiotics tested except for vancomycin, ability to survive in the simulated gastrointestinal conditions of both acid and bile salt solution, and ability to inhibit growth of E. coli O157: H7 and V. cholerae. Bacterial species identification revealed that all five isolates were firmly identified as Lactobacillus rhamnosus species.*
Conclusion: *The L. rhamnosus strains that were isolated and characterized in this study could be considered as probiotic strains, and then used for further probiotic characterization in human cell cultures or animal models.*

Keywords: *Breast-fed infant, Lactic acid bacteria, Lactobacillus rhamnosus, Probiotic*

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According to the Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics are live micro-organisms that when administered in adequate amounts confer health on the host[1]. Probiotics can be yeast and bacteria (both gram-positive and gram-negative bacteria) but most are members of lactic acid bacteria (LAB), especially in the genus *Lactobacillus.* Many *Lactobacillus* species including *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, and *Lactobacillus acidophilus* have been evaluated for their probiotic properties[2] such as modulation of human immune responses, improvement of the human gut microbiota and intestinal function[3,4], reduction of the risk of colon cancer[5], and competitive nature against microbial pathogen[6].

Lactobacillus species are microflora in various environments, including foods (meat, vegetable, and milk and other dairy products), and the mucosa of animals and humans. However, the most potential probiotic strains have been often isolated from human origins[7]. Several in vitro tests used for initial screening and selection of the potential probiotic included the test of survival during gastrointestinal transit, antibiotic susceptibility profile, antimicrobial activity, and species identification[8]. These tests have been widely accepted to reflect the probiotic properties in the host including humans.

The present study aimed to isolate, characterize, and identify the LAB for potential use as a probiotic. The probiotic tests included evaluation of hemolysis activity, survival in a simulated human

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gastrointestinal tract, antibiotic susceptibility profile, and bacterial species identification.

Material and Method

Ethical approval

The experiment was approved by the Human Ethics Committee of Ubon Ratchathani University, Ubon Ratchathani, Thailand (2/2556). All experiments throughout this study were performed under the declaration of Helsinki.

Isolation of LAB

An eight-day-old male was a donor of feces. Five g of the feces was suspended and homogenized in 45 ml of phosphate buffered saline (PBS) pH7.4 (Sigma, Singapore). The homogenized feces were used to prepare the 10-fold serial dilutions. 100 µl of an appropriate dilution were spread-plated on Man, Rogosa and Sharpe agar (MRS) (LAB, United Kingdom) containing 0.5% (w/v) $CaCO_3$ and incubated at 37°C under anaerobic conditions in an anaerobic jar with gas pack (AnaeroPack®-Anaerobe, Mitsubishi, Japan). After 48 hour of incubation, a single colony with a clear zone was randomly selected. Based on rod shape morphology, gram positivity, and catalase negativity, all isolates were identified as LAB. An LAB isolate was selected and inoculated in MRS broth at 37°C for 18 hour. The culture stock was prepared in skimmed milk containing 20% glycerol and stored at -20°C until use.

Hemolysis activity

A single colony of each LAB isolate was picked up from the MRS agar plate and then streaked on blood-based agar supplemented with 5.0% human blood. The plate was incubated at 37°C for 48 h and then observed for the appearance of hemolysis types, beta-hemolysis (clear zone around bacterial colony), alpha-hemolysis (green zone and partial clear zone around bacterial colony), and gamma-hemolysis (no clear zone around colony). LAB isolates with beta-and alpha-hemolysis types were used for further probiotic tests.

Survival under simulated human gastrointestinal conditions

A single colony of LAB isolate was grown in 10 ml MRS broth and incubated at 37°C for 18 hour. After incubation, the bacterial cells were harvested by centrifugation at 3,000 x g for 10 minute at an ambient temperature. The bacterial cells were then washed twice in PBS pH 7.4. The amount of bacterial cells was adjusted to $1.5x10^8$ CFU/ml by comparing to the McFarland No. 0.5.

