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# Isolation, Identification and Analysis of Probiotic Properties of Lactic Acid Bacteria from Selective Various Traditional Thai Fermented Food and Kefir

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**Abstract:** A total of 499 lactic acid bacteria were isolated from 93 samples traditional Thai fermented food (91 samples) and kefir (2 samples). Antimicrobial activities of all isolates grown under oxygen-restricted conditions to eliminate the effect of hydrogen peroxide were tested against 20 intestinal and urogenital pathogenic bacterial using directed agar and spot on lawn technique. Thirty one isolates exhibited antimicrobial activity against at least one indicator strain tested. We observed that *Shigella boydii* and *Shigella flexneri* were the most inhibited pathogen. The characterization of the microorganism as probiotic were tested by growth in wide temperature ranges (25-45°C), salt, bile salt and acid tolerant, antibiotic sensitivity, cell surface hydrophobicity, hemolysis pattern and cell free agar diffusion method. Results showed that there were 4 isolates which showed effective antimicrobial activities and probiotic properties. Diversity of lactic acid bacteria with antimicrobial activities was studied by identification to the species level of the 4 isolates by using API 50 CHL test kit. Of the 4 isolates, 2 isolates were identified as *Lactobacillus plantarum*, the other 2 isolates were identified as *Lactobacillus pentosus* (1 isolates) and *Lactobacillus brevis* (1 isolate). Therefore, these strains may have potential use as an alternative to antibiotics. The strains can also be used to produce antimicrobial compounds which can be a substitute for chemical preservatives in food industry.

**Key words:** Lactic acid bacteria, probiotic, traditional Thai fermented food, Kefir, antimicrobial activity

## INTRODUCTION

Microorganisms play an essential role in the food fermentations. Lactic acid bacteria (LAB) have been well known for centuries about their responsible mainly used in food preservation including dairy, meat, vegetables and bakery products due to their fermentative capacities and safety either separately or in combination with other conventional treatment. LAB has been isolated from several foods, including dairy products, meat products, plants, sewage, manure animals and also humans (Gharaei and Eslamifar, 2011).

Health benefits of LAB are known to give positive influence in the gastrointestinal of humans (Hafidh *et al*., 2010). The inhibitory properties of LAB is due to produce variety of antimicrobial compounds includes lactic and acetic acids, ethanol, formic acid, acetone, hydrogen peroxide, diacetyl and bacteriocins which confer preservative ability on them as a natural competitive means to overcome other microorganisms sharing the same niche (Oliveira *et al*., 2008). This effect depends on the species and loads of pathogenic bacteria, sanitation processes as well as the number of LAB in fermented foods. Currently, the only bio-preservative compound that could be added to food and considered as functional foods (prebiotics, probiotics or nutraceuticals) as well as in human therapy is the one produced by lactic acid bacteria (Gardiner *et al*., 2000; Corcoran *et al*., 2004; De Vuyst and Leroy, 2007). Nowadays, there were many typed of LAB isolated from fermented food including *Lactobacillus* spp., *Bifidobacterium* spp. (Yateem *et al*., 2008) and *Enterococcus* spp. (Ljungh and Wadstrom, 2006) which most widely used and commonly studied probiotic bacteria.

Fermentation was one of the ancient methods used to preserved food worldwide. Most fermented foods owe their origin to the fact that the processes used in their production are inhibitory to many microorganisms. As a result that fermented products generally have a longer shelf life than their original substrate and their ultimate spoilage is different in character (Adams and Mitchell, 2002). Since Thailand has many methods used to preserved and fermented food, both meat and vegetable products. There are wide varieties of fermented food available on market. It is interesting that probiotic strains is important to satisfy the increasing market demand and to obtain highly active probiotic cultures for improved products with probiotic characteristics that are superior to those presently on the market.

