Assessment of Probiotic Properties in Lactic Acid Bacteria Isolated from Fermented Vegetables

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Abstract
The present study was conducted in order to screen lactic acid bacteria which have the probiotic properties. A total of 82 isolates of lactic acid bacteria was preliminarily screened from fermented vegetables. Most strains exhibited autoaggregation ability, cell surface hydrophobicity and antimicrobial activity against food-borne pathogens Escherichia coli O157:H7 DMST 12743 and Salmonella Typhimurium ATCC 13311. The results revealed that 23 isolates possess some desirable probiotic properties and were selected to determine other probiotic properties including antibiotic resistance, coaggregation ability with E. coli O157:H7 DMST 12743 and S. Typhimurium ATCC 13311, heat tolerance at 65°C for 60 min and the ability to survive under gastrointestinal tract condition pH 2.0 and 8.0. From probiotic properties determination mentioned above, it was observed that the strain KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-21 were found to meet all the criteria and could be considered as potential probiotic. The identification of the strains based on 16S rDNA sequencing analysis indicated that the strain KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-were identified to be Pediococcus pentosaceus.

Keywords: *Probiotic properties, Pediococcus pentosaceus, Lactic acid bacteria, Screening, Fermented vegetables*

1 Introduction

Probiotic cultures have been exploited extensively by the dairy industry as a tool for the development of novel functional products [1]. The global probiotics market which included foods supplements and ingredients was valued at US$21.6 billion in 2010 and was expected to reach US$31.1 billion by 2015 [2]. Traditionally, most probiotic products are marketed in the form of yoghurt and fermented milk. However, an increase in the vegetarianism throughout the developed countries, there is also a demand for the vegetarian probiotic products [3]. Therefore, a number of carriers (such as cereals, fruits, and vegetables) for probiotics have been developed to determine their suitability for designing new non-dairy probiotic foods [4-6].

Probiotic is generally used to name the microorganisms associated with the beneficial effects for the humans and animals [3]. The majority

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of probiotic microorganisms belong to lactic acid bacteria genera *Lactobacillus* and *Bifidobacterium*. However, strains of *Pediococcus* and some yeasts have also been found as suitable candidates [7]. Probiotics are defined by FAO/WHO [8] as ''live microorganisms which, when administered in adequate amounts, confer health benefit on the host.'', To achieve health benefits, probiotic foods need to contain an adequate amount of live microorganisms and available at high concentrations of at least 10^6-10^7 CFU/g at the time of consumption [9,10]. The beneficial effects of probiotics on human health has been claimed by several studies such as alleviation of lactose intolerance, prevention and reduction of diarrhea symptoms, reduction of the risk associated with mutagenicity and carcinogenicity, inhibition of intestinal pathogens, prevention of inflammatory bowel disease and modulation of the immune system [1,11-14]. In order to use as probiotic, the microorganism must fulfill several criteria including the ability to survive passage through the upper gastrointestinal tract, tolerance to human gastric juices, alkaline bile secretions in the presence of hydrolytic and proteolytic digestive enzymes during their transit through the gut. Additionally, probiotic bacteria must have the ability to adhere to epithelial surfaces, antibiotic resistance and antagonistic activity toward pathogens [1,8,11,15]. Several technological aspects have to be considered in probiotic selection such as viability during processing and stability in production and during storage [16,17]. These properties make it possible to screen and select specific probiotic strains for both food and technological uses. Taheri et al. [18] also suggested that aggregation and cell surface hydrophobicity could be used for preliminary selection of probiotic bacteria which have been proposed as an indirect method for evaluation the adhesion ability of bacteria [32]. In addition, coaggregation ability of lactic acid bacteria with pathogenic microorganisms may serve as a barrier which blocks colonization by pathogens in the intestines [19-21]. Therefore, the objective of this study was to select potential probiotics from fermented vegetables which possessed probiotic traits including autoaggregation, cell surface hydrophobicity, antimicrobial activity, antibiotic resistance, coaggregation ability, heat tolerance and tolerance to gastrointestinal tract conditions.

2 Materials and Methods

2.1 *Microorganisms*

Lactic acid bacteria isolated from fermented vegetables were preserved in de Man, Rogosa, Sharpe (MRS) broth (Difco, Detroit, MI, USA) containing 20% (v/v) of glycerol (Panreac Quimica SAU, Barcelona, Espana) and stored at -20°C. For routine analysis, the strain was subcultured twice in MRS broth and was incubated at 37°C for 24 h. The pathogenic bacteria *Escherichia coli* O157:H7 DMST 12743 and *Salmonella* Typhimurium ATCC 13311 were purchased from the Department of Medical Science, Ministry of Public Health, Thailand. The indicator strains were grown in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) at 37°C. All strains were subcultured twice and incubated at 37°C for 24 h under microaerobic-static condition and then used as inoculum. According to safety guidelines for the treatment of pathogenic bacteria, *E. coli* O157:H7 DMST 12743 and *S.* Typhimurium ATCC 13311 were destroyed by moist heat (autoclave) at 121°C for 15 min before disposal.

2.2 *Preparation of fermented vegetables*

The vegetables including Chinese cabbage, pumpkin, carrot and spring onion were selected for fermentation. All vegetables were chopped, shredded or sliced into small pieces and mixed with 5% (w/w) salt, 2.5% (w/w) table sugar and 2.5% (w/w) water from washing rice. Then, mixed vegetables were placed in a glass jar and poured with hot water until full. A chopstick or knife was used to poke down into the jar (around the sides) to remove air bubbles. Finally, the glass lid was closed over the jar. The fermentation was carried out at 40°C for 5 days.

