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NOVEL *RUMMELIIBACILLUS* SP. ISOLATED FROM FERMENTED VEGETABLE PRODUCTS AS THE POTENTIAL PROBIOTICS

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ABSTRACT

The novel lactic acid bacteria has potential probiotics which were selectively screened from different fermented vegetable products by antagonistic activity against *Escherichia coli* ATCC 25922 as a food-borne pathogenic bacteria. From 40 isolates on MRS agar, some isolates had anti-*E. coli* activity. Surprisingly, isolated STR 0103f and STR 0404f strains demonstrated bile salt-tolerance at 0.3% bile salt for 24 hours with survival rate of 67.77±0.38 and 68.72±0.45%, respectively. Both strains also survived under acidic conditions (pH 3-5) with 60.53±0.08-76.87±0.09% of survival rate. STR 0103f and STR 0404f strains elicited the broad spectrum against both Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311 and *Salmonella paratyphi* A) and Gram-positive (*Staphylococcus aureus* ATCC 25923) pathogenic strains which appeared inhibition zone of 7.33±0.58-8.33±0.58 mm and revealed a susceptibility to various antibiotics. Moreover, two selected strains were also able to adhere to stainless steel plates at least 30% of adhesion rate and without any detectable hemolytic activity. Morphological, biochemical characteristics and 16S rRNA gene sequences were used as a tool for bacterial identification. Both STR 0103f and STR 0404f isolates were identified as *Rummeliibacillus* sp. which are *Rummeliibacillus pycnus* STR 0103f and *Rummeliibacillus stabekisii* STR 0404f, respectively. Thus, *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f would be the novel bacterial strains from fermented vegetable products that act as the potential probiotic candidates for safety food bio-preservation.

Keywords: Antimicrobial activity, *Rummeliibacillus pycnus*, *Rummeliibacillus stabekisii*, Lactic acid bacteria, Fermented vegetables, acid- and bile salt-tolerance, Probiotic properties

INTRODUCTION

Thailand is an agricultural country that produces fermented foods from various sources such as seafood, vegetables, fruits and cereals. Fermented vegetables or pickles are seasonal home-made indigenous product. The major of pickled vegetables are onion, radish, beans, bamboo shoots, cucumber, and garlic. Lactic acid bacteria (LAB) play a pivotal role in the fermentation. These products results in fermentation. LAB strains are also important in foods that act as potential probiotics by producing antimicrobial compounds such as lactic acid, bacteriocin and other organic acids. Their role is to improve shelf life and flavor of the fermented food products **(Perez *et al.*, 2014)**. Probiotic strains including *Lactobacillus* sp. and *Bifidobacterium* sp. have been isolated from traditionally fermented foods and drinks. Probiotics are added to starter culture for large-scale production of daily products as a functional supplement for improving gut health, antimicrobial activity and reducing lactose intolerance **(Perez *et al.*, 2014; Kimura & Yokoyama, 2018)**. Yet, there are some concerns of potential health-associated probiotics. The potential probiotics should survive under acidic conditions and bile salts in the mammalian gastrointestinal tract. Epithelial attachment is a probiotic characteristic that allows them to colonize the human intestine to prevent the invasion of pathogenic microorganisms **(Piano *et al.*, 2006)**. Therefore, acid and bile salt resistance tests should be a primary screening method to identify potential probiotics from various bacterial strains **(Guo *et al.*, 2009)**. The antagonistic activity of intestinal pathogens is also an important functional requirement of probiotics. Furthermore, hydrophobicity of bacterial cell surface indicates the nonspecific interaction that depended on physical and chemical characteristics of the bacterial cell surface. *In vitro* models have been thus established in many studies using mammalian epithelial cells to determine the adhesion of probiotics to the gastrointestinal tract. **(Kos *et al.*, 2003; Klingberg *et al.*, 2005; Han *et al.*, 2017)**. Recently, *Bacillus* is an interesting genus due to its ability to produce inhibitory substances such as bacteriocins (subtilin and ericin S) and peptide antibiotics (bacilysin, surfactin) that represented different basic chemical structures **(Bizani *et al.*, 2005; Abriouel *et al.*, 2011; Nithya and Halami, 2012; Dimkic *et al.*, 2017)**. Furthermore, many tremendous researches have been undertaken on probiotic and lactic acid bacteria (LAB) of the *Bacillus* spp. from food and other sources. *B. amyloliquefaciens*, *B. coagulans*, *B. licheniformis*, *B. safensis* and *B. subtilis* are Generally Recognized As Safe (GRAS) in food industry and agriculture **(Nyangale *et al.*, 2014; Shobharani *et al.*, 2015; Elshaghabee *et al.*, 2017)**. The morphology of *Bacillus* genus are Gram-positive bacilli, aerobic and facultative anaerobic and ubiquitous in nature. *Bacillus* strains produce endospores to survive extreme environmental conditions including in food processing. Species of *Bacillus* are also presented in several fermented food products, such as cured sausages, cheeses, traditional fermented milks, sourdough, *etc* **(Manhar *et al.*, 2016; Kimura & Yokoyama, 2018)**. The relationship of these bacteria to contamination during processing and natural contamination of raw materials was also found. **(Magnusson *et al.*, 2007; Eglezos *et al.*, 2010; Kimura and Yokoyama, 2019)**. Moreover, the *Bacillus* spp. collaborates with other microorganisms by releasing metabolites including antimicrobial substances and enzymes (amylases, lipases and proteases) during fermentation for bioactive activities **(Chantawannakul *et al.*, 2002; Ghani, 2013)**. The genus *Rummeliibacillus* is a member of *Bacillaceae* family. Both of *Rummeliibacillus stabekisii* and *Rummeliibacillus pycnus* as members of this genus that have been isolated from soil **(Vaishampayan *et al.*, 2009)**. The *Rummeliibacillus pycnus* was reclassified from *Bacillus pycnus* due to closely relation on the basis of polyphasic taxonomy **(Nakamura *et al.*, 2002; Vaishampayan *et al.*, 2009)**. *Rummeliibacillus stabekisii* and *Rummeliibacillus pycnus* are Gram-positive rods, aerobic and round-spore-forming bacteria. These species were isolated from different geographical locations **(Nakamura *et al.*, 2002; da Mota *et al.*, 2006; Vaishampayan *et al.*, 2009)**. There are only a limited number of studies investigating *R. stabekisii* and *R. pycnus* and even fewer investigating their probiotic properties. Therefore, the aims of the current work were to isolate lactic acid bacteria (LAB) from various fermented foods with antimicrobial properties. The isolated strains were identified by 16S rRNA sequencing analysis. Probiotic properties of candidate strains were evaluated according to survival in a low pH environment, tolerance to bile salts, adhesion ability, antibiotic resistance, hemolytic activity and antagonistic activity towards pathogenic bacteria for using as food bio-preservatives.