Kefir is a traditional popular Middle Eastern beverage. Kefir grains look like pieces of coral or small clumps of cauliflower, which contain a complex mixture of both bacteria (including various species of lactobacilli,

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lactococci, leuconostocs and acetobacteria) and yeasts (both lactose-fermenting and non-lactose-fermenting) such that beneficial yeast as well as friendly probiotic bacteria found in yogurt (Libudzisz and Piatkiewicz, 1990). The grains cause its fermentation that results numerous components in the kefir including lactic acid, acetic acid, CO, ethyl alcohol and aromatic compounds. Many studies have shown that kefir has many biological activities e.g. anti-inflammatory activity, immune-modulating activity, antimicrobial activity and anti-proliferative activity and it has the potential to become a type of functional food (Diniz *et al*., 2003; Vinderola *et al*., 2005; Liu *et al*., 2006; Silvia *et al*., 2009). However, few study were identified antimicrobial and probiotic properties of bacteria which colonies in this community organism.

To our knowledge, a specific LAB species which has the inhibitory activity should be well characterized. The present study aimed at studying the key probiotic properties of isolated LAB from various sources including Thai traditional fermented food and kefir. Assess antimicrobial activities against important gastrointestinal and urogenital pathogenic bacteria of some LAB were determined. Physical and chemical characterize for their potential application as a probiotic properties supplement based on resistance in conditions of the intestinal tract and antimicrobial activity and identify the LAB isolates to species level were elucidated.

## MATERIALS AND METHODS

**Sample collection:** Ninety-one samples containing of 10 samples each of various Thai traditional fermented food such as meat products e.g. *sai-krog-prieo* (fermented sausage), *mam* (fermented beef), *nham* (fermented pork); fish, shrimp and shell fish products e.g. *kung-chom* (fermented fresh water shrimp), *pla-som* (fermented fresh water fish with rice), *som-fak* (fermented fresh water fish), *pla-ra* (fermented fresh water fish with salt), *phu-dong* (fermented fresh water crab), *sai pra mun* (fermented fresh water fish intestinal), *tai pra* (fermented fish intestinal), *hoi dong* (fermented shell fish); vegetable products e.g. *toa hu yee* (fermented soy bean paste), *toa jeaw* (fermented soy bean); fruit products including *phakgard-dong* (pickled green mustard), *mamuang-dong* (fermented mango), *bai maeng* (fermented tea leaves) and 2 samples of kefir were collected. The samples were stored on ice until delivery to the laboratory.

**Isolation, identification and culture conditions of bacteria:** Lactic acid bacteria were isolated by the dilution plate method in de Man Rogosa Sharpe (MRS) agar + 0.04% bromocresol blue. Briefly, Homogenized samples were 10-fold serially diluted by sterile 0.85% NaCl and incubated under microaerophilic for 24-48 h at 30°C. Single bacterial colonies produced yellow zone were initially separated according to their morphological differences. All isolates were presumptively identified as lactic acid bacteria, based on gram stain affinity, motility and catalase and oxidase production. Cells morphology and colonial characteristics on MRS agar were also examined. All isolates were re-streaked out on MRS agar medium to get the pure culture. The culture was maintained in MRS agar stored at 4°C and activated in MRS agar for 24 h before experimental use.

**Pathogenic organisms:** The pathogenic indicator microorganisms which use for screening of antimicrobial substance producing LAB including 20 of important pathogenic bacteria for gastrointestinal and urogenital pathogenic bacteria were prepared (Table 2). All tested microorganisms were obtained from Faculty of Medical Technology, Rangsit University Culture Collection.

**Determination of antimicrobial activity by spot on lawn technique:** All pathogenic indicator microorganisms were grown with nutrient agar (NA) at 37°C for 24 h. The cultured slants were stored at 4°C and subcultured every two weeks. The indicator microorganisms were prepared by transfer the microorganisms from 24 h culture to sterile 0.85% NaCl. The optical density of culture was observed at 625 nm and adjusted absorbance to 0.08-0.1 which the viable cells of that density is equal to $1.0 \times 10^8$ cells/ml.

The inhibitory activity of the selected LAB isolates against the pathogenic indicator microorganisms was assayed by the spot on lawn technique described by Schillinger and Lücke (Schillinger and Luke, 1989). The LAB isolates were spotted onto the surface of bacteriocin screening medium agar (BSM) plates and incubated at 30°C for 24 h to allow colonies to develop. The pathogenic indicator microorganisms were inoculated into 5 mL of trypticase soy broth + 0.6% yeast extract + 1% soft agar made their density equal to $1.0 \times 10^7$ cells/ml and poured over the plate on which the LAB isolates were grown. After incubation at 37°C for 24-48 h under microaerophilic, the plates were examined for the presence of inhibition zones. Inhibition was considered positive when the width of the clear zone around the colonies of the LAB isolates was 1 mm or larger.