2.3 *Autoaggregation assay*

Autoaggregation assays were performed according to Del Re et al. [22]. Overnight cultures were harvested by centrifugation at 4000 *g* for 15 min. The cell pellets were washed twice and resuspended in sterile phosphate buffer saline (PBS) to give viable cell counts of approximately 10^8 CFU/mL. The cell suspensions (4 mL) were vortexed for 10 s. During incubation

at room temperature for 5 h, 0.1 mL of the upper suspensions was transferred to another tube containing 3.9 mL of PBS and the optical density (OD_t) was measured at 600 nm. The autoaggregation percentage is expressed as $\left[1-\left(\frac{OD_t}{OD_0}\right)\right] \times 100$, where OD_t represents the optical density at 5 h and OD_0 is the optical density at $t = 0$.

2.4 *Cell surface hydrophobicity*

Cell surface hydrophobicity was determined by the method of Kos et al. [23] with minor modifications. Overnight cultures were harvested by centrifugation at 4000 g for 15 min. The cell pellets were washed twice and resuspended in sterile 0.85% NaCl solution to give an optical density of 0.5 at 600 nm (A_0). To test tubes containing 3 mL of washed cells, 1 mL of toluene (Panreac Quimica SAU, Barcelona, Espana) was added. The mixtures were vortexed for 90 s. After incubation at room temperature for 15 min, the aqueous phase was removed and its optical density at 600 nm (A_1) was then measured. The percentage of cell surface hydrophobicity was calculated as $1-\left(\frac{A_1}{A_0}\right) \times 100$].

2.5 *Antimicrobial activity*

Antimicrobial activity of lactic acid bacteria strains were tested using an agar well diffusion method. Overnight culture (200 µL) of *E. coli* O157:H7 DMST 12743 or *S.* Typhimurium ATCC 13311 was mixed with 20 mL of melt tryptic soy agar (approximately 10^6 CFU/mL) and poured onto sterile Petri-dishes. Wells (7 mm-diameter) were punched out of the solid agar with a sterile cork borer. Overnight culture of lactic acid bacteria (50 µL) was introduced into the wells and the plates were incubated at 37°C for 24 h [24]. Antimicrobial activity was measured by examining the diameters of the inhibition zones around the wells by vernier caliper. The inhibitory activity corresponding to the diameters of the inhibition zones were expressed in mm.

2.6 *Antibiotic resistance assay*

Antibiotic sensitivity of lactic acid bacteria was determined by the Bauer-Kirby method. The optical density at 600 nm of the overnight culture was adjusted to 0.08-0.1 (1-2 × 10^8 CFU/mL). The inoculums were spread evenly over the entire surface of the MRS agar plates. Subsequently, paper discs containing the antibiotics of Penicillin 10 IU/IE/UI, Cepfoxitin 30 µg, Bacitracin 10 IU/IE/UI, Kanamycin 30 µg, Rifampin 5 µg, Streptomycin 10 µg, Clindamycin 2 µg, Nalidixic acid 30 µg, Trimethoprim 5 µg, Vancomycin 30 µg, Gentamicin 10 µg, Oxacillin 1 µg, Ciprofloxacin 5 µg, Novobiocin 30 µg, Ampicillin 10 µg, Chloramphenicol 30 µg, Tetracycline 30 µg and Erythromycin 15 µg (BD BBL™, Becton Dickinson, MD, USA) were laid on the plates. After incubation at 37°C for 24 h, the inhibition zones were measured inclusive of the diameter of the discs (7 mm-diameter). Results were expressed as sensitive, S (≥ 21 mm); intermediate sensitive, I (16-20 mm) and resistant, R (≤ 15 mm) [25].

2.7 *Coaggregation assay*

Coaggregation between selected lactic acid bacteria and *E. coli* O157:H7 DMST 12743 or *S.* Typhimurium ATCC 13311 was determined. The cell suspensions were prepared by the same manner as described in the autoaggregation assay. Equal volumes (2 mL) of lactic acid bacteria and pathogen cell suspension were mixed together in pair by vortexing for 10 s. Control tubes were set up at the same time, containing 4 mL of each bacterial suspension on its own. The optical density at 600 nm of the suspensions was measured after 5 h of incubation at room temperature. Samples were taken using the same procedure as in the autoaggregation assay. The percentage of coaggregation was calculated using the equation of Handley et al. [26]:

$$\text{Coaggregation}(\%) = \frac{\left(\frac{A_x + A_y}{2}\right) - A_{(x+y)}}{\left(\frac{A_x + A_y}{2}\right)} \times 100$$

where x and y represent lactic acid bacteria and pathogen, respectively and (x+y) represents the mixture of lactic acid bacteria and each pathogen.

2.8 *Heat tolerance*

Heat tolerance of the strains was determined according to Ding and Shah [27]. Overnight cultures were incubated at 65°C. The viable cell number was monitored at 0, 30 and 60 min.

2.9 *Simulated gastrointestinal tract tolerance determination*

2.9.1 Preparation of washed cell suspension

Lactic acid bacteria was grown in MRS broth at 37°C for 24 h. Cell culture of each test strain was centrifuged at 4000 *g* for 10 min. After washing twice with sterile saline, the cell pellet was resuspended in the same solution. The viable cell count of washed cell suspension was determined prior to assay of transit tolerance [28].

2.9.2 Preparation of simulated gastric and small intestinal juices

Simulated gastric juice was prepared by means of suspension of pepsin (1:10,000, ICN, Sigma, Basingstoke, Hampshire, UK) in sterile 0.5% NaCl to a final concentration of 3 g/L and adjusted to pH 2.0 with concentrated HCl. Simulated small intestinal juice was prepared by suspension of pancreatin USP (P-1500, Sigma, Basingstoke, Hampshire, UK) in a sterile 0.5% NaCl to a final concentration of 1 g/L with 0.45% bile salt content (Oxoid, Basingstoke, Hampshire, UK) and adjusted to pH 8.0 with sterile 0.1 mol/L NaOH [28].