MATERIAL AND METHODS

Samples for isolation of bacterial strains

The four fermented vegetable products including; pickled cabbage (01), canned pickled cabbage (02), kimchi (03) and pickled bamboo shoots (04) were randomly collected from local markets of Bangkok and Nonthaburi province, Thailand.

Isolation of potential probiotic strains

Isolation of bacterial stains from the selected samples was screened by using the microbial pour-plating method. Ten grams of each sample were aseptically transferred into 90 ml of saline (0.85% NaCl) solution. Homogenized samples were serially diluted in saline solution, respectively. Appropriate dilutions (10^{-6}-10^{-7}) were inoculated on de Man, Rogosa and Sharpe (MRS) agar and Tryptic soy agar (TSA). All plates were then incubated at 37ºC for 24-48 hours. Different morphology colonies were picked up and inoculated in MRS and TSA agar and purified their selected isolated by re-streaking on agar plates until single type appearance. These cultures were preserved at 5°C on MRS and TSA agar plate for further studies. The pure strains were stored in broth containing 30% sterile glycerol at -20°C.

Preliminary Screening for Probiotic Properties by Antimicrobial activity test

The preliminary anti-*E. coli* activity of pure isolated strains was tested by agar well diffusion method as previously described by **Tinrat *et al*. (2018)**. The overnight culture of *Escherichia coli* ATCC 25922 as pathogenic strain was prepared in TSB broth at 37°C for 18 hours and then was adjusted to bacterial cell concentration about 1.0 x 10^{8-9} CFU/mL (OD_{600} = 0.5). Next, an aliquot of *E. coli* culture was mixed into TSA soft agar (0.75%) and poured on lower layer of TSA agar (1.5%). Finally, supernatants of isolated strains (80 µL; overnight cultures in MRS or TSB broth) were added into the punched wells (∅ = 5 mm by cork borer). The inhibition zones (IZ) around the wells were evaluated in millimeters (mm) after 24-48 hours of incubation at 37ºC. The IZ of ≥ 1 mm was considered as a positive inhibition **(Zhang *et al*., 2016)**

In *Vitro* Characterization of Probiotic Properties

The common methods for in *vitro* analysis of probiotic properties include tolerance to low pH and bile salt, antibiotic susceptibility, hemolytic activity, antimicrobial activity and bacterial adherence to stain steel plates.

Acid tolerance patterns test

Simulated gastric juice (3 g/L pepsin, 7 mM KCl, 45 mM $NaHCO_3$, and 125 mM NaCl) was prepared and adjusted to pH values of 2.0, 3.0 and 5.0 by using 1M HCl and 1M NaOH for creating the gastric environment. Each of the selected cultures (10^{8-9} CFU/mL as initial concentration) was centrifuged at 5,000 rpm for 15 min at 4°C. Cell pellets were washed twice in the phosphate buffered saline (PBS; pH 7.2) and resuspended in sterile MRS broth under simulated gastric juice at pH of 2.0, 3.0 and 5.0 compared with pH 7.0 as control **(Hassanzadazar *et al*., 2012; Tinrat *et al*., 2018)**. 1 mL of 9 log CFU/mL of each overnight culture was inoculated in to simulated gastric juice (10 mL; log 8 CFU/mL as an initial inoculum concentration) at 2.0, 3.0, 5.0 (assay) and 7.0 (control). After incubation at 37°C, growth of tested cultures were monitored at 0, 3, 6 and 24 hours of incubation by plate count method and compared with the starting bacterial concentration. The grown isolated colonies were expressed as colony-forming units per milliliter (CFU/mL). The percentage of survival rate of isolated strains was determined by the plate count method compared with the initial bacterial concentration:

Survival rate (%) = [(log CFU*assay* / log CFU*control*) x 100

Bile salt tolerance patterns test

The selected strains was determined the bile salt tolerance under 0.00 (control), 0.15, 0.30 and 1.0% (assay) of oxagall bile salt at pH 8.0. 1 mL of cell suspensions of selected strains (10^{8-9} CFU/mL) was resuspended in the broth supplemented with different oxagall bile salt concentrations. After 24 hours of incubation at 37°C, growth of tested cultures were monitored at 0, 3, 6 and 24 hours after incubation by plate count method and compared with the starting bacterial concentration **(Hassanzadazar *et al*., 2012; Tinrat *et al*., 2018)**. Isolated strains counts were expressed in colony-forming units per milliliter (CFU/mL). The percentage of survival rate was determined by the plate count method compared with the initial bacterial concentration:

Survival rate (%) = [(log CFU*assay*/ log CFU*control*) x 100

Assessment of antimicrobial activity

Bacterial strains and growth condition

The antimicrobial activity of all isolated strains was tested against eight pathogens as the indicator strains including; Gram-Negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311 and *Salmonella paratyphi* A) and Gram-positive bacteria (*Bacillus cereus* DMST 5040, *Bacillus subtilis* and *Staphylococcus aureus* ATCC 25923). The bacterial strains were obtained from the laboratory of the Department of Biotechnology, King Mongkut's University of Technology North Bangkok, Thailand. All the tested strains were maintained on TSB agar (Difco) at 37ºC for 18-24 hours prior to use. For preparation of cell-free supernatants, all isolated strains were incubated in MRS broth for 24-48 hours at 37°C. Bacterial cells were removed by centrifugation at 10,000xg for 20 min at 4°C. The supernatants were sterilized by filtration through sterile syringe filter (Millipore, 0.22 µm) and stored at 4°C.