**Determination the inhibitory activity of Cell free supernatants by agar diffusion method**

**Preparation of cell free supernatants:** The strain of the selected LAB isolates which showed inhibition zone for at least two indicator microorganisms were used for further studies. Cell free supernatants (CFS) from these strains were obtained and their inhibitory activity against the indicator microorganisms was assayed. CFS were

obtained by inoculated the selected LAB isolates into 5 ml of MRS broth cultures. After 48 h of incubation at 30°C, the culture was centrifugation at 10,000 g for 10 min at 4°C. To rule out inhibition due to pH reduction caused by organic acids, the pH of the supernatants was adjusted to 6.2 using 1 N NaOH. The supernatants were filter-sterilized through 0.22 µm pore-size filters. Serial two fold dilutions of CFS were done by diluted with MRS broth from undiluted to 1: 128.

To determine the inhibitory activity of the CFS, the pathogenic indicator microorganisms were added into 5 mL of soft trypticase soy broth + 0.6% yeast extract + 1% soft agar made theirs density equal to 1.0 x $10^7$ cells/ml and poured over the plate containing nutrient agar. 10 µl of each dilution of CSF were filled onto agar. After diffusion at 4±1°C for 1 h, the plates were incubated at 30°C for 24 h. The antimicrobial activity of each isolate was evaluated based on the formation of a clear zone around wells and measured with a caliper.

**Determination of growth on various temperature and NaCl tolerance:** For the determination of growth on various temperatures, one colony of fresh overnight culture of LAB isolated was inoculated into sterile MRS broth tubes of varying temperatures (25, 37 and 45°C) for 24 h. Then 0.1 ml inoculums from each tube was poured to MRS agar medium by spread plate method on MRS agar and incubated at 30°C for 24 h. The growth of LAB on MRS agar was used to designate isolates as temperatures tolerant (Tambekar and Bhutada, 2010). The test was performed in triplicates.

For the determination of NaCl tolerance of LAB, MRS broth was adjusted with different concentration (4, 8 and 10% (w/v)) NaCl. After sterilization, one colony of fresh overnight culture of LAB isolated were inoculated into sterile MRS broth and incubated at 30°C. The growth was evaluated by spread plate method on MRS agar and monitors their growth at 0, 3, 6 and 24 h. The test was performed in triplicates.

**Determination of acid and bile salt tolerance:** Determination of acid and bile salt tolerance have modified from Tambekar and Bhutada (Tambekar and Bhutada, 2010). Briefly, one colony of fresh overnight culture of LAB isolated was inoculated into MRS medium with various acid conditions (pH 1, 2 and 3). To study bile salt tolerance, the experiments were performed using similar procedure as described above, but percentage of bile salt was adjusted with different concentration (1.5, 3 and 4.5%). The growth was evaluated by spread plate method on MRS agar and growth at 0, 3, 6 and 24 h. Acid and bile resistance bacteria were indicated by the presence of colonies on plates. The concentrations that the isolated was able to grow were recorded. Triplicates of each sample were performed.

**Determination of hemolysis:** One colony from a 1 day culture was restreak into blood agar. After incubation at 30°C for 24 h under microaerophilic, the hemolysis pattern was evaluated according to β-hemolysis, α-hemolysis, or γ-hemolysis pattern. The assay was performed in duplicate.

**Cell surface hydrophobicity:** In order to assess the degree of hydrophobicity, the Microbial Adhesion to Hydrocarbons (MATH) method was used as a measure of their hydrophobicity using three different hydrophobic solvents: octane, xylene and toluene as with a little modification as described by Rosenberg *et al.* (Rosenberg *et al.*, 1980). Briefly, bacteria were harvested during the exponential growth phase by centrifugation at 4000 g for 15 min, washed twice with PBS (pH = 7) and resuspended in the same buffer. The suspension was adjusted to approximately $10^8$ CFU/ml cell densities (by optical density at 625 nm (A1)). Samples (3.5 ml) of bacterial suspensions were placed in tubes and 500 µl of hydrophobic solvents were added, mixed using a vortex mixer for 2 min. The tubes were allowed to stand until the phase separated (10-15 min). The lower aqueous phase was carefully removed and the OD at 625 nm (A2) was measured. The hydrophobicity index (HPBI) was calculated as the decrease in the optical density of the initial bacterial suspension due to cell partitioning into a hydrocarbon layer as the following equation:

$$HPBI = [(A1-A2)/A1] \times 100$$

According to the hydrophobic characteristics of the bacterial surface, strains were classified into three categories. Isolates with a HPBI greater than 70% was arbitrarily classified as highly hydrophobic. Isolates with HPBI between 50 and 70 classified as moderate and isolates with HPBI lower than 50 classified as low hydrophobic.

**Antimicrobial susceptibility test:** The antimicrobial susceptibility test of the isolated strains to antimicrobial agent was determined by using the agar plate method according to the agar overlay diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS, 1997). All isolates were screened for their susceptibility to Trimethoprim (SXT) 25 µg, Tetracycline (TE) 30 µg, Penicillin (P) 6 µg, Amoxicillin (AMC) 20/10 µg, Erythromycin (E) 15 µg, Chloramphenicol (C) 30 µg and Gentamicin (GM) 10 µg. Susceptibility testing was studied in triplicates. MRS agar was used as a basal medium for bacterial growth. LAB isolates were adjusted absorbance to 0.08-0.1 at 625 nm and spread on the surface of MRS agar by three way swabs. Seven standard antibiotic discs (Oxoid) were placed on the inoculated plates with sterile conditions and incubated

anaerobically at 30°C for 24 h. The diameter of the inhibition zone was measured. Breakpoints for the interpretation of inhibition zone were those defined by expressed in terms of resistance (R), moderate susceptibility (MS) and susceptibility (S).

**Identification of lactic acid bacteria using API 50CHL system:** Each sample was inoculated in MRS broth tubes. Then, carbohydrate fermentation patterns of each LAB isolates were determined using API 50 CH (bioMerieux, France) for final identification of isolated species. The experiment was performed at 30°C. The results of biochemical test and carbohydrate fermentation were determined after 24 and 48 h. The strains were identified using the APILAB Plus software version 3.3.3 from bioMerieux.

## RESULTS

A total of 499 microaerophilic colonies were isolated from 93 various samples on MRS agar and cultured under microaerophilic conditions. All isolates were primary observing their colony morphology as well as some biochemical characteristics. Microscopically, we observed well-defined gram-positive bacilli and coccobacilli, which were distributed either in groups or individually. Biochemical characteristics have shown non-motile, catalase negative, oxidase-negative and non-spore forming. The number of LAB in traditional Thai fermented food (91 samples) and kefir (2 samples) was in the range of $1.2 \times 10^4$ to = $3.0 \times 10^6$ CFU/g. By total LAB, samples from *mam*, *nham*, *pla-som* and *sai-krog-prieo* exhibited the largest LAB count, while samples from *bai maeng* have shown a small number of LAB count (Table 1). Moreover, a sample from *phu-dong* have 6 isolates which shown antimicrobial activity.

**Spectrum of inhibitory activity:** The spot on lawn technique was used to assess the production of antimicrobial compounds initially screened for antagonistic activity against 20 of important pathogenic bacteria for gastrointestinal and urogenital pathogenic bacteria. The inhibitory spectrum of the LAB produced by selected strains is presented in Table 2. Thirty one (6.21%) of 499 LAB isolates was able to inhibit growth of at least one of the 20 indicator strains. The inhibitory zone varied from 4 to 10 mm. The selected LAB strains showed a relatively wide inhibition spectrum, inhibiting the growth of a number of intestinal and urogenital pathogenic bacterial both Gram-positive and Gram-negative bacteria including species of the genera Shigella, Salmonella, Staphylococcus, Escherichia, Pseudomonas and Klebsiella. This is probably because LAB could produce various antimicrobial compounds. Holzapfel *et al.* (1995) mentioned that LAB can produce various metabolic products with antimicrobial properties (Holzapfel *et al.*, 1995). Isolate FI 1/5 and Fsh 2/3