2.9.3 Determination of gastrointestinal tract tolerance

Aliquot (0.2 mL) of each washed cell suspension was transferred to a sterile tube, mixed with 0.3 mL sterile 0.5% NaCl and finally blended with 1.0 mL of simulated gastric juice (pH 2.0). The viable cell number was determined after exposure for 60, 90 and 180 min. Then, cell suspension after exposure to simulated gastric juice (pH 2.0, 180 min) was centrifuged at 4000 *g* for 10 min. After washing twice with sterile saline, cell pellets were subsequently resuspended in simulated small intestinal juice (pH 8.0 with 0.45% bile salt) [29]. The suspensions were incubated at 37°C for 240 min. The samples were taken at 60 and 240 min during exposure to simulated small intestinal juice.

2.10 *Analytical procedure*

2.10.1 Determination of viable cell counts

Viable cell counts were determined on MRS agar supplemented with 0.5% $CaCO_3$. The plates were incubated at 37°C for 24 h. The viable cell counts were expressed as log10 value/mL. The percentage of cell survival was defined as follows: survival rate (%) = $\left(\frac{N}{N_0}\right) \times 100$, where N represents the number of viable cells (CFU/mL) after exposure and N_0 denotes the initial viable cell count (CFU/mL) prior to exposure [30].

2.10.2 Identification of lactic acid bacteria

DNA extraction was described by Marmur [31]. PCR amplification of 16S rRNA genes was carried out. The amplified genes were sequenced and analyzed according to the method of Yukphan et al. [32]. Two primers, 27F (5'–AGA GTT TGA TCC TGG CTC A–3') and 1492R (5'–GGT TAC CTT GTT ACG ACT T–3'), were used. The sequences were aligned with sequences in GenBank using the BLAST program.

2.10.3 Statistical analysis

Each result was expressed as the mean ± S.D. of three determinations. The data were assessed using analysis of variance (ANOVA) with a level of significance at $P < 0.05$. Significant divergences among mean values were determined with Duncan's multiple range tests. All statistical analyses were performed using SPSS Software, version 12 (SPSS, now a part of IBM Corp.; White Plains, NY, USA).

3. Results and Discussion

A total of 82 isolates of lactic acid bacteria was preliminarily screened from fermented vegetables for their ability to produce acid on MRS agar supplemented with $CaCO_3$ used as an indicator for acid-producing strains [33]. All isolates were Gram-positive, coccus, facultative anaerobic, catalase-negative and homofermentative characteristic. Their optimum growth temperature was 37°C, however all isolates grew slowly at 45°C. Also, it was observed that the addition of salt resulted in decrease growth of lactic acid bacteria strains and all strains could not grow at 10% NaCl.

3.1 *Autoaggregation ability and cell surface hydrophobicity*

Aggregation and cell surface hydrophobicity were used to preliminary screen for probiotic properties

which have been proposed as an indirect method for evaluation the adhesion ability of bacteria [18]. Autoaggregation of probiotic strains means the clumping of bacterial cells from the same strain [20] and appeared to be necessary for adhesion to intestinal epithelial cells [23]. Furthermore, autoaggregation capability could be useful in forming biofilms in gastrointestinal (GI) tract colonization, which are ways to form a barrier against colonization by pathogens [21]. As shown in Table 1, most isolates exhibited a strong autoaggregation after 5 h incubation. The strain KMUTNB 5-8 showed the highest autoaggregation ability of 96.09%, while KMUTNB 5-27 did not show autoaggregation ability. According to Lee et al. [20], *P. pentosaceus* D56 isolated from Jeotgals exhibited autoaggregation ability of 45.2% followed by *P. pentosaceus* F66 (41.1%) and *P. pentosaceus* A24 (40.4%) after 5 h incubation. Moreover, *P. pentosaceus* (15a) isolated from cooked meat products showed autoaggregation capacity of 43.83% after 24 h incubation [21].

Bacterial adhesion to hydrocarbon indicates bacterial cell surface hydrophobicity that plays a key role in first non-specific interaction between microbial cells and mucus or epithelial cells [34]. The percentage of cell adhering to toluene, apolar solvent, demonstrated hydrophobic cell surface property. Of all test strains, a significant difference ($P < 0.05$) in cell surface hydrophobicity was observed. The strain KMUTNB 5-9 exhibited the greatest cell surface hydrophobicity ability of 54.69% followed by KMUTNB 5-22 (33.22%) and KMUTNB 6-17 (30.18%), respectively. However, 9 isolates (KMUTNB 5-2, KMUTNB 5-5, KMUTNB 5-8, KMUTNB 5-12, KMUTNB 5-17, KMUTNB 5-21, KMUTNB 6-26, KMUTNB 6-27 and KMUTNB 7-3) did not show this ability. Osmanagaoglu et al. [34] revealed that *P. pentosaceus* OZF isolated from human breast milk exhibited cell surface hydrophobicity of 34% in n-hexadecane. Furthermore, *P. pentosaceus* D56 and *P. pentosaceus* F66 showed 33.71% and 19.93% cell surface hydrophobicity in xylene, respectively [20]. Lapsiri et al. [35] also reported that 7 strains of *L. plantarum* TISTR 2072, TISTR 2073, TISTR 2074, TISTR 2075, TISTR 2079, TISTR 2081 and TISTR 2082 exhibited cell surface hydrophobicity in toluene ranging from 47.14 to 99.79%. Hydrophobic property is generally thought to be correlated with bacterial adhesion to intestinal mucosa [20]. It has been suggested that bacterial cells with a high hydrophobicity usually form strong interactions with mucosal cells or adhere strongly to epithelial cells or mucus [34]. Mechanisms of adherence to an epithelial surface involve both receptor-specific binding and charge and hydrophobic interaction [36]. These differences in cell surface hydrophobicity could be due to variation in the level of expression of cell surface protein among strains of a species as well as due to environmental conditions which could affect the expression of surface protein [37].