To determine the survival of LAB in gastric juice solution, 100 µl of $1.5x10^8$ CFU/ml of each LAB isolate was added into 900 µl of synthetic gastric juice (NaCl 2 g, pepsin 3.2 g, dissolved in 1 L of water, pH3.0) and incubated at 37°C for 3 hour. After incubation, 50 µl of each bacterial solution was collected and used to prepare a 10-fold serial dilution. The viable population was calculated by plate counting on the MRS agar. The percentage (%) of cell survival was calculated by formulation as follow:

- % of cell survival = $(\log CFU_T/\log CFU_C) \times 100$

- where CFU_C and CFU_T represent the total viable count of LAB before and after, respectively, incubated under the simulated gastrointestinal condition (gastric juice or bile salt solution).

To determine the survival of LAB in bile salt solution, 100 µl of 1.5×10^8 CFU/ml was added into 900 µl of 0.3% (w/v) bile salt solution pH 8.0 (Sigma, Singapore). The bacterial solution was then incubated at 37°C for 4 hour. The percentage (%) of cell survival was calculated as previously described.

Antibiotic susceptibility test

The minimal inhibition concentration (MIC) assay using the Etest® (bioMerieux, France) was used to determine the antibiotic susceptibility of the LAB isolates. Three antibiotics, erythromycin, tetracycline, and vancomycin, were tested. The LAB isolates were grown in MRS broth at 37°C for 18 hour. The bacterial culture was adjusted to obtain $1.5x10^8$ CFU/ml. 100 µl of adjusted bacterial culture was spread onto a fresh MRS agar plate. The standard strips containing gradient of antibiotic concentrations ranging from 0 to 256 µg were placed on the MRS agar plate. The plate was incubated at 37°C for 48 hour. The MIC value was determined according to the manufacturer's instructions. The criteria for interpretation of susceptible (S), intermediate (I), or resistant (R) bacteria were performed according to standards of the National Committee for Clinical Laboratory Standards (NCCLS)[9].

Antimicrobial activity against pathogenic bacteria

LAB isolates were grown in 10 ml MRS both at 37°C for 24 hour. After incubation, the cell-free supernatant (CFS) from the culture was collected by centrifugation at 3,000 x g for 10 minute. The CFS was consequently sterilized by filtration through a 0.45 µm

pore size filter (Corning, USA). Three pathogenic bacteria, *E. coli* O157:H7, *S. typhimurium*, and *V. cholerae* were used as indicator strains and grown in 2 ml brain heart infusion (BHI) medium at 37°C for 24 hour. An amount of 20 ml of warmed BHI agar medium was mixed with 200 µl of overnight culture of each indicator and the mixtures was poured into Pedti dish plates. A central well (diameter of 5 mm) was made on an agar plate and filled with 50 µl of the CFS of each LAB isolate. The plate was incubated at 37°C for 24 hour. The presence and absence of inhibition zones around the wells were recorded as positive and negative results, respectively.

Identification of LAB

The bacterial species identification was performed by the determination of sugar utilization and 16S rRNA sequencing. Sugar utilization was performed by using API® 50 CH kit (BioMerieux, France) according to the manufacturer's instructions. The data were analyzed through the website at https://apiweb.biomerieux.com/. For the 16S rRNA sequencing, the partial sequence of 16S rRNA gene with the length of 1,500 base pair (bp) was amplified with the universal prokaryotic primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-'3) and 1492R (5'-GGTTACCTTGTTACGACTT-3') as previously described by Tan et al (2013)[8]. The sequencing results were compared with reported sequences held in NCBI databases at http://www.ncbi.nlm.nih.gov.

Results

Isolation of LAB

Fifty single bacterial colonies with a clear zone around their colonies were selected from MRS agar medium containing calcium carbonate at 37°C under anaerobic conditions. Gram staining revealed that all bacterial isolates were gram-positive with a bacilli shape. All isolates were catalase negative. These preliminary results indicated that they were LAB. Thus, these fifty LAB isolates were further used for probiotic characterization.

Hemolysis activity

The fifty LAB isolates exhibited non-hemolytic activity when they were grown in blood agar medium containing human blood.

Survival in simulated human gastrointestinal tract condition

Five of the fifty LAB isolates, UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37 strains, showed their high resistance to gastric juice with low pH and to bile salt, comparable to that of the reference strain, *Lactobacillus rhamnosus* GG (ATCC53103).

All five LAB isolates showed the viability of more than 80% after 3 hour exposure to gastric juice (Fig. 1). In addition, all five strains showed their viability at the percentage of an approximately 80% after 4 hour exposure to 0.3% bile salt, except for UBU-37 giving only 63.65 percentage of cell survival (Fig. 1).