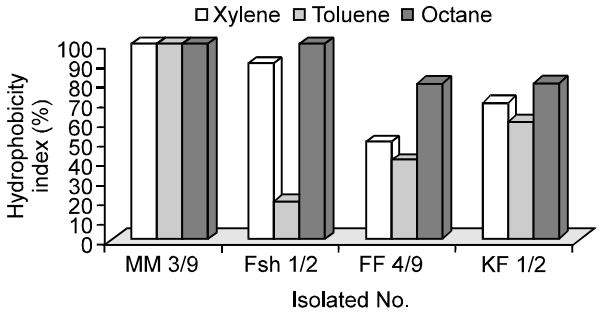


Fig. 1: Cell surface hydrophobicity of the selected LAB isolates

showed the largest antimicrobial spectrum, exhibiting inhibitory activity against 5 pathogens. Interestingly, effective LAB which we choose showed most inhibitory to *S. flexneri* (5 isolates) *S. bondii* (5 isolates), *E. agglomerans* (4 isolates) and *C. freundii* (4 isolates), respectively (data not showed). Therefore 31 isolates were subject to further evaluated probiotic properties.

**Determination the inhibitory activity of cell free supernatants by agar diffusion method:** The inhibitory activity of cell free supernatant was present on 3 selected isolates from 31 isolates. There were KF 1/3, MM 3/9 and FF 4/9 which showed antimicrobial activity when calculated inhibitor concentration as 6,000, 500 and 1,400 AU/ml to *Citrobacter frundii*, *Vibrio cholarae* and *Streptococcus agalactiae*, respectively (Table 3).

**Selection of various temperature, sodium chloride, acid, bile salt tolerance and hemolysis test:** From the study of various temperature, sodium chloride, acid and bile salt tolerance isolates, it was shown that on 0, 3, 6 and 24 h observation, all 31 LAB isolates (100%) were capable of growing at 25, 37 and 45°C. The similar results were present on the study of various sodium chloride and bile salt tolerance. It was revealed that all 31 LAB isolates (100%) were able to grow at wide range of sodium chloride concentration (4, 8 and 10% w/v) and various concentrations of bile salts (1.5, 3 and 4.5% w/v) at interested time point. However, the result of acid tolerance found that, many LAB isolates (74.2%) were capable of growing at pH 3 in MRS broth adjusted pH with hydrochloric acid, only 41.9% of LAB isolates could grow at pH 2. Whereas, LAB isolates could not grow in MRS broth adjusted at pH 1. The hemolysis test was shown that, most of LAB isolates (96.8%) were not produced hemolysis on blood agar. However, only 1 isolate (MI 3/3) was produced γ-hemolysis (data not showed). Therefore, the selected LAB isolates with probiotic properties used in this study may be used as starter culture in fermented food (Table 4).

**Hydrophobic characteristics:** Among the selected LAB isolate, only 1 LAB isolates, namely, MM 3/9, showed

Table 1: Isolation of bacteriocin-producing LAB from various souces

| Sources | No.of samples | Total LAB count | No.of isolates | No.of bacteriocin producing LAB | Code | Sources | No.of samples | Total LAB count | No.of isolates | No.of bacteriocin producing LAB | Code |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Sai-krog prieo* | 10 | $> 3 \times 10^8$ | 30 | 1 | SS | *Tai pha* | 4 | $3.4 \times 10^6$ | 34 | 0 | FK |
| *Mam* | 5 | $> 3 \times 10^8$ | 30 | 4 | MM | *Hoi dong* | 6 | $3.8 \times 10^6$ | 38 | 0 | PS |
| *Nham* | 10 | $> 3 \times 10^8$ | 30 | 0 | FP | *Toa hu yee* | 3 | $1.7 \times 10^4$ | 17 | 0 | FC |
| *Kung-chom* | 5 | $4.6 \times 10^6$ | 46 | 3 | Fsh | *Toa jeaw* | 4 | $2.4 \times 10^4$ | 24 | 3 | FB |
| *Pla-som* | 5 | $> 3 \times 10^8$ | 30 | 4 | FS | *Phakgard-dong* | 10 | $2.8 \times 10^6$ | 28 | 0 | FV |
| *Som-fak* | 2 | $1.6 \times 10^6$ | 16 | 1 | PA | *Bai maeng* | 4 | $1.2 \times 10^4$ | 12 | 2 | MI |
| *Pla-ra* | 5 | $1.9 \times 10^5$ | 19 | 0 | FF | *Mamuang-dong* | 8 | $2.2 \times 10^5$ | 22 | 0 | MA |
| *Phu-dong* | 7 | $5.5 \times 10^6$ | 55 | 6 | CR | Kefir | 2 | $8 \times 10^7$ | 25 | 3 | KF |
| *Sai pra mun* | 3 | $4.3 \times 10^5$ | 43 | 4 | FI | Total | 93 | | 499 | 31 | |