3.2 *Antimicrobial activity*

As shown in Table 1, most strains exhibited antimicrobial activity against foodborne pathogen *E. coli* O157:H7 DMST 12743 and *S.* Typhimurium ATCC 13311. The strain KMUTNB 5-11 displayed the highest antimicrobial activity against *E. coli* O157:H7 DMST 12743 with inhibition zone of 10.45 mm while the strain KMUTNB 6-2 showed the greatest antimicrobial activity against *S.* Typhimurium ATCC 13311 with inhibition zone of 15.40 mm. Lin et al. [38] suggested that the antimicrobial activity of lactic acid bacteria relies on acidity, lactic acid or other organic acids produced. Other possible factors might be some bacteriocins which play roles at low pH values [39,40]. From preliminarily determination of probiotic properties including autoaggregation ability, cell surface hydrophobicity and antimicrobial activity, 23 isolates of lactic acid bacteria were found to complete these probiotic properties. The results revealed that the selected strains exhibited high autoaggregation ability of > 75%, cell surface hydrophobicity ability and antimicrobial activity against both foodborne pathogens *E. coli* O157:H7 DMST 12743 and *S.* Typhimurium ATCC 13311 with average diameter of inhibition zones > 8.5 and 9.0 mm, respectively. Therefore, these 23 isolates (KMUTNB 5-3, KMUTNB 5-4, KMUTNB 5-6, KMUTNB 5-9, KMUTNB 5-10, KMUTNB 5-11, KMUTNB 5-13, KMUTNB 5-14, KMUTNB 5-24, KMUTNB 5-34, KMUTNB 5-35, KMUTNB 5-36, KMUTNB 6-1, KMUTNB 6-2, KMUTNB 6-3, KMUTNB 6-6, KMUTNB 6-21, KMUTNB 6-23, KMUTNB 6-28, KMUTNB 6-29, KMUTNB 6-33, KMUTNB 6-34 and KMUTNB 6-39) were further evaluated on probiotic properties including antibiotic resistance, coaggregation ability, heat tolerance and tolerance to gastrointestinal tract conditions.

Table 1: Autoaggregation ability, cell surface hydrophobicity and antimicrobial activity of 82 isolates of lactic acid bacteria

Isolates	Autoaggregation ability* (% ± S.D.)	Cell surface hydrophobicity* (% ± S.D.)	Antimicrobial activity* (mm. ± S.D.)	
			E. coli O157:H7 DMST 12743	*S.* Typhimurium ATCC 13311
KMUTNB 5-1	83.89 ± 0.44	10.99 ± 0.12	8.95 ± 0.05	9.15 ± 0.05
KMUTNB 5-2	10.71 ± 0.13	0.00 ± 0.00	9.25 ± 0.05	9.80 ± 0.70
KMUTNB 5-3	82.09 ± 0.35	12.33 ± 0.75	10.20 ± 0.50	10.65 ± 0.30
KMUTNB 5-4	81.83 ± 0.11	13.34 ± 0.81	9.55 ± 0.85	11.43 ± 0.73
KMUTNB 5-5	89.04 ± 0.10	0.00 ± 0.00	9.20 ± 0.70	9.63 ± 0.13
KMUTNB 5-6	76.57 ± 1.23	9.34 ± 1.85	9.30 ± 0.20	10.35 ± 0.85
KMUTNB 5-7	10.69 ± 0.10	7.19 ± 0.68	9.43 ± 0.23	9.18 ± 0.27
KMUTNB 5-8	96.09 ± 0.30	0.00 ± 0.00	8.48 ± 0.23	9.43 ± 0.33
KMUTNB 5-9	82.20 ± 0.10	54.69 ± 1.84	8.65 ± 0.08	10.53 ± 0.23
KMUTNB 5-10	86.68 ± 0.21	18.69 ± 1.17	9.78 ± 0.68	10.58 ± 0.33
KMUTNB 5-11	78.95 ± 0.52	12.34 ± 1.26	10.45 ± 0.25	10.50 ± 0.30
KMUTNB 5-12	79.43 ± 0.52	0.00 ± 0.00	9.60 ± 0.20	11.85 ± 1.55
KMUTNB 5-13	80.13 ± 0.21	20.60 ± 2.49	9.50 ± 0.00	9.30 ± 0.90
KMUTNB 5-14	75.38 ± 0.53	12.42 ± 0.65	9.58 ± 0.38	10.60 ± 0.50
KMUTNB 5-15	65.37 ± 0.32	14.60 ± 0.34	9.25 ± 0.85	8.95 ± 0.15
KMUTNB 5-16	64.58 ± 0.42	17.69 ± 2.89	9.40 ± 0.20	7.80 ± 0.40
KMUTNB 5-17	73.12 ± 0.21	0.00 ± 0.00	9.35 ± 0.25	9.90 ± 0.30
KMUTNB 5-18	70.42 ± 0.68	22.63 ± 2.23	9.00 ± 0.20	9.78 ± 0.43
KMUTNB 5-19	65.19 ± 0.34	17.86 ± 0.56	9.15 ± 0.35	8.68 ± 0.43
KMUTNB 5-20	67.46 ± 0.52	22.77 ± 2.11	8.10 ± 0.00	8.10 ± 0.10
KMUTNB 5-21	69.48 ± 0.95	0.00 ± 0.00	8.65 ± 0.05	9.13 ± 0.43
KMUTNB 5-22	74.50 ± 0.21	33.22 ± 3.32	8.50 ± 0.40	9.83 ± 0.08
KMUTNB 5-23	65.76 ± 0.00	13.41 ± 0.86	8.58 ± 0.33	9.98 ± 0.88
KMUTNB 5-24	81.81 ± 0.19	11.76 ± 0.17	8.85 ± 0.05	9.85 ± 0.75
KMUTNB 5-25	82.14 ± 0.11	16.99 ± 0.52	8.10 ± 0.70	9.85 ± 0.35
KMUTNB 5-26	75.43 ± 0.11	14.44 ± 0.74	8.05 ± 0.85	8.38 ± 1.38
KMUTNB 5-27	0.00 ± 0.00	4.44 ± 1.45	7.90 ± 0.60	8.75 ± 0.35
KMUTNB 5-28	73.72 ± 0.31	12.79 ± 0.74	7.53 ± 0.28	8.48 ± 0.23
KMUTNB 5-29	76.63 ± 0.41	15.88 ± 1.47	7.63 ± 0.33	9.75 ± 0.00
KMUTNB 5-30	77.72 ± 0.11	5.81 ± 0.78	7.45 ± 0.45	7.20 ± 0.10
KMUTNB 5-31	74.78 ± 0.09	17.80 ± 0.17	7.00 ± 0.00	11.13 ± 0.82
KMUTNB 5-32	75.98 ± 0.33	19.49 ± 0.17	7.00 ± 0.00	11.15 ± 0.80
KMUTNB 5-33	72.49 ± 0.52	15.93 ± 0.74	7.00 ± 0.00	11.50 ± 0.20
KMUTNB 5-34	84.91 ± 0.41	17.88 ± 0.33	9.93 ± 0.02	10.35 ± 0.15
KMUTNB 5-35	85.16 ± 0.44	18.89 ± 0.65	10.00 ± 0.50	9.90 ± 0.80
KMUTNB 5-36	81.20 ± 0.71	13.13 ± 0.81	9.40 ± 0.10	11.50 ± 1.30
KMUTNB 5-37	76.70 ± 0.53	7.56 ± 0.31	9.30 ± 0.40	7.55 ± 0.45
KMUTNB 5-38	68.28 ± 0.10	3.96 ± 0.62	9.23 ± 0.98	8.95 ± 0.15
KMUTNB 5-39	70.34 ± 0.50	20.00 ± 0.00	8.33 ± 0.08	8.58 ± 0.08
KMUTNB 5-40	71.92 ± 0.33	11.84 ± 0.14	7.00 ± 0.00	12.83 ± 0.43
KMUTNB 6-1	75.93 ± 0.11	13.08 ± 0.12	8.35 ± 0.45	14.60 ± 0.65
KMUTNB 6-2	78.55 ± 0.00	8.80 ± 0.36	8.23 ± 0.68	15.40 ± 0.20
KMUTNB 6-3	77.92 ± 1.35	11.41 ± 0.12	8.75 ± 0.05	11.55 ± 1.65
KMUTNB 6-4	55.04 ± 0.44	4.69 ± 0.47	7.65 ± 0.55	8.03 ± 0.48
KMUTNB 6-5	55.99 ± 0.00	2.75 ± 0.33	7.85 ± 0.05	10.00 ± 0.90
KMUTNB 6-6	80.76 ± 0.25	13.40 ± 0.00	8.45 ± 0.25	11.45 ± 0.25