Antimicrobial activity by agar well diffusion method

In vitro antagonistic activity of isolated strains was evaluated by agar well diffusion method against eight pathogenic strains as previously described by **Tinrat (2015)**. The overnight cultures of pathogenic strains were adjusted the OD_{600} to 0.5 (10^{8-9} CFU/mL) with spectrophotometer. The indicator strains were mixed into 0.75% TSA and poured on the surface of the basal layer of TSA (1.5% agar). 5 mm-diameter wells were drilled into agar surface using a sterilized cork-borer after solidification of the agar. Approximately 80-100 µL of cell-free supernatants from isolated strains was added in each well. Finally, the inhibition zones (IZs) around the well were measured and reported the diameter in millimeters±SD after incubation at 37°C for 18-24 hours. A positive control was penicillin (10 µg).

Antibiotic Susceptibility Tests

The antibiotic susceptibility test was evaluated by agar diffusion method with ten antibiotics, including penicillin, ampicillin, bacitracin, gentamicin, streptomycin, erythromycin, chloramphenicol amikacin, nalidixic acid and norfloxacin. Then, the overnight cultures of the selected strains (OD_{600} = 0.5; 10^{8-9} CFU/mL) were swabbed on the surface of MRS agar (**Tinrat and Singhapol, 2021)**. After dying, the antibiotic discs were placed on the surface of agar plates before incubating at 37°C for 24-48 hours. Antibiotic susceptibility of testes strains was considered by the average of diameters of inhibition zones (millimeters±SD) around antibiotic discs **(NCCLS, 2002)**.

Bacterial adhesion assay

The bacterial adhesion assay of isolated strain was assessed on stainless steel plates with slight modifications from protocol of **El-Jeni *et al*. (2015)**. Briefly, the 500 µL of overnight bacterial culture was deposited in a tested tube containing sterile stainless steel plate and then filled with 450 µL of MRS. After incubating for 24 hours at 37°C, the stainless steel plate was removed under aseptic conditions and washed with 10 mL of 1% peptone water (sterile) and left for 5 min in tube. The stainless steel plate was then washed in the same conditions and vortexed for 2-3 min in 6.0 mL of sterile 1% peptone water tube consecutively to separate the bacterial cells from surface of the steel plate. Finally, the survival cell was determined by plate counting method on MRS agar after 24 hours incubation at 37°C. The percentage of adhered bacterial cells for each isolates was calculated by comparing with total initial survival cells.

Phenotypic characterization of isolated strains

Observations of colony morphology and Gram staining from different pure cultures were done after getting a pure culture in a petri dish by streak plate technique at 37°C for 24 hours. The bacterial colonies were evaluated for size, sharp, pigmentation, form, margin, elevation and texture under standard microbiology methods and protocols. The cultures in MRS broth were studied the biochemical tests which included fermentation of different carbon sources, acid and gas production from glucose, catalase test and growth at different temperatures (25°C, 37°C, 45°C and 55°C). For hemolytic activity, the selected strains were subsequently cultured on Columbia blood agar plates containing 5% (v/v) sheep blood and incubated for 24-48 hours at 37ºC. The results were detected by appearance of halo around the colony (α-hemolysis: green zone, β-hemolysis: clear zone and γ-haemolysis: no halo) **(Pieniz *et al*., 2014)**.

16S rDNA sequencing and sequence analysis

The universal oligonucleotide primers in this reaction were UFUL (5'-GCC TAA CAC ATGCAA GTC GA-3') and URUL (5'CGT ATT ACC GCGGCT GCT GG-3') (**Tinrat *et al*., 2018**). The thermocycle program was as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 5 min, 55°C for 30 s, and 72°C for 30 s and a final extension step at 72°C for 5 min. After amplification, the bacterial 16S rRNA amplicons

were purified using a QIAquick PCR purification kit (QIAGEN, Hilden,Germany) according to the manufacture's instructions. Then, the 16S rDNA gene was sequenced using a BigDye v.3.1 cycle sequencing kit (Applied Biosystems) with the previous primer sets **(Thompson *et al*., 1997)**. The nucleotide sequencing of 16S rDNA was determined with automated sequencing. Finally, sequence homology was analyzed and aligned using the BLAST and ClustalW program. A phylogenetic tree was constructed using MEGA X with a level of 1000 x bootstrap **(Kumar *et al*., 2018)**.

Statistical analysis

All experiments were carried out in triplicate. The results were analyzed for statistical significance at the 95%confidence level ($p \leq 0.05$) by one-way ANOVA and followed by *t*-tests.

RESULTS AND DISCUSSION

Isolation and Preliminary Screening for Probiotic Properties

Approximately sixty isolated strains from four various types of Thai fermented vegetable products including; pickled cabbage (No. 01), canned pickled cabbage (No. 02), kimchi (No. 03) and pickled bamboo shoots (No. 04) were screened on MRS (40 isolated strains) and TSB medium (40 isolated strains) for antimicrobial activity against pathogenic strains (Table 1).