Table 2: Antimicrobial activity by spot on lawn technique

| | | | | | Indicator pathogen (inhibition zone diameter in mm) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Urogenital pathogenic bacteria | | | | | | | | Gastrointestinal pathogenic bacteria | | | | | | | | | | | |
| Code | *C.F* | *E.A* | *S.R* | *S.M* | *S.S* | *S.A* | *K.P* | *P.A* | *P.M* | *P.R* | *A.H* | *E.T* | *E.C* | *S.T* | *S.P.A* | *S.F* | *S.B* | *S.A* | *V.C* | *Y.E* | Total |
| MM 3/9 | | | | | | 4.5 | | | | | | | | | | | | | 10 | | 2 |
| FSh 2/3 | 4 | | | | | 4 | | 6 | | | | | | | 7.5 | | | | 6 | | 5 |
| KF 1/3 | 6 | | | | | | | | | | | | | | | | | | | | 1 |
| FF 4/9 | | 4 | | | | 4 | | | | | | | | | | | | | | | 2 |

*CF: C. freundii* *EA: E. agglomerans* *SR: S. rubidaea* *SM: S. marcescens* *SS: S. saprophyticus* *SA: S. agalactiae* *KP: K. pneumoniae* *PA: P. aeruginosa*
*PM: P. mirabilis* *PR: P. reftgeriA. hydrophila* *ET: E. tarda* *EC: E. coli* *ST: S. typhi* *SP: S. paratyphi A* *SF: S. flexneri* *SB: S. boydii*
*SA: S. aureus* *VC: V. cholerae* *YE: Y. enterocolitica*

hydrophobicity value as 100% with Xylene, Toluene and Octane. The high to medium degree of hydrophobicity with Xylene (hydrophobicity index 100-50%) was observed in Fsh2/3, FF 4/9 and KF 1/3 (90, 50 and 70%, respectively). Similar result was observed on degree of hydrophobicity with Octane. The high to medium degree of hydrophobicity with Octane (hydrophobicity index 100-50%) was observed in Fsh2/3, FF 4/9 and KF 1/3 (100, 80 and 80%, respectively). However, the high to low degree of hydrophobicity with Toluene (hydrophobicity index 100-20%) was shown in Fsh2/3, FF 4/9 and KF 1/3 (20, 40 and 60%, respectively) (Fig. 1).

**Antimicrobial profile:** Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes (Florez *et al*., 2005). Our results were agreed with this observer. Since the MIC values of 7 tested antibiotics (Table 5), it was found that selected LAB strains were resistant to penicillin and amoxicillin. Most of the selected strains were susceptible to chloramphenicol.

**Identification of lactic acid bacteria using API 50CHL system:** Four effective isolates from above experiment were selected for further study. Carbohydrate fermentation patterns of these isolates were tested using API50 CHL kit. Based on carbohydrate fermentation pattern analysis, Initial identifications made by API database correlation indicated that 2 isolates (MM 3/9 and FSh 2/3) were identified to *Lactobacillus plantarum*. Isolate FF 4/9 and KF 1/3 were identified to *Lactobacillus pentosus* and *Lactobacillus brevis*, respectively.