Table 1: Autoaggregation ability, cell surface hydrophobicity and antimicrobial activity of 82 isolates of lactic acid bacteria *(Continued)*

Isolates	Autoaggregation ability* (% ± S.D.)	Cell surface hydrophobicity* (% ± S.D.)	Antimicrobial activity* (mm. ± S.D.)	
			E. coli O157:H7 DMST 12743	*S.* Typhimurium ATCC 13311
KMUTNB 6-7	80.12 ± 0.88	21.62 ± 0.63	8.80 ± 0.70	7.50 ± 0.50
KMUTNB 6-8	80.83 ± 0.11	18.97 ± 4.88	8.10 ± 0.20	8.30 ± 0.40
KMUTNB 6-9	73.33 ± 0.44	8.09 ± 0.12	9.90 ± 0.00	8.10 ± 1.10
KMUTNB 6-10	82.44 ± 0.11	13.67 ± 0.60	9.40 ± 0.30	7.00 ± 0.00
KMUTNB 6-11	70.89 ± 0.43	4.01 ± 0.41	8.15 ± 0.15	8.20 ± 0.40
KMUTNB 6-12	63.55 ± 0.32	11.72 ± 0.00	7.00 ± 0.00	7.95 ± 0.65
KMUTNB 6-13	70.15 ± 0.31	12.73 ± 0.38	7.00 ± 0.00	10.08 ± 0.77
KMUTNB 6-14	77.54 ± 0.23	12.76 ± 0.14	7.00 ± 0.00	10.75 ± 0.65
KMUTNB 6-15	78.02 ± 0.82	12.50 ± 0.13	8.70 ± 0.10	10.60 ± 0.40
KMUTNB 6-16	75.49 ± 0.21	8.39 ± 0.61	7.40 ± 0.40	9.43 ± 0.33
KMUTNB 6-17	49.04 ± 0.33	30.18 ± 0.74	7.60 ± 0.20	13.78 ± 1.33
KMUTNB 6-18	34.19 ± 0.24	19.69 ± 0.27	7.00 ± 0.00	12.55 ± 1.25
KMUTNB 6-19	39.74 ± 0.12	2.12 ± 0.40	7.00 ± 0.00	12.50 ± 0.20
KMUTNB 6-20	52.83 ± 0.34	14.36 ± 0.00	7.60 ± 0.20	12.80 ± 0.85
KMUTNB 6-21	77.60 ± 0.22	9.93 ± 0.61	8.85 ± 0.05	14.40 ± 0.50
KMUTNB 6-22	62.19 ± 0.12	9.86 ± 0.38	7.45 ± 0.05	12.35 ± 1.05
KMUTNB 6-23	75.19 ± 0.21	6.10 ± 0.12	8.20 ± 0.10	12.15 ± 0.95
KMUTNB 6-24	66.77 ± 0.45	2.00 ± 0.38	7.38 ± 0.23	9.00 ± 0.90
KMUTNB 6-25	65.98 ± 0.41	2.74 ± 0.37	7.30 ± 0.10	7.98 ± 0.98
KMUTNB 6-26	58.15 ± 0.00	0.00 ± 0.00	7.10 ± 0.10	10.50 ± 0.30
KMUTNB 6-27	62.63 ± 0.11	0.00 ± 0.00	7.55 ± 0.25	9.60 ± 0.10
KMUTNB 6-28	77.07 ± 0.33	15.25 ± 0.50	8.00 ± 0.10	10.60 ± 0.60
KMUTNB 6-29	78.89 ± 0.21	5.92 ± 0.71	8.80 ± 0.10	10.40 ± 0.30
KMUTNB 6-30	75.41 ± 0.00	2.24 ± 0.95	7.45 ± 0.05	11.10 ± 0.00
KMUTNB 6-31	76.45 ± 0.51	6.60 ± 0.60	7.75 ± 0.35	11.15 ± 0.55
KMUTNB 6-32	69.81 ± 0.20	10.98 ± 0.77	7.78 ± 0.48	10.65 ± 0.15
KMUTNB 6-33	86.75 ± 0.00	11.94 ± 0.47	9.15 ± 0.05	11.05 ± 0.25
KMUTNB 6-34	75.65 ± 0.46	20.43 ± 0.16	9.40 ± 0.50	10.35 ± 0.15
KMUTNB 6-35	63.66 ± 0.69	6.75 ± 0.49	8.00 ± 0.80	9.30 ± 0.20
KMUTNB 6-36	61.74 ± 0.50	2.48 ± 0.15	7.20 ± 0.10	7.25 ± 0.25
KMUTNB 6-37	68.32 ± 0.31	3.30 ± 0.00	7.70 ± 0.20	7.00 ± 0.00
KMUTNB 6-38	60.51 ± 0.00	9.59 ± 0.80	7.50 ± 0.20	7.00 ± 0.00
KMUTNB 6-39	64.26 ± 0.30	12.34 ± 0.46	7.00 ± 0.00	7.00 ± 0.00
KMUTNB 7-1	60.64 ± 0.61	7.56 ± 1.44	8.65 ± 0.25	7.98 ± 0.33
KMUTNB 7-2	52.91 ± 0.53	2.39 ± 0.48	8.25 ± 0.05	7.05 ± 0.05
KMUTNB 7-3	51.78 ± 0.12	0.00 ± 0.00	8.25 ± 0.15	8.98 ± 0.98

* Values in the same column of each property were significant differences ($P < 0.05$).

3.3 *Antibiotic resistance*

Antibiotics are utilized by the medical and pharmacological industries to fight pathogenic bacteria. Resistance of probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections [41]. In order to be used as probiotics, lactic acid bacteria must show an ability to resist various antibiotics and subsequently exhibit profitable effects on the health of the host [42]. From the results, it was observed that all selected isolates resisted to Tetracycline, Cepfoxitin, Bacitracin, Kanamycin, Streptomycin, Nalidixic acid, Trimethoprim, Vancomycin, Gentamicin, Oxacillin, Ciprofloxacin

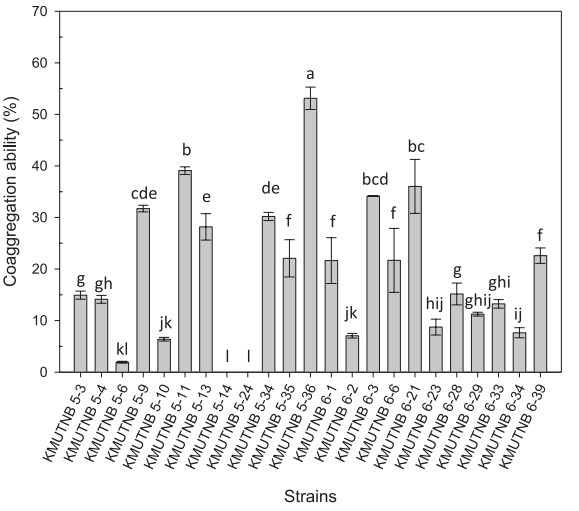
and Novobiocin with the inhibition zone of < 15 mm and intermediate resistance to Rifampin and Ampicillin. However, all isolates were susceptible to Clindamycin, Chloramphenicol, Penicillin and Erythromycin with the inhibition zone of > 21 mm. These results also agree with those of Borges et al. [43] that *P. pentosaceus* SB83 was resistant to Tetracycline and Vancomycin and sensitive to Chloramphenicol, Penicillin and Ampicillin. Klare et al. [44] also reported that 29 isolates of *P. acidilactici* and 20 isolates of *P. pentosaceus* were susceptible to Erythromycin and Clindamycin. The susceptibility and resistance to antibiotics of various strains were variable depending on the species [42].

3.4 *Coaggregation ability*

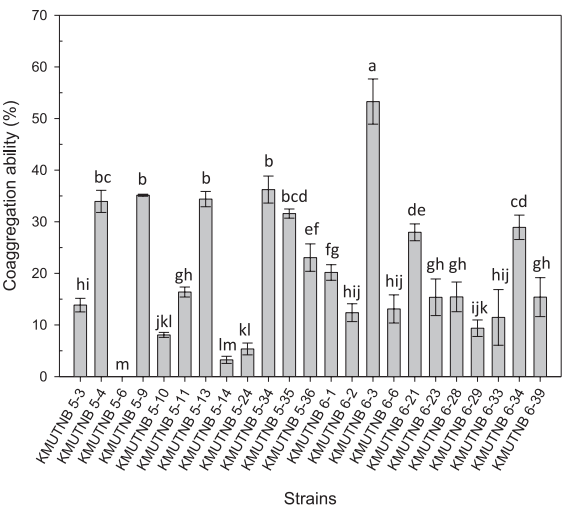
Of 23 isolates, most strains exhibited coaggregation ability with both *E. coli* O157:H7 DMST 12743 (1.92-53.12%) and *S.* Typhimurium ATCC 13311 (3.25-53.28%) (Figure 1). However, KMUTNB 5-14 and KMUTNB 5-24 did not show coaggregation ability with *E. coli* O157:H7 DMST 12743 and KMUTNB 5-6 did not have coaggregation ability with *S.* Typhimurium ATCC 13311. It has been suggested that probiotic microorganisms that have the ability to coaggregate with pathogens may be better able to kill undesirable bacteria because they could produce antimicrobial substances in very close proximity to them [34]. This result was in close agreement with the finding of Osmanagaoglu et al. [34] that *P. pentosaceus* OZF exhibited coaggregation ability with two enteropathogens *E. coli* LMG 3083 (ETEC) (12.99%) and *S.* Typhimurium SL 1344 (6.26%). Furthermore, Kos et al. [23] revealed that the coaggregation percentage of *L. acidophilus* M92 and pathogens were 15.11% with *E. coli* 3014 and 15.70% with *S.* Typhimurium, respectively. It could be indicated that coaggregation mechanisms between probiotic and pathogen could be involved in the reduction in pathogen adhesion to mucus. Additionally, Reid et al. [19] suggested that coaggregation ability of probiotic may enable the formation of a barrier to prevent colonization by pathogens.

3.5 *Heat tolerance*

A major challenge associated with the application of probiotic cultures in functional foods is the retention of viability during processing [16]. In this respect, the drying of live probiotic strains is a critical step in the preparation of concentrated probiotic food ingredients. The heat tolerance of selected lactic acid bacteria incubated at 65°C for up to 60 min was shown in Table 2. It was found that a slight decrease in viable



(a)



(b)

Figure 1: Coaggregation ability between lactic acid bacteria and *E. coli* O157:H7 DMST 12743 (a) and *S.* Typhimurium ATCC 13311 (b) Values with different lowercase letter (a–m) were significant differences by Duncan's multiple range test ($P < 0.05$).

cell number of approximately 0.15-1.78 log CFU/mL was detected after exposure to 65°C for 60 min. The survival rate of all strains was in the range of 1.66–70.49%. The highest survival rate of 70.49% was observed in KMUTNB 5-11 which was not significant difference ($P > 0.05$) from survival rate of KMUTNB 5-9 (65.93% survival rate). Kim et al. [45] suggested that a temperature at 60°C was considered as the lethal temperature because the viability of *L. acidophilus* was significantly reduced but not all cells were killed. Additionally, Champagne et al. [46] suggested that temperatures over 65°C are highly detrimental to probiotic cultures. Heat tolerance of lactic acid bacteria is a complex process involving proteins with different roles in cell physiology, including chaperone activity, ribosome stability, stringent response mediation, temperature sensing and control of ribosomal functions [47,48]. From the results, 6 isolates of lactic acid bacteria (KMUTNB 5-3, KMUTNB 5-4, KMUTNB 5-9, KMUTNB 5-11, KMUTNB 5-36 and KMUTNB 6-21) that exhibited the ability to tolerate to high temperature at 65°C for 60 min (survival rate of 45.87-70.49%, 0.15-0.34 log reduction) and also had coaggregation ability with both *E. coli* O157:H7 DMST 12743 and *S.* Typhimurium ATCC 13311 were selected for further evaluation of gastrointestinal tract tolerance.

Table 2: Viable cell number of 23 strains of lactic acid bacteria after exposure to 65°C for 60 min

Strains	Viable cell number (log CFU/mL ± S.D.)			Survival rate after exposure at 60 min (% ± S.D.)
	Initial	After exposure to heat at 65°C for 60 min		
		30 min	60 min	
KMUTNB 5-3	8.32 ± 0.01	8.19 ± 0.04	8.12 ± 0.05	62.98 ± 7.48^{b}
KMUTNB 5-4	8.42 ± 0.02	8.15 ± 0.10	8.19 ± 0.01	59.96 ± 1.90^{b}
KMUTNB 5-6	8.28 ± 0.02	8.25 ± 0.04	7.85 ± 0.06	37.08 ± 5.17^{e}
KMUTNB 5-9	8.43 ± 0.06	8.35 ± 0.04	8.26 ± 0.01	65.93 ± 10.4^{ab}
KMUTNB 5-10	8.66 ± 0.07	8.33 ± 0.06	7.55 ± 0.05	7.88 ± 0.93^{h}
KMUTNB 5-11	8.47 ± 0.01	8.39 ± 0.01	8.32 ± 0.01	70.49 ± 2.38^{a}
KMUTNB 5-13	8.54 ± 0.01	8.12 ± 0.07	6.76 ± 0.04	1.66 ± 0.17^{h}
KMUTNB 5-14	8.38 ± 0.05	7.96 ± 0.01	7.84 ± 0.04	28.63 ± 2.35^{f}
KMUTNB 5-24	8.28 ± 0.02	8.21 ± 0.05	7.82 ± 0.01	34.47 ± 1.12^{ef}
KMUTNB 5-34	8.46 ± 0.01	8.19 ± 0.01	6.90 ± 0.06	2.78 ± 0.37^{h}
KMUTNB 5-35	8.35 ± 0.13	8.02 ± 0.07	6.79 ± 0.35	3.11 ± 2.25^{h}
KMUTNB 5-36	8.66 ± 0.03	8.20 ± 0.04	8.32 ± 0.03	45.87 ± 2.65^{d}
KMUTNB 6-1	8.35 ± 0.05	8.21 ± 0.09	6.76 ± 0.03	2.51 ± 0.19^{h}
KMUTNB 6-2	8.37 ± 0.03	8.22 ± 0.04	7.56 ± 0.04	15.53 ± 1.50^{g}
KMUTNB 6-3	8.17 ± 0.01	7.95 ± 0.05	6.89 ± 0.07	5.27 ± 0.83^{h}
KMUTNB 6-6	8.33 ± 0.01	8.10 ± 0.14	6.98 ± 0.03	4.48 ± 0.33^{h}
KMUTNB 6-21	8.39 ± 0.02	8.24 ± 0.01	8.11 ± 0.08	52.93 ± 9.14^{c}
KMUTNB 6-23	8.35 ± 0.04	7.70 ± 0.11	6.94 ± 0.01	3.93 ± 0.10^{h}
KMUTNB 6-28	8.35 ± 0.07	7.50 ± 0.02	6.77 ± 0.04	2.61 ± 0.25^{h}
KMUTNB 6-29	8.40 ± 0.06	8.01 ± 0.01	6.67 ± 0.08	1.89 ± 0.33^{h}
KMUTNB 6-33	8.40 ± 0.02	7.71 ± 0.02	6.73 ± 0.07	2.14 ± 0.34^{h}
KMUTNB 6-34	8.27 ± 0.07	8.08 ± 0.07	7.08 ± 0.01	6.31 ± 0.09^{h}
KMUTNB 6-39	8.37 ± 0.13	8.01 ± 0.10	6.85 ± 0.05	2.96 ± 0.35^{h}

Values in the same column of with different lowercase letter (a–h) were significant differences by Duncan's multiple range test ($P < 0.05$).

3.6 *Viability of lactic acid bacteria during sequential exposure to simulated gastric and small intestinal juice*

An important step toward the selection of potential probiotic candidates is to determine their resistance to the extreme conditions of the gastrointestinal tract. Barriers that test strains must overcome are stomach with low pH and digestive enzyme (i.e. pepsin) and the upper intestine which contains bile salt [28,49,50]. The results revealed that all of selected strains were found to exhibit the tolerance ability to simulated gastric juice at pH 2.0 for 180 min. After exposure to simulated gastric juice, the reduction in viable cell number of 1.91-2.55 log CFU/mL (0.29-1.25% survival rate) was observed (Figure 2). The strain KMUTNB 6-21 exhibited the highest survival rate of 1.25% followed by KMUTNB 5-9 (0.69% survival rate) and KMUTNB 5-36 (0.68% survival rate). Then, the test strains were subsequently evaluated for their ability to tolerate under simulated small intestinal juice pH 8.0 with 0.45% bile salt which was considered as sufficient concentration to determine any resistant strains [51]. The capability to survive under small intestinal juice in the human intestine in order to take up residence and multiply in human large intestine is an important characteristic of lactic acid bacteria to be used as probiotic dietary adjuncts [52]. The results indicated that all strains showed the ability to survive under simulated small intestinal juice pH 8.0 for 240 min. A small decrease in viable cell count of 0.30-0.40 log CFU/mL was achieved. As shown in Table 3, it was found that KMUTNB 6-21 showed the greatest survival rate of 0.50% which was significant difference (P<0.05) from the survival rate of KMUTNB 5-36 (0.34% survival rate) and KMUTNB 5-9 (0.33% survival rate). However, there was no significant difference (P>0.05) in survival rate of the strain KMUTNB 5-3, KMUTNB 5-4 and KMUTNB 5-11. From the results mentioned above, KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-21 were able to withstand a high temperature of 65°C for 60 min which is a desirable characteristic for industrial strains as it could have a better chance of remaining viable during the drying process required for prolonged storage. Also, these 3 strains have the capability to maintain their high viable cell number after sequential exposure to simulated gastric and small intestinal juice.

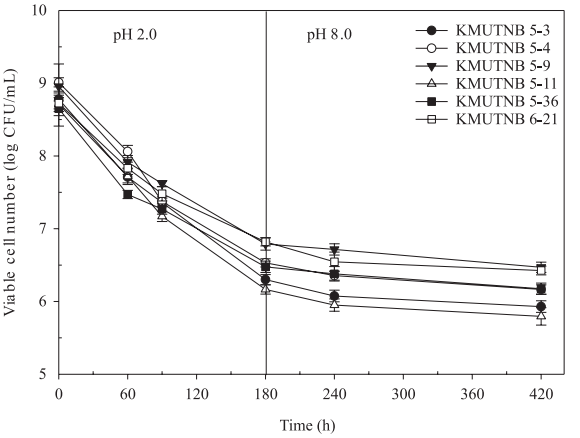


Figure 2: Viable cell number of selected strains during sequential exposure to simulated gastric and small intestinal juice.

Consequently, the strain KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-21 were selected to identify their species based on 16S rDNA sequencing analysis. It was indicated that the strain KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-21 was found to be *Pediococcus pentosaceus* with 99.58%, 99.72% and 99.42% similarity, respectively.

Table 3: Survival rate of the selected strains after sequential exposure to simulated gastric and small intestinal juice

Strain	Survival rate after exposure to simulated gastric juice pH 2.0 for 180 min (% ± S.D.)	Survival rate after exposure to simulated small intestinal juice with 0.45% bile salt pH 8.0 for 420 min (% ± S.D.)
KMUTNB 5-3	0.33 ± 0.05^{c}	0.14 ± 0.03^{c}
KMUTNB 5-4	0.33 ± 0.04^{c}	0.14 ± 0.02^{c}
KMUTNB 5-9	0.69 ± 0.13^{b}	0.33 ± 0.05^{b}
KMUTNB 5-11	0.29 ± 0.04^{c}	0.12 ± 0.03^{c}
KMUTNB 5-36	0.68 ± 0.12^{b}	0.34 ± 0.06^{b}
KMUTNB 6-21	1.25 ± 0.16^{a}	0.50 ± 0.07^{a}

Values in the same column of with different lowercase letter (a-c) were significant differences by Duncan's multiple range test (P < 0.05).

4 Conclusions

In this study, *P. pentosaceus* KMUTNB 5-9, KMUTNB 5-36 and KMUTNB 6-21 isolated from

fermented vegetables were found to meet all probiotic criteria observed in vitro and could be considered as probiotic. However, further *in vivo* evaluation of immunomodulatory function and the production of antagonistic substances such as bacteriocin studies will boost the application of the strain.

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