Table 1 Isolation of Anti- *E. coli* ATCC 25922 from Thai fermented vegetables on different mediums

Thai fermented vegetable products	No. of isolates		No. of anti-*E. coli* ATCC 25922 isolates		Name
	TSB	MRS	TSB	MRS	
Pickled cabbage (01)	10	10	0	2	STR 0103f and STR 0104f
Canned pickled cabbage (02)	11	12	0	4	STR 0202f, STR 0203f, STR 0206f and STR 0208f
Kimchi (03)	7	6	0	2	STR 0302f and STR 0303f
Pickled bamboo shoots (04)	12	12	0	2	STR 0404f, and STR 0405f

All isolated bacterial strains were preliminary screened for antimicrobial activity against *Escherichia coli* ATCC 25922 and tolerance to low pH. A total of 40 isolates on TSB from various types of fermented vegetables product did not show the anti-*Escherichia coli* ATCC 25922. Based on MRS agar screening, ten out of forty isolates (25%) could successfully inhibit *Escherichia coli* ATCC 25922 growth under aerobic condition (Table 1). Moreover, all of them on MRS medium was used to simultaneously evaluated the tolerance to various pH levels. The acid tolerance patterns of isolated strains on MRS medium at different pH values are showed in the Table 2. Out of 40 isolates, 12 isolates (30%), 14 isolates (35%), 15 (37.5%) isolates can survive at pH 2, 3 and 5 respectively, after exposure for 1.5 hours. The survival rate of tested strains was reduced at 2, 3 and 5 after 3.0 and 6.0 hours of incubation, respectively. The STR 0103f, STR 0203f, STR 0302f and STR 0404f isolates were representative of the isolated strains from each fermented products because they were shown the highest antibacterial activity and had the high tolerance of acidity. All the selected strains were further evaluated as a potential probiotic.

In *Vitro* Characterization of Probiotic Properties

Acid tolerance test

The acid and bile tolerance determination have been used as preliminary screening methods to identify potential probiotics from various bacterial strains **(Ding *et al*., 2017)**. Probiotic strains must survive in the gastrointestinal tract at pH 3 or below during the 2-3-hours incubation period of food before passing through small intestinal tract. **(Nath *et al*., 2020)**. The isolated strains were assessed the survival in gastric juices (acid) by culturing in simulated gastric juice at various pH (2, 3, 5 and 7 as control) and the results are showed in the table 2. The percentage survival of 4 isolated strains at different pH levels for creating the gastric environment after 1.5, 3.0, 6.0 and 24.0 hours exposure are shown in the figure 1A. The results showed that the survival rate of the 4 selected strains was ranged from 31.67±0.09 to 76.87±0.09% at different pH values after 24 hours of incubation periods. The STR 0103f isolated strains was significantly presented the highest the survival rate, and followed by STR 0404f and STR 0302f isolated strains in all tested pH levels, respectively ($p \leq 0.05$). Both of STR 0103f and STR 0404f strains were displayed highly tolerant and persistent above 50% for 1.5, 3.0, 6.0 and 24.0 hours under all acidic conditions (Figure 3A). On the other hand, isolated STR 0203f and STR 0302f strains are not able to grow above 50% at pH 2.0 and 3.0 after 24 hours of incubation. Moreover, the survival rate of all selected strains (4 isolates) was reduced after 6 hours of incubation at pH 2.0.

Table 2 Acid tolerance patterns of isolated strains on MRS medium

Acid tolerance patterns		Percentage of surviving isolated bacteria on MRS medium			
		Pickled cabbage (01)	Canned pickled cabbage (02)	Kimchi (03)	Pickled bamboo shoots (04)
1.5 hr	**pH 2**	2 (20%)	5 (41.67%)	3 (50%)	2(16.67%)
	pH 3	3 (30%)	6 (50%)	3 (50%)	2(16.67%)
	pH 5	4 (40%)	6 (50%)	3 (50%)	2(16.67%)
3.0 hr	**pH 2**	2 (20%)	4 (33.33%)	2 (33.33%)	2(16.67%)
	pH 3	2 (20%)	5 (41.67%)	2 (33.33%)	2(16.67%)
	pH 5	2 (20%)	5 (41.67%)	3 (50%)	2(16.67%)
6.0 hr	**pH 2**	2 (20%)	4 (33.33%)	2 (33.33%)	2(16.67%)
	pH 3	2 (20%)	4 (33.33%)	2 (33.33%)	2(16.67%)
	pH 5	2 (20%)	4 (33.33%)	2 (33.33%)	2(16.67%)
Number of bacterial isolates		10	12	6	12

Bile salt tolerance test

All selected strains were determined *in vitro* survival in small intestinal juices by culturing in 0.15, 0.30 and 1.0% bile salt comparing with control without bile salt. The percentage survival of 4 selected strains at different bile salt levels after 1.5-, 3.0-, 6.0- and 24.0-hours exposure are shown in the Figure 1B. The results showed that isolated STR 0404f strains was found the most tolerant to 0.15-1.0% bile salt with 64.48±0.06 - 69.90±0.37% after 24 hours incubation (Figure 1B). 2 out of the 4 selected strains, including STR 0103f and STR 0404f could survive above 60% in the presence of 0.15-1.00% bile salt while survival rates of STR 0203f and STR 0302f isolates were 40.11±0.08 and 38.75±0.19% under this conditions (0.15, 0.30 and 1.00% bile salt), respectively. Additionally, survival in bile of STR 0103f and STR 0404f isolates at a concentration of 0.3% was 71.71±0.15 and 72.84±0.06% and also strongly grow at 1.0% bile salt with 70.85±0.09 and 71.38±0.08 of survival rate for 3 hours, respectively (Figure 1B). The STR 0302f isolate was the most bile salt-sensitive strain in this study ($p \leq 0.05$). In this study, all of the selected strains were able to grow under 0.15-1.0% bile salts pressure, especially isolated STR 0103f and STR 0404f strains showed the longest adaptation time of approximately 6 and 24 hour with survive rate of above 70 and 63%, respectively. In previous studies, several species of lactic acid bacteria strains with different bioactive potentials such as antimicrobial effects have been identified from various types of fermented and pickled vegetables **(Tamang *et al*., 2005; Bao *et al*., 2010; Lee *et al*., 2010; Kumar *et al*., 2012; Zhang *et al*., 2016**) which was consistent with this study. In other words, the present study was aimed to screen acid and bile salt-tolerance strains or lactic acid bacterias (LAB) as the potential probiotics and evaluate antimicrobial properties and antibiotic susceptibility of bacterial isolates from some fermented vegetables.