## DISCUSSION

Consumption of food with live probiotic bacteria may mediate various health effects such as regulation of intestinal microbial homeostasis to the modulation of immune responses, decrease of cholesterol and cancer risk, improvement of the clinical outcome in many intestinal disease targets and improvement of immune and mucosal barrier function (Salminen *et al*., 1999). In the last two decades, research in the probiotic area has achieved major progresses in the selection and characterization of specific probiotic cultures and confirmed the health benefits associated with them. Traditionally, fermented foods are the main source of probiotics. Of the 499 isolates of LAB obtained from Thai traditional fermented food and kefir, 31 isolates produced antimicrobial substances which inhibited the growth of at least two of twenty of important pathogenic bacteria for gastrointestinal and urogenital pathogenic bacteria by varying degrees. The main revelation in this study was that strains were active both against gram positive and gram negative bacteria. Most LAB isolates showed wide inhibition zone to *S. boydii*,

Table 3: Inhibitory activity of Cell free supernatants by agar diffusion method

| Code | Indicator pathogen | Dilution | Inhibition zone diameter in mm | Inhibitor concentration (AU/ml) |
|---|---|---|---|---|
| KF 1/3 | *Citrobacter frundii* | 1:64 | 6 | 6,000 |
| MM 3/9 | *Vibrio cholarae* | 1:8 | 5 | 500 |
| FF 4/9 | *Streptococcus agalatiae* | 1.16 | 4 | 1,400 |

*E. agglomerans* and *S. saprophyticus*, respectively. LAB strains able to produce the best antibacterial compounds were isolated from *kung-chom*. However, when evaluated the inhibitory activity of cell free supernatant by agar diffusion method, only 3 isolated; KF 1/3, MM 3/9 and FF 4/9 can produced inhibition zone to *C. frundii*, *V. cholarae* and *S. agalactiae*, respectively. Inhibitor concentration was correlated with the width of inhibition zone by spot on lawn technique which use to screen antimicrobial activity. We suggest that the antimicrobial activity of LAB is mainly due to organic acids; such as lactic acid, formic acid and acetic acids, reuterin, proteinaceous compounds, cyclic dipeptides, ethanol, acetone, hydrogen peroxide, diacetyl and bacteriocins (Delgado *et al*., 2001; Oliveira *et al*., 2008). This finding was similar to previous reported that LAB from Thai traditional fermented food and freshwater fish have an antimicrobial activity against pathogenic bacteria (Siripornadulsil *et al*., 2014).

The growth rate at different temperatures are limiting factors for the persistence and competitiveness of the starter culture over the entire fermentation and ripening process. Also NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. The current interested isolates revealed that there are wide ranges of different temperatures. Since all 4 LAB grew at wide range of temperatures (25 to 45°C) and NaCl concentration (4-10% w/v) for 3 to 24 h.

High tolerance to acid and bile helps probiotic bacteria survive the harsh physical-chemical conditions of GI tract and thus, is a prerequisite for bacteria to be used as probiotics. Before reaching intestinal tract, it must first survive during the transit through the stomach where the pH can be as low as 1.5 to 3.0 (Dunne *et al*., 2001) and remain viable for 4 h or more (Ouwehand *et al*., 1999). Also in the intestinal tract, the average bile concentration is around 0.3% and may range up to an extreme of 2.0% during the first hour of digestion (Havenaar *et al*., 1992; Gotcheva *et al*., 2002). All 4 interested LAB were tolerance to the strong acid condition at pH 2 up to 3 and also tolerance to 0.5-1.5% bile acid for 3 to 24 h. In addition, most of them showed non-hemolysis on blood agar. Altogether most of them were resistant only to penicillin, amoxicillin and gentamicin, however and exhibited sensitivity to chloramphenicol. This was possibly due to widely use of antibiotics in veterinary medicine and agriculture which could be contributing to the dissemination of resistances. By the results of hydrophobic property, which was good probiotic bacteria should be attached to organic solvent. Our results showed that the interested LAB isolates revealed various degree of hydrophobicity.