Antibiotic susceptibility

The selected five LAB isolates with acid and bile salt tolerance were used to evaluate for their antibiotic susceptibility. As shown in Table 1, they were susceptible to erythromycin and tetracycline but resistant to vancomycin.

Antimicrobial activity

Five LAB isolates were examined for their antimicrobial activity against selected pathogens including *E. coli* O157: H7, *S. typhimurium*, and *V. cholerae*, by the agar diffusion method. As shown in Table 2, the five isolates showed growth inhibition activities to *V. cholerae*. Two LAB isolates, UBU-03 and UBU-06, showed growth inhibition to *E. coli* O157: H7. No LAB isolates showed growth inhibition to

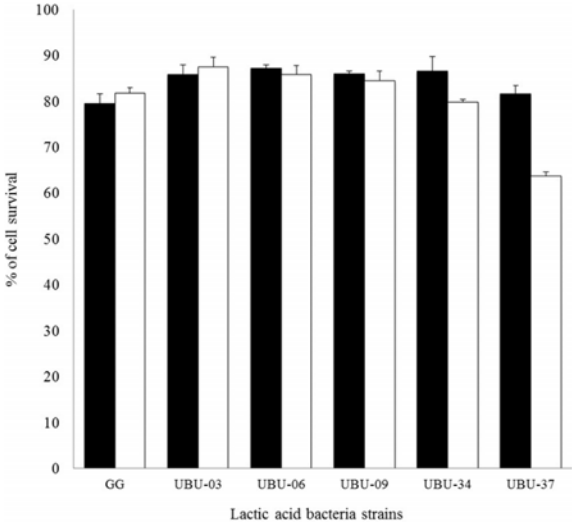

Fig. 1 Percentage of survival of LAB isolates after exposure to gastric juice solution pH3.0 for 3 hour at 37°C (■) and 0.3% (w/v) bile salt solution pH8.0 for 4 hour at 37°C (□). The experiment was performed in triplicate. GG, *Lactobacillus rhamnosus* ATCC53103.

S. typhimurium.

Bacterial identification

Based on the result of sugar utilization, all LAB isolates showed the same profile of carbohydrate utilization after 5 days of incubation. They utilized D-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, N-acetyl-glucosamine, amygdaline, arbutin, esculin, salicin, cellobiose, maltose, trehalose, melezitose, gentiobiose, D-tagatose, L-fucose, and gluconate. These sugar utilization profiles showed similarity to that of the *L. rhamnosus* GG[10]. To assure the identity of UBU strains, 16S rRNA gene amplification and sequencing were performed. Sequence analysis and comparison of UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37 showed more than 95% identity with 16S rRNA of several *L. rhamnosus* strains in database (Table 3). In addition, among the five strains they showed more than 90% identity (data not shown). Therefore, based on these results it was concluded that all five LAB isolates belonged to the *L. rhamnosus* species.

Table 1. Antibiotics susceptibility of five LAB isolates

Strains	MIC (μg/ml) of antibiotics		
	Erythromycin	Tetracycline	Vancomycin
UBU-03	0.125 (S)	0.75 (S)	>256 (R)
UBU-06	0.380 (S)	0.50 (S)	>256 (R)
UBU-09	0.380 (S)	0.50 (S)	>256 (R)
UBU-34	0.094 (S)	0.50 (S)	>256 (R)
UBU-37	0.094 (S)	0.50 (S)	>256 (R)

S = sensitive; R = resistant

Table 2. Antimicrobial activity of the five LAB isolates against selected pathogenic bacteri

Isolates	Antimicrobial activity (diameter of inhibition zone, mm)		
	E. coli	*V. cholerae*	*S. typhimurium*
UBU-03	+ (15)	+ (15)	-
UBU-06	+ (11)	+ (17)	-
UBU-09	-	+ (13)	-
UBU-34	-	+ (9)	-
UBU-37	-	+ (9)	-

+ = giving inhibition zone; - = no inhibition zone

Discussion

Currently, there has been much interested in using LAB as probiotics in humans. Several LAB strains have been widely isolated from various origins, such as foods, vegetables, beverages, and human mucosal surfaces (gastrointestinal and urogenital tracts). For probiotic use in humans, the isolated LAB strains were first screened for their potential probiotic properties in in vitro experiments, which could be reflected, by the bacterial properties in humans. These experiments included the determination of LAB strains to survive in human gastrointestinal tracts (gastric juice with low pH and bile tolerance), toxicity to human cells called hemolysis, antibiotic susceptibility test,