The isolation of several new *Bacillus* spp. exhibiting potential antibacterial activity from acidic to alkaline food sources have been reported **(Inatsu *et al*., 2006; Nath *et al*., 2015; Silva *et al*., 2018)**. The tolerance of pH and bile salt assays were used as the preliminary screening methods to identify potential probiotics. The optimum concentration of bile salts used in the screening probiotic bacteria for human application is approximately 0.15-0.3% **(Goldin and Gorbach, 1992)**. Selected STR 0103f and STR 0404f strains could be classified as acid and bile tolerant strains. Because they were able to grow in presence of acid (pH = 2-5) and bile salt (0.15, 0.30 and 1.0%) which could give them the potential to resist during their transit through the gastric and small intestine host compartments. Both the selected strains (STR 0103f and STR 0404f) had a high survival rate in 0.3% bile salt conditions of up to 73% and were able to grow well under 1.0% bile salt conditions with 71% rate of survival for 3 hours.

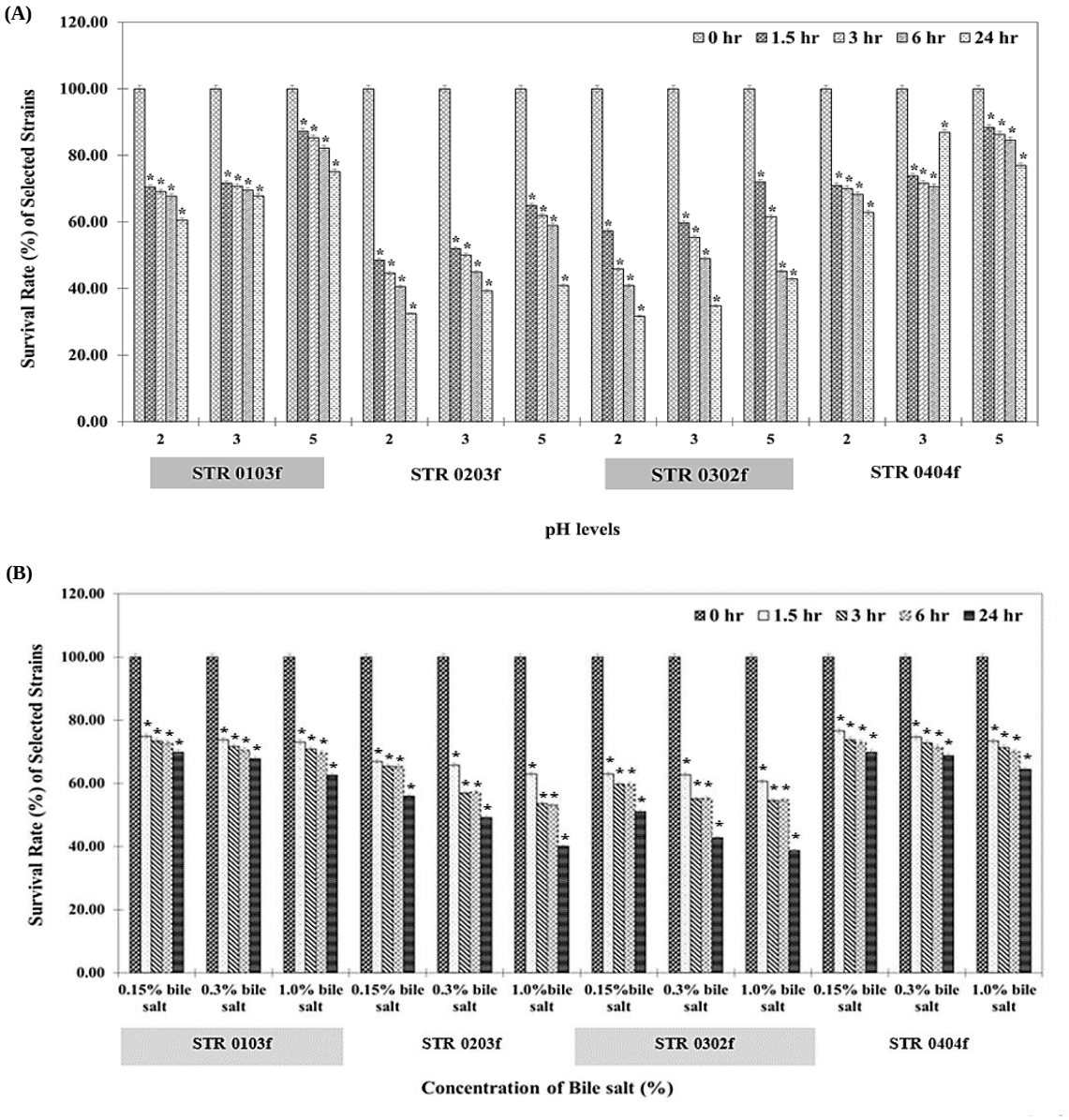

Figure 1 Percentage survival of selected strains at different pH levels (A) and bile salt level (B) (* = p≤0.05 by paired *t*-test; compared to bacterial concentration under without acid and bile salt conditions)