Four effective isolates from above experiment were selected for further study. All isolates were gram positive, nonmotile, nonspore forming and not catalase producing. The isolate FSh 2/3 can inhibit 5 tested pathogenic bacteria whereas the isolate MM 3/9 FF 4/9 and KF 1/3 have the ability to inhibit tested pathogenic bacteria even CSF dilution. All isolates fulfilled the probiotic properties such as temperature, NaCl, acid and bile tolerance, none hemolysis and broad range antibiotic resistant. By biochemical analysis, isolate FF 4/9 and KF 1/3 were identified to *Lactobacillus pentosus* and *Lactobacillus brevis*, respectively. The isolates MM 3/9 and FSh 2/3 were determined to be belonged to *Lactobacillus plantarum*. The species *L. pentosus* isolated from plants has proven to produce the highest bacteriocin titers in environmental conditions (Delgado *et al*., 2005), which suggest its technological interest as a starter culture. *L. plantarum* has been isolated from different ecological niches, such as cereal products, fruits, meat, fish, vegetables, milk, many fermented food products as well as anaerobic plant matter. It has been used as a starter culture in various food fermentation processes, contributing to improve food quality and sensory properties, like flavor, consistency and texture. *L. brevis* can found in the fermented foods and as normal microflora. It is the most widely LAB species used in many fermentation system. This species have probiotic benefits because of their ability to tolerate low pH, bile acids and have antimicrobial activity against potentially harmful organism (Ogunbanwo *et al*., 2003). Since *L. brevis* found in study were isolate from kefir which is one the famous variety of probiotic organisms. Previous study found that certain bacteria in the kefir culture shown to be effective antimicrobial and anti-inflammatory agents for improved wound healing (Rodrigues *et al*., 2005). And some *Lactobacillus* spp., an important group from the LAB, is usually found as commensal bacteria and is commonly used as probiotics in humans and animals (Ouwehand *et al*., 2002).

Although more *in vitro*, *in vivo* or clinical data would be needed before any conclusion on the probiotic properties of the strains can be drawn, our results demonstrate that some of the tested strains that were isolated from various Thai traditional fermented food and kefir may have good probiotic potential for their inclusion in products targeting commercial used. As cultures originating from Thai traditional fermented food and kefir itself are better adapted to the ecology of their fermentation, those LABS with potential probiotic properties and antimicrobial activity in this study could be further developed as the probiotic starter culture or

Table 4: Probiotic properies of the selected LAB isolates

| | | Temp. (°C) | | | NaCl % (w/v) | | | | | | | | pH | | | | | | | | | | Bile salt % (w/v) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 4 (time h) | | | 8 (time h) | | | 10 (time h) | | | 1 (time h) | | | 2 (time h) | | | 3 (time h) | | | 1.5 (time h) | | | 3 (time h) | | | 4.5 (time h) | | |
| Code | H | 25 | 37 | 45 | 0 | 3 | 6 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 | 0 | 3 | 6 | 24 |
| MM 3/9 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Fsh 2/3 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| KF 1/3 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| FF 4/9 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

H: Hemolysis test, Temp: Temperatures

Table 5: Antimicrobial sensitivity of the selected LAB isolates

| | Antibiotics | | | | | | |
|---|---|---|---|---|---|---|---|
| Code | Amik. (30 µg) | Tetr. (30 µg) | Pen. (10 µg) | Amox. (30 µg) | Erythr. (15 µg) | Gent. (10 µg) | Cipr. (5 µg) |
| MM3/9 | S | R | R | R | R | S | S |
| FSh2/3 | R | R | R | R | R | I | S |
| KF1/3 | R | S | R | R | S | R | R |
| FF 4/9 | R | R | R | R | R | I | S |

Amik: Amikacin, Tetr: Tetracycline, Pen: Penicillin, Amox: Amoxicillin, Erythr: Erythromycin, Gent: Gentamicin, Cipr: Ciprofloxacin

protective cultures to provide significant health benefits and contribute to enhance the hygienic quality of the products.

**Conclusions:** A total of 499 LAB isolates were isolated and selected from various traditional Thai fermented foods and kefir. Of the 31 antimicrobial producing isolates, 4 isolates displayed best antimicrobial activity against important gastrointestinal and urogenital pathogenic bacteria. All selected isolates showed the most potential probiotic properties. They are tolerant to heat, acid, bile salt, NaCl, non RBC hemolysis, broad range antibiotic resistant and adhesion property to organic solvent suggesting the ability to survive through the stringent conditions in the stomach and small intestine. Based on antimicrobial activity and probiotic properties of selected LAB, these species could be further developed as the good candidates for potential application as probiotic or protective cultures and may be advantageous to the producing strains for their establishment and competition in the gastrointestinal and urogenital tract.

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