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Probiotic and Antioxidant Properties of Lactic Acid Bacteria Isolated from Indigenous Fermented Tea Leaves (Miang) of North Thailand and Promising Application in Synbiotic Formulation

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Abstract: Miang, a traditional fermented tea from Northern Thailand, potentially hosts beneficial probiotic bacteria. A total of 133 isolates of lactic acid bacteria (LAB) isolated from Miang were evaluated for probiotic potential. Among them, 5 strains showed high tolerance to bile and acidic conditions and were selected for further evaluation. All selected strains showed inhibitory activity against human pathogens, including *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella* ser. Typhimurium. Nucleotide sequences analysis of the 16S rRNA gene revealed that 3 isolates were identified as *Lactobacillus pentosus*; the remaining were *L. plantarum* and *Pediococcus pentosaceus*, respectively. All 5 strains showed a high survival rate of more than 90% when exposed to simulated gastrointestinal conditions and were also susceptible to antibiotics such as erythromycin, tetracycline, and gentamycin, and resistant to vancomycin, streptomycin, and polymycin. In addition, the selected isolates exhibited different degrees of cell surface hydrophobicity (58.3–92.9%) and auto-aggregation (38.9–46.0%). The antioxidant activity reflected in DPPH scavenging activities of viable cells and their cell-free culture supernatants (CFCS) were also found in selected LAB isolates. Moreover, selected LAB isolates showed ability to grow on commercial prebiotics (GOS, FOS or XOS). The preliminary study of spray-drying using cyclodextrin as thermoprotectant suggested that all strains can be designed as a powdered formulation. *L. pentosus* A14-6 was the best strain, with high tolerance against simulated gastrointestinal conditions, high cell surface hydrophobicity, effective response to tested commercial oligosaccharides, especially XOS, and the highest cell antioxidant properties. *L. pentosus* A14-6 was therefore targeted for further applications in food and synbiotic applications.

Keywords: probiotic potential; fermented tea; Miang; synbiotic; antioxidant activity

1. Introduction

Probiotics are live microorganisms which exert beneficial effects on host health when consumed in adequate amounts [1]. Some lactic acid bacteria (LAB) have been widely

used as probiotics in humans and animals, and the strains most used as probiotics belong to either the genus *Bifidobacterium* or *Lactobacillus* [2,3]. The approval process for probiotics requires certain imperative characteristics such as the resistance to bile and low pH, antibiotic susceptibilities, and antimicrobial activity [2]. Additionally, other properties are also beneficial for probiotic cultures, such as desirable technological, sensorial, and safety features [4]. Moreover, the probiotic culture should be well-adapted to fermented dairy product environments (i.e., presence of curing salts, acidity, and temperature) in order to compete with the endogenous microbiota and grow to levels that enable the display of health-promoting effects [5]. Although dairy products are the most commonly used food vehicles for probiotic delivery, probiotics can also be included in different fermented or unfermented foods [6]. However, the classical obstacle of living probiotic applications in various food vehicles is the survival of probiotic microbes. The vehicle food products require specific temperatures to preserve and sustain the survival rate of probiotics [7]. Besides the direct application of living probiotic LAB using food vehicles, the applications of LAB in the form of either nutraceuticals or food supplements are also gaining interest [8]. The commercial product formulation of probiotics combined with specific growth-promoting carbon sources called prebiotics is also well accepted among consumers and physicians [9,10]. Some oligosaccharides are commercially produced for use as prebiotics, including xylooligosaccharides (XOS), fructooligosaccharide (FOS), and galactooligosaccharides (GOS) [11].

Traditional fermented products constitute an alternative and readily available delivery matrix for LAB starter cultures with attractive functional characteristics particularly with additive probiotic properties [12]. Miang is a traditional fermented food product made of tea leaves (*Camellia sinensis* var. *assamica*) which is commonly produced and consumed in northern Thailand and neighboring countries [13]. The manufacturing process of Miang includes many steps following the inherited protocol depending on local communities, and the most important step of Miang production process is the natural fermentation for a few weeks or up to one year without the use of any preservatives [14,15]. LAB is a key group of microorganisms having an important role in Miang fermentation. Various strains of LAB, including *Lactobacillus* sp., *Pediococcus* sp. and an *Enterococcus* sp., have been isolated from Miang [16–20]. Recent studies exploring the microbial community during Miang fermentation by non-filamentous growth-based fermentation (NFP process) [21], and filamentous growth-based fermentation (FFP process) [22] have confirmed the important role of LAB and their diversity in Miang samples. This indicates the relevance of Miang as potential source of probiotic bacteria.

Therefore, the objectives of this study were to screen for beneficial probiotics from LAB derived from Miang tea fermentation process and to investigate their characteristics and capabilities for use as human probiotics. This study also provides the rationale for targeting specific LAB strains and aligning them to commercial prebiotics to advance commercial synbiotic products as functional foods or nutraceuticals.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The LAB strains isolated from Miang samples were collected from different locations in Upper-Northern Thailand including Chiang Mai, Chiang Rai, Nan, and Phrae, as described in a previous study [16]. A total of 133 LAB were maintained in de Man, Rogosa, and Sharpe (MRS) broth (HiMedia, Mumbai, India) containing 30% (*v*/*v*) glycerol and stored at −80 °C. Four pathogenic bacteria were used as antimicrobial activity indicators including *Bacillus cereus* TISTR 747, *Salmonella* ser. Typhimurium TISTR 1472, *Staphylococcus aureus* TISTR 746, and Lactobacillus acidophilus TISTR 2365 was used as the reference probiotic strains. To prepare seed inoculum, 1 mL aliquot of LAB was added to 10 mL MRS broth vials and statically incubated at 37 °C for 12 h. The LAB isolates were spread on MRS agar supplemented with 125 ppm bromocresol purple. Plates were incubated for 48 h at 37 °C.

A single colony of LAB was inoculated into 5 mL of the MRS broth and further incubated at 37 °C under static conditions for 18 h.

2.2. Screening and Selection of Acid and Bile Salt Tolerant LAB as the Potent Probiotic

All LAB isolates were tested for their tolerance to acidic conditions and bile salts. Tolerance to acidic conditions was determined using the method of Argyri, et al. [23], with some modification. Briefly, an overnight culture of LAB strains in MRS broth at 37 °C was harvested by centrifugation at 8000× *g* for 10 min at 4 °C, then washed twice with phosphate-buffered saline (PBS), pH 7.2. The washed cell pellets were resuspended in PBS to approximately 10^8 CFU/mL. A total 0.1 mL of LAB cell suspension was transferred into 10 mL of PBS pH 2.0 adjusted by 1.0 M hydrochloric acid, and cells suspended with PBS (pH 7.2) were used as control. All mixtures were incubated at 37 °C for 3 h. A viable cell count was determined by plating on MRS agar and incubated at 37 °C for 12 h. The viable cell count was expressed as the log value of colony-forming units per mL (logCFU/mL).

The bile salts tolerance of LAB isolates was determined according to the method of García-Hernández, et al. [24]. Briefly, 0.1 mL of LAB cell suspension was inoculated into 10 mL of PBS supplemented with 0.3% (*w*/*v*) bile salts (Oxgall, Merck, Germany) and PBS without bile salts served as the control; all cultures were incubated at 37 °C for 3 h. The viable cell count was determined, and the survival rate was calculated as follows:

$$\text{Survival (\%)} = [\text{final (logCFU/mL)/control (logCFU/mL)}] \times 100 \quad (1)$$

2.3. Identification and Characterization of Lactic Acid Bacteria

The selected LAB isolates showing the acid and bile salt tolerance properties were identified based on morphological characteristics and 16S rDNA sequence analysis. The genomic DNA was extracted using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA) following the manufacturer's protocol. Each genomic DNA obtained from the pure culture was used as a template with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) for 16S rRNA gene amplification. The PCR products were purified by GF-1 PCR clean-up gel extraction kits (Vivantis, Malaysia), visualized by electrophoresis on 1.0% (*w*/*v*) agarose gels, and were sent for sequencing service at a sequencing service provider (1st BASE Laboratory Company, Singapore). The sequenced 16S rRNA gene was employed to search the closest sequences using basic local alignment search tool (BLAST) available at the National Center for Biotechnology Information (NCBI) GenBank databases (http://www.ncbi.nlm.nih.gov (accessed on 28 August 2021)). A multiple sequence alignment was performed, and the phylogenetic tree was constructed by the neighbor-joining method using MEGA (Molecular Evolution Genetic Analysis) software, version 4.0 [25]. In order to identify the LAB isolates of the *L. plantarum* group, the isolates were subjected to *recA* gene analysis using species-specific PCR. A multiplex PCR assay was performed with the *recA* gene-based primers paraF, pentF, planF, and pREV, the annealing temperature was 56 °C [26]. The expected sizes of the amplicons were 318 bp for *L. plantarum*, 218 bp for *L. pentosus*, and 107 bp for *L. paraplantarum*. The identified sequences were submitted to NCBI GenBank with the following accession number: MW564014-MW564018.

2.4. Antimicrobial Activity against Pathogens

The selected LAB isolates were investigated for their antagonistic activity in cell-free culture supernatant (CFCS) against a variety of gastrointestinal pathogenic bacteria, including *B. cereus*, *S.* Typhimurium, and *S. aureus* by the agar well diffusion method. Briefly, an overnight culture of pathogenic bacteria (approximately 10^6–10^8 CFU/mL) was gently swabbed on the surface of NA plate. Sterile filter paper discs (8.0 mm diameter) containing 20 µL of the unneutralized CFCS and neutralized CFCS (neutralized to pH 7 by addition of 5 N NaOH) obtained from the MRS culture broth of the LAB isolates were placed on the surface of the swabbed agar plates. The presence of growth inhibition was

observed from the appearance of clear zone around the well after plates were incubated at 37 °C for 24 h.

2.5. Resistance to Simulated Gastrointestinal Conditions

Gastrointestinal tolerance was determined as described by Sriphannam, et al. [27], with some modification. The selected LAB isolates and reference probiotic strains (*L. acidophilus*) were inoculated in MRS broth, incubated at 37 °C for 18 h, and harvested by centrifugation at 8000× *g* with 4 °C for 10 min. After washing twice with sterile PBS, the cell pellets were resuspended in electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, and 1.2 g/L $NaHCO_3$) for an initial viable cell count of 10^8 CFU/mL. One milliliter aliquot was aseptically removed, serially diluted in 0.85% (*w*/*v*) NaCl, and spread on MRS agar to determine the number for the surviving bacterial count (CFU/mL) at time 0. To simulate the dilution and possible hydrolysis reaction of bacteria in the human oral cavity, 5 mL of cell suspension in electrolyte solution was mixed with the same volume of sterile electrolyte solution supplemented with lysozyme (Wako Pure Chemical Industries Ltd., Japan) to obtain a final concentration of 100 ppm, and the sample was then incubated for 5 min at 37 °C. The mixture solution was subsequently diluted 3:5 with an artificial gastric fluid consisting of 0.3% (*w*/*v*) pepsin (Fluka Biochemika, Germany) in the electrolyte solution, with the pH adjusted to 2.5. After 1 h of incubation at 37 °C, a sample was taken to measure the viable cells on an MRS agar plate. For simulation of the conditions in the small intestine, the remaining volume was then diluted 1:4 using an artificial duodenal secretion (6.4 g/L $NaHCO_3$, 0.24 g/L KCl, 1.28 g/L NaCl, 0.5% (*w*/*v*) bile salts, and 0.1% (*w*/*v*) pancreatin) (Fluka Biochemika, Germany) at pH 7.2 and was incubated at 37 °C. At 2 and 3 h of the incubation time, samples were taken for measurement of viable cells by plate count technique on MRS agar plates. The viable cell count was determined and the survival rate was calculated.

2.6. Auto-Aggregation

An auto-aggregation study was carried based on the Xu, et al. [28] method, with some modification. A total of 10 mL of LAB cell suspension in PBS (10^8 CFU/mL) was vortexed homogeneously for 10 s, and 1 mL of cell suspension was measured at 600 nm ($A_{initial}$). After being incubated at 37 °C without disturbing for 2 h, the absorbance of the upper fraction (1 mL) was measured (A_{final}). The auto-aggregation percentage was calculated using the following equation:

$$\text{Auto-aggregation (\%)} = [(A_{initial} - A_{final})/A_{initial}] \times 100 \quad (2)$$

where $A_{initial}$ and A_{final} are the absorbance at 0 and 2 h, respectively.

2.7. Cell Surface Hydrophobicity

The cell surface hydrophobicity of the selected LAB was determined in terms of the bacterial cell adhesion to solvents based on the ability of cells to bind to hydrocarbons, according to the methodology described by García-Hernández, et al. [24]. Chloroform was chosen as a nonpolar solvent because it reflects cell surface hydrophobicity and hydrophilicity. Briefly, an overnight culture of selected LAB was harvested by centrifugation at 8000× *g* with 4 °C for 10 min, washed twice with PBS buffer (pH 7.2), and resuspended with PBS to an absorbance of 1.0 at 600 nm ($A_{initial}$). An equal volume of chloroform (BDH Chemicals, Ltd., Poole, England) was added and mixed by vortex mixer for 5 min. After 1 h of incubation at 37 °C, the optical density of aqueous phase was measured at 600 nm (A_{final}). Isolates with cell surface hydrophobicity above 50% were considered hydrophobic. Cell surface hydrophobicity was calculated using the following equation:

$$\text{Cell surface hydrophobicity (\%)} = [(A_{initial} - A_{final})/A_{initial}] \times 100 \quad (3)$$

2.8. Antibiotic Resistance

The LAB strains were tested for antibiotic susceptibilities by the disc diffusion method. Antibiotic discs including erythromycin (15 µg/disc), tetracycline (30 µg/disc), gentamycin (10 µg/disc), kanamycin (30 µg/disc), vancomycin (30 µg/disc), polymycin (30 µg/disc), and streptomycin (10 µg/disc) were used [29]. Briefly, 50 µL of overnight-grown LAB culture on MRS broth (approximately 10^7–10^8 CFU/mL) was spread on the MRS agar plate and the antibiotic discs (HiMedia, Mumbai, India) were placed on it. The plates were incubated at 37 °C for 24 h and the inhibition zone diameters were measured. The results were expressed as sensitive, S; intermediate, I; or resistant, R as described by the Clinical and Laboratory Standard Institute [30].

2.9. Hemolytic Activity Test

The hemolysis assay of the LAB strains was evaluated using Columbia blood agar (Himedia, Mumbai, India) supplemented with 5% (*v*/*v*) sheep blood according to Angmo, et al. [31], with some modification. The strains were streaked on a blood agar plate and incubated at 37 °C for 48 h. The characteristics of hemolysis, shown as clear zones around colonies on blood agar, were recorded and classified as hemolytic (β-hemolysis); green-hued zones around colonies (α-hemolysis) and no clear zones around colonies (γ-hemolysis) were considered non-hemolytic.

2.10. DPPH Free Radical Scavenging Ability

The overnight culture of LAB strains in MRS broth was centrifuged at 8000× *g* for 10 min at 4 °C to separate the CFCS and cells. The CFCS was harvested and filtered through 0.2 µm Acrodisc® syringe filters (Pall Netherlands B.V., Medemblik, The Netherlands). The cell pellets were resuspended in PBS to reach a concentration of 10^8 CFU/mL and served as intact cells. The radical scavenging capacities of LAB strains were evaluated using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) free radical assays [32]. The assay was carried out by mixing 800 µL of freshly prepared 0.2 mM of DPPH solution in 80% methanol with 400 µL of CFCS or intact cells and vortexed for 30 s. The mixture was left at room temperature in the dark for 30 min. Scavenging ability was measured by decrease in absorbance at 517 nm. Uninoculated MRS broth and PBS were used as control samples for the DPPH scavenging measurement of CFCS and intact cells, respectively. The percentage scavenging potential was estimated using the following equation:

$$\text{DPPH scavenging capacity (\%)} = (A_{control} - A_{sample})/A_{control} \times 100 \quad (4)$$

2.11. Prebiotic Utilization

The ability to utilize different prebiotics of LAB strains was evaluated and presented as a prebiotic score as described by Kondepudi, et al. [33]. Commercial FOS, GOS, and XOS purchased from Wako Pure Chemical Industries (Tokyo, Japan) were used as the carbon sources for LAB cultivation. An overnight culture of LAB strains in MRS broth at 37 °C was harvested by centrifugation (8000× *g* for 10 min at 4 °C) and the cell pellets were washed twice with PBS buffer. The washed cell pellets were resuspended in PBS. The inoculum size of the bacterial viable cell (approximately 10^8 CFU) was transferred into 10 mL of modified MRS medium containing 10 g/L of individual prebiotics (GOS, FOS, or XOS) as a sole carbon source and statically incubated at 37 °C for 24 h. The modified MRS broth containing 10 g/L of glucose as the sole carbon source was used as positive control. Prebiotic utilization was determined by measuring the viable cells (logCFU/mL) at 24 h. The prebiotic score is the highest growth achieved by a strain in the presence of MRS supplemented with prebiotics relative to their growth in glucose, which was considered 100%. The prebiotic score was calculated as below:

$$\text{Prebiotic score (\%)} = (A/B) \times 100 \quad (5)$$

where A and B are the mean viable cell values (logCFU/mL) of a strain grown in the presence of each prebiotic (GOS, FOS, or XOS) and glucose, respectively, after 24 h cultivation.

2.12. Viability of Probiotic Strains after Spray-Drying

An overnight culture of LAB strains in MRS broth at 37 °C was harvested by centrifugation at 8000× *g* for