Detection of antimicrobial activity

The importance of probiotics LAB for protecting the host from pathogenic infections is their antibacterial activity. The antimicrobial effect of isolated strain was evaluated by agar well diffusion. The inhibition zones (IZs) results of tested strains against pathogenic strains were showed in table 3. Out of 10 isolates on MRS medium, 4 isolates of STR 0103f, STR 0203f, STR 0302f and STR 0404f were showed the highest antagonistic effect in each of fermented vegetables. All of the selected strains (STR 0103f, STR 0203f, STR 0302f and STR 0404f) were susceptible to *B. cereus* DMST 5040, *B. subtilis* and *K. pneumoniae*. STR 0103f and STR 0404f isolated strains could inhibit the broad spectrum in Gram positive and Gram negative strains. Both of them had antimicrobial effect to 5 out of 8 pathogenic strains. *E. coli* ATCC 25922 (7.33±0.58 - 8.33±0.58 mm) and *S. typhimurium* ATCC 13311 (7.33±0.58 - 8.33±0.58 mm) were highly susceptible to 4 of pure isolates ($p \leq 0.05$). *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were significantly most susceptible to isolated STR 0103f and STR 0404f strains with IZs of 8.66±0.58 mm ($p \leq 0.05$). However, only STR 0103f and STR 0404f isolated strains represented the best inhibitory activity that were selected to assess the susceptibility of antibiotics, identified the morphology/biochemical test and 16sRNA sequencing analysis, respectively.

Table 3 Antimicrobial activity of isolated strains by agar well diffusion

Pathogenic strains		Inhibitory Zone (∅ = 5 mm; mm±SD)				Penicillin (10 µg)
		STR 0103f	STR 0203f	STR 0302f	STR 0404f	
Gram positive strains	***B. cereus* DMST 5040**	R	R	R	R	20.67±0.52
	B. subtilis	R	R	R	R	20.17±0.41
	***S. aureus* ATCC 25923**	8.66 ± 0.58^{aAB}	R	R	8.66 ± 0.58^{aAB}	26.50±1.05
Gram negative strains	***E. coli* ATCC 25922**	8.00 ± 0.00^{abAB}	7.33 ± 0.58^{bA}	7.33 ± 0.58^{bA}	8.33 ± 0.58^{aA}	R
	K. pneumoniae	R	R	R	R	R
	***P. aeruginosa* ATCC 27853**	8.66 ± 0.58^{aA}	R	R	8.66 ± 0.58^{aA}	15.33±4.18
	***S. typhimurium* ATCC 13311**	8.33 ± 0.58^{aB}	7.66 ± 0.58^{aA}	7.33 ± 0.58^{aA}	7.66 ± 0.58^{aAB}	12.83±0.41
	***S. paratyphi* A**	7.33 ± 0.58^{aB}	R	R	7.66 ± 0.58^{aAB}	11.67±0.52

R = Resistant, ^{AB} = Values on the same column with different superscripts were significantly different ($p \leq 0.05$), ^{ab} = Values on the same row with different superscripts were significantly different ($p \leq 0.05$)

Identification of selected strains (*Rummeliibacillus* sp.)

Based on the acid/bile salt tolerance test and antimicrobial activity, 0103f and STR 0404f isolates will be selected to study the characterization and subsequent species identification by 16S rRNA sequencing analysis and then were assessed the in vitro characterization of probiotic properties, respectively. The bacterial strains were studied the biochemical test and identified by using Bergey's Manual of Determinative Bacteriology **(Gibson *et al.*, 1985)**. The phenotypic identifications of STR 0103f and STR 0404f selected strains were shown in Table 4. Both of isolated bacterial strains were *Rummeliibacillus* sp. that were Gram-positive, rod-shaped and endospore-forming. After incubation of 24 hours on MRS agar, colonies of both isolated strains on agar had non-pigmented, opaque, smooth, circular, entire and 0.5 to 1 mm in diameter (data not shown). Both strains was catalase-positive, oxidase-negative, grows in pH of 3-5, aerobic acid production from D-glucose, D-lactose and D-mannitol. Additionally, STR 0103f and STR 0404f could grow in temperature of 25-55°C and provided non-hemolytic activity. The rod-like morphologies under a compound microscope (100X) of *Rummeliibacillus* sp. were shown in Figure 2A-B. The STR 0103f and STR 0404f isolates were the heat-tolerant strains due to high survival rate at 45-55°C (above 6.3log CFU/mL; data not shown). The viability of STR 0103f and STR 0404f also remained stable throughout the 12 and 16 hours of incubation at 15°C (6.21 ± 0.07 and 6.23 ± 0.08 log CFU/mL, respectively) and 45°C (6.49 ± 0.06 and 6.53 ± 0.03 log CFU/mL, respectively) when compared with control (37 °C; 7.78 ± 0.1 CFU/mL at 12 hours of incubation and 7.85 ± 0.06 log CFU/mL at 16 hours of incubation). The different temperature conditions does not significantly affect the viability of the both isolated strains.

Table 4 Phenotypic characteristics and biochemical tests of isolated strains

Character	Observations	
	STR 0103f	**STR 0404f**
Colony morphology / Shape	Circular, white / Short rod	Circular, white / Rod
Gram stain	Positive	Positive
Cell arrangement	Single and Chain	Single and Chain
Spore formation	+	+
Motility test	-	-
Catalase	+	+
Oxidase	-	-
Acid & gas from glucose (Triple sugar iron; TSI)	+/-	+/-
Fermentation		
Glucose	w/+	w/+
Fructose	-	-
Sucrose	-	-
Lactose	w/+	w/+
Mannitol	w/+	w/+
Sorbitol	-	-
Effects of temperature on the growth		
25°C	+	+
37 °C	+	+
45°C	+	+
55°C	+	+
Hemolytic activity	γ-haemolysis	γ-haemolysis