Table 3. Bacterial identification by 16S rRNA sequencing

Isolates	Gene similarity in NCBI data base*	% of nucleotide identity	Identified bacteria
UBU-03	*Lactobacillus rhamnosus* strain Z5 16S ribosomal RNA gene, partial sequence (accession No. KM350174.1)	97	*Lactobacillus rhamnosus*
UBU-06	*Lactobacillus rhamnosus* strain HT2 16S ribosomal RNA gene, partial sequence (accession No. JF414108.1)	97	*Lactobacillus rhamnosus*
UBU-09	*Lactobacillus rhamnosus* strain FT218 16S ribosomal RNA gene, partial sequence (accession No. KM207836.1)	97	*Lactobacillus rhamnosus*
UBU-34	*Lactobacillus rhamnosus* strain LC2 16S ribosomal RNA gene, partial sequence (accession No. KM350164.1)	96	*Lactobacillus rhamnosus*
UBU-37	*Lactobacillus rhamnosus* strain 20300 16S ribosomal RNA gene, partial sequence (accession No. HM162419.1)	97	*Lactobacillus rhamnosus*

* The result shows only the gene which was highest similar to the sequencing result

antimicrobial activity against pathogens, and bacterial species identification. In this study, fifty LAB isolates were totally isolated from infant feces.

The survival ability in a human gastrointestinal tract (GI) is a major desirable property required for probiotics. In the human body, approximately 2.5 L of gastric juice[11] and 1 L of bile[12], are produced and secreted daily into the GI tract. Thus, to act as probiotics in humans, the introduced strain must survive during transit through the harsh environment found in the human GI tract. After screening of LAB isolates for their tolerance to gastric juice with low pH and to bile salt, five of the fifty LAB isolates, UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37 strains, showed their high resistance to gastric juice with low pH and to bile salt, comparable to that of the reference strain *Lactobacillus rhamnosus* GG (ATCC53103), a well-characterized probiotic strain. In addition, this result was accordance with many studies that have also been demonstrated that LAB, especially *Lactobacillus* and *Bifidobacterium*, isolated from a human's intestine had the ability to resist bile salt better than those of strains isolated from another sources, including foods[13, 14].

The five LAB isolates showed susceptibility to erythromycin and tetracycline but resistance to vancomycin, indicating native resistance to vancomycin. This result was consistent with a previous report that the LAB strains of *L. plantarum, L. casei, L rhamnosus, Pediococci,* and *Leuconostoc* species were resistant to vancomycin[15]. It was reported that vancomycin resistance in LAB was associated with a gene located on a chromosome; thus, it could not be transferable to other bacterial species[16].

In the present study, the five LAB isolates, UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37 strains, showed antimicrobial activity against *V. cholerae,* whereas two strains, UBU-03 and UBU-06, could inhibit the growth of *E. coli* O157: H7. All five LABs had no antimicrobial activity against *S. typhimurium*. These results were similar to a previous study, which found that *Lactobacillus rhamnosus* strains isolated from infant feces could inhibit the growth of gastrointestinal pathogens[17]. In addition, it has been suggested that the antimicrobial activity produced by LAB were caused by metabolic substances produced by LAB during their growth, such as lactic acid, fatty acid, H_2O_2, and bacteriocin[9,14,15]. Based on phenotypic profiling via carbohydrate utilization and molecular identification, it was proved that the five LAB isolates belonged to the *L. rhamnosus* species. Comparison of partial sequence of 16S rRNA among the five LAB strains revealed that they were closely related with each other and might have originated from the same clone, especially, UBU-03 and UBU-06, as they gave the similar probiotic properties such as acid and bile tolerances and antimicrobial activity.

Conclusion

In conclusion, five of the fifty LAB isolates isolated from infant feces, UBU-03, UBU-06, UBU-09, UBU-34, and UBU-37, were identified as *Lactobacillus rhamnosus* species and showed good probiotic properties that were comparable to the reference strain, *L. rhamnosus* GG. These five isolates showed gamma-hemolysis, susceptibility to all antibiotics type, tolerance and survive under acid and bile salt conditions, and produce substances to inhibit growth of *E. coli* O157: H7 and *V. cholerae*. Thus, the five LAB strains isolated in this study could be considered as probiotic strains, and can be used for the further probiotic characterization in human cell cultures or animal models.

What is already known on this topic?

We have previously studied on the isolation, probiotic characterization, and identification of many strains of lactic acid bacteria such as *Lactobacillus casei* from chicken and *Bifidobacterium* species from human.

What this study adds?

The present study shows the isolation of new strains of *Lactobacillus rhamnosus* with good probiotic properties, isolated from human source (feces). These strains can be further used for evaluating their probiotic properties in humans. In addition, the *L. rhamnosus* UBU-06 and UBU-37 were recently being used as host for heterologous expression that will be used as a vaccine vector.

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Potential conflicts of interest

None.

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การคัดแยก จำแนก และประเมินคุณสมบัติโพรไบโอติกของเชื้อแบคทีเรียกรดแลคติกที่คัดแยกได้จากอุจจาระของเด็กแรกเกิดที่ดื่มนมแม่

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วัตถุประสงค์: เพื่อทำการคัดแยก จำแนก และประเมินคุณสมบัติโพรไบโอติกของแบคทีเรียกรดแลคติกที่คัดแยกได้จากอุจจาระของเด็กแรกเกิดที่ดื่มนมแม่

วัสดุและวิธีการ: ทดสอบคุณสมบัติความเป็นโพรไบโอติกของแบคทีเรียกรดแลคติกที่คัดแยกได้จากอุจจาระของเด็กแรกเกิดที่ดื่มนมแม่ คุณสมบัติโพรไบโอติก ที่ศึกษาได้แก่ การย่อยสลายเม็ดเลือดแดงของคน การทนต่อสภาวะที่พบในระบบทางเดินอาหาร ได้แก่ การทนกรดและเกลือน้ำดี การทดสอบความไว ต่อยาปฏิชีวนะ การสร้างสารยับยั้งจุลินทรีย์ชนิดอื่นๆ ได้แก่ เชื้อ Escherichia coli O157: H7 Vibrio cholerae และ Salmonella enterica subsp. enterica serovar Typhimurium และการจำแนกชนิดแบคทีเรีย

ผลการศึกษา: สามารถคัดแยกแบคทีเรียกรดแลคติกที่มีคุณสมบัติเป็นโพรไบโอติกที่ดี จำนวน 5 ไอโซเลต จากจำนวนเชื้อทั้งหมดที่แยกได้ คือ 50 ไอโซเลต ได้แก่ UBU-03, UBU-06, UBU-09, UBU-34 และ UBU-37 โดยทุกไอโซเลตไม่ย่อยสลายเม็ดเลือดแดงของคน ไวต่อยาปฏิชีวนะทุกชนิดยกเว้น vancomycin สามารถทนต่อสภาวะความเป็นกรดและสภาวะที่มีเกลือน้ำดี สร้างสารเพื่อยับยั้งการเจริญของเชื้อ E. coli O157: H7 และ V. cholerae ได้ผลการจำแนกชนิดแบคทีเรียในระดับสปีชีย์ด้วยวิธีการทดสอบ ความสามารถในการใช้น้ำตาลชนิดต่างๆ และด้วยวิธีการเพิ่มจำนวนและตรวจสอบลำดับเบสของจีน 16S ribosomal RNA พบว่าเชื้อแบคทีเรียกรดแลคติกทั้ง 5 ไอโซลเลตเป็นเชื้อ Lactobacillus rhamnosus

สรุป: เชื้อ L. rhamnosus ทั้ง 5 สายพันธุ์ ได้แก่ UBU-03, UBU-06, UBU-09, UBU-34 และ UBU-37 ที่คัดแยกได้จากอุจจาระของเด็กแรกเกิดที่ดื่มนมแม่ ซึ่งผ่านการทดสอบในระดับหลอดทดลองแล้วว่ามีคุณสมบัติโพรไบโอติกที่ดี ดังนั้นเชื้อเหล่านี้จึงมีความเหมาะสมที่จะนำไปทดสอบความเป็นโพรไบโอติกในขั้นสูงต่อไป คือ การทดสอบกับเซลล์เพาะเลี้ยงหรือกับสัตว์ทดลอง