+ = positive; - = negative; w = weakly positive

PCR amplification with UFUL and URUL primers as an universal bacterial primer. Based on nucleotide sequences of 16S rDNA, two selected strains of MRS 0103f and MRS 0404f strains had a nucleotide sequence of 492 bp. The 16S rDNA sequencing analysis indicated that both MRS 0103f and MRS 0404f strains belong to the genus *Rummeliibacillus* sp. by aligning with nucleotide–nucleotide BLAST (blastn) program of the NCBI (http:// www.ncbi.nlm.nih.hov). The selected STR 0103f and STR 0404f strains were grouped most closely with *Rummeliibacillus pycnus* SCTB112 (99% similarity; GenBank accession number JN650277.1) and *Rummeliibacillus stabekisii* B1-37c-21 (100% similarity; GenBank accession number JX517270.1), respectively. Hence, 16S rRNA sequencing analysis showed that STR 0103f and STR 0404f isolated strains were the LAB which were the member of the *Rummeliibacillus* genus, including *Rummeliibacillus pycnus* STR 0103f and *Rummeliibacillus stabekisii* STR 0404f (Figure 2C). *Rummeliibacillus* strain was members of the genus *Bacillus* which gram-positive rod-shaped bacterium, spore-forming, motile, oxidase-negative and catalase-positive. Both the isolated strains had temperature range for growing at 25-55°C (at 37-45°C as an optimum temperature). While the range of temperature for growth of *Rummeliibacillus* gen. nov. were at 28-55°C. The optimum growth temperature of *Rummeliibacillus stabekisii* was at 28-32°C. **(Nakamura *et al*., 2002; Vaishampayan *et al*., 2009)**. It was strictly aerobic, oxidase-negative **(da Mota *et al*., 2016)**. It was previously reported that the characteristics of *Rummeliibacillus pycnus* comb. Nov. (JCM 11075T) and *Rummeliibacillus stabekisii* gen. nov., sp. nov (KSC-SF6gT) was successful in isolating from soil in USA on TSA agar **(Vaishampayan et al., 2009)** but, *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f were screened from fermented vegetables on MRS agar in this study. In addition, the hemolytic activity is needed to assess probiotic and to confirm the infectivity and absence of pathogenicity. In the current study, the *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f were non-hemolytic strains because both strains did not hydrolyze sheep blood on Columbia blood agar supplement with 5% (v/v) sheep blood. Therefore, both of these bacteria are likely safe for using in humans or animals.

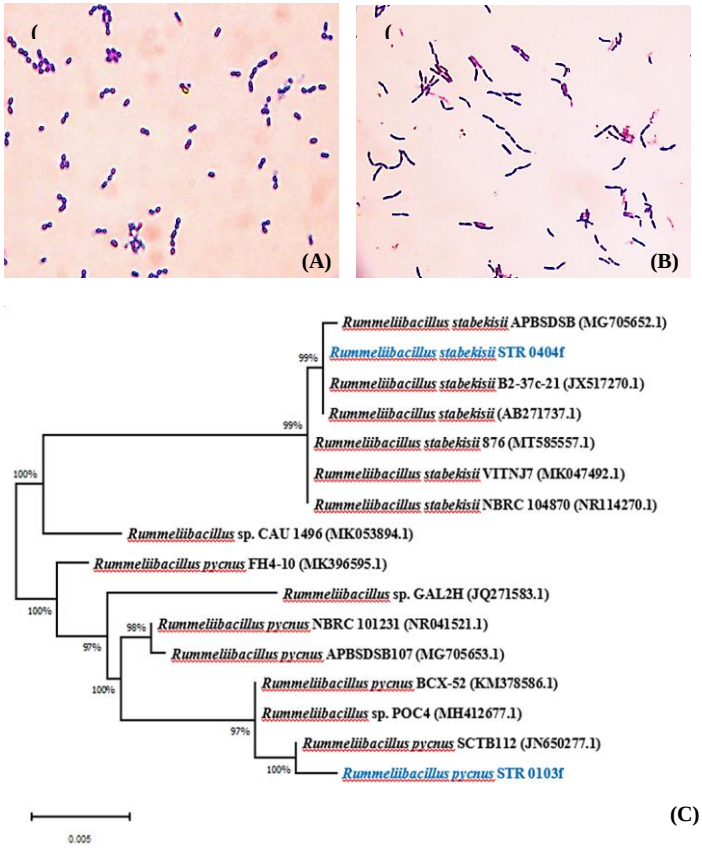

Figure 2 Morphologies of bacteria isolated from fermented vegetables under 100×microscope (Olympus CX21 Microscope, Japan), MRS 0103f (A; short rod); MRS 0404f (B; rod) and Phylogenic tree of *Rummeliibacillus pycnus* STR 0103f and *Rummeliibacillus stabekisii* STR 0404f (C)

Susceptibility of antibiotics test

Although LAB has been obtained the status "generally recognized as safe" (GRAS), these bacterial strains can exchange of antibiotic resistance genes to enhance their survival in antibiotic-containing environments. Importantly, they are also able to transfer antibiotic resistance genes among bacteria of different genera (**Doucet-Populaire et al., 1992; Jacobsen et al., 2007; van Reenen and Dicks 2011**). Hence, non-resistance to antibiotics is one of preliminary stage for the selection of potential probiotic strains. For the safe use of novel isolated probiotic LAB strains, their antibiotic resistance characteristics must be identified in advance. In this study, *Rummeliibacillus pycnus* STR 0103f and *Rummeliibacillus stabekisii* STR 0404f strains was determined the safety evaluation of selected potential probiotic strains by agar disc diffusion with 3 antibiotic groups which were shown in Table 5. Selected STR 0103f and STR 0404f strains were shown susceptibility to various antibiotics by disc diffusion.

Table 5 The susceptibility of antibiotics to the isolated strains

Antibiotic names	Antibiotic content (µg/piece; ∅ = 6 mm)	Inhibition zone (IZ; mm)		Antibiotics pattern	
		R. pycnus STR 0103f	*R. stabekisii* STR 0404f	*R. pycnus* STR 0103f	*R. stabekisii* STR 0404f
Group I Inhibiting cell wall synthesis					
Ampicillin (Am 10)	10	15.63±0.23	16.67±0.28	I	I
Penicillin (P 10)	10	16.00±0.00	19.00±0.00	I	I
Bacitracin (B 10)	10	7.50±0.50	7.50±0.50	R	R
Group II Inhibiting the synthesis protein					
Gentamycin (CN 10)	10	10.67±0.58	12.33±0.58	R	R
Streptomycin (S 10)	10	16.50±0.50	18.50±0.50	I	I
Erythromycin (E 15)	15	22.00±0.00	23.00±0.00	S	S
Chloramphenicol (C 30)	30	28.50±0.50	30.00±0.00	S	S
Amikacin (AK 30)	30	21.00±0.00	22.00±0.00	S	S
Group III Inhibiting the synthesis nucleic acid					
Norfloxacin (NOR 10)	10	31.67±0.58	34.00±0.00	S	S
Nalidixic acid (NA 30)	30	24.56±0.58	27.67±0.58	S	S

R = Resistant (0 mm ≤ DIZ ≤ 15 mm); I = Intermediate (16 mm ≤ DIZ ≤ 20); S = Sensitive (DIZ ≥ 21 mm)

Both selected strains was highly sensitive to norfloxacin and chloramphenicol as revealed by inhibition zone over 30 mm while, *R. stabekisii* STR 0404f was susceptible strains to only norfloxacin. Most interestingly, amikacin, norfloxacin, nalidixic acid, erythromycin and chloramphenicol were able to inhibit growth of both isolated strains in this study (Table 5). These results show that when antibiotic treatment is required, it is likely that the probiotics residing in the colon will be eliminated, therefore, it will be necessary to re-take the probiotics after the end of the treatment period to build up the army of probiotics in colon. However, *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f were resistance to gentamycin and bacitracin. The main safety concern about probiotics is antibiotic resistance since these strains may transfer antibiotic resistance genes to pathogenic bacteria which could represent a serious risk for treating bacterial infections. (Doucet-Populaire et al., 1992; Jacobsen et al., 2007; van Reenen and Dicks 2011).

Bacterial Adhesion on stainless steel plates

Among the main important characteristics of probiotic bacteria, adhesion to the intestinal mucosa is required. The current results showed that the screened probiotic LAB isolates possessed in vitro adherence property to stainless steel plates with the adhesion rate ranging from 32.75 to 36.30%. *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f strains were significantly stronger adhesion than all pathogenic strains ($p \leq 0.05$) (Figure 3). *Rummeliibacillus pycnus* STR 0103f strain showed the highest adherence rate with (34.12±0.46%), followed by *R. stabekisii* STR 0404f strains with 32.60±0.57%. In the pathogenic strains, *E. coli* ATCC 25922 had the highest adherence ability of 22.95±0.81% while *S. paratyphi* A had the least adherence ability of 20.01±0.75% on stainless steel plate surface.

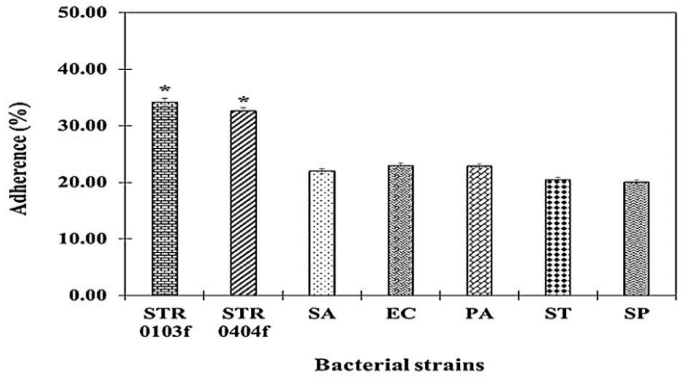

Figure 3 Adhesion of two isolated strains and pathogenic strains on stainless steel plate surface (SA = *S. aureus* ATCC 25923; EC = *E. coli* ATCC 25922; PA = *P. aeruginosa* ATCC 27853; ST = *S. typhimurium* ATCC 13311; SP = *S. paratyphi* A; * = p≤0.05 by paired *t*-test comparing with pathogenic strains)

Among the main characteristics of probiotic bacteria, the adhesion to the intestinal mucosa is required. Thus, in vitro models were used such as cell lines **(Gilliland *et al.* 1984; Tinrat *et al.*, 2011; Tinrat *et al.*, 2018)** and stainless steel **(Winkelstroter *et al.*, 2011; El-Jeni *et al.*, 2016; Mulaw *et al.*, 2019)**. The results showed that *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f possessed in vitro adhesion properties which suggested that both strains may have a potential capacity to colonize the gastrointestinal tract mucosa. They were showed the adhesion rate to stainless steel plates range from 32.75 to 36.30%. The previous study of **El-Jeni *et al.* (2016)** reported that lactic acid bacteria could to stainless steel plates of 32-35% which is consistent with our current results. The positive results of this test showed that selected potential probiotic LAB strains may have a potential capacity to colonize the gastrointestinal tract mucosa. However, further studies on the binding ability of Caco-2 intestinal epithelial cells should be studied to prove their potential as a probiotic in vivo.

CONCLUSION

Thai fermented vegetable products are versatile sources of bioactive microorganisms. Outstandingly, the probiotic characteristics of the *Rummeliibacillus* isolates from fermented vegetable products have not been studied before. In this study, the *Rummeliibacillus pycnus* STR 0103f and *Rummeliibacillus stabekisii* STR 0404f from Thai fermented vegetable sources were the novel isolated strains. They were acid- and bile-salt resistant strains and showed highly sensitive to various antibiotics as well as displayed non-hemolytic activity. Moreover, both of them also exhibited the adhesion ability and had inhibitory activity against a broad spectrum of food-borne pathogens which is of crucial important of bio-preservative application in food industry. Therefore, the findings indicated that *R. pycnus* STR 0103f and *R. stabekisii* STR 0404f were considered as probiotic strains because of their in vitro probiotic and safety properties. However, *in further vivo* studies will be conducted to confirm the potential for probiotics of both the *Rummeliibacillus* strains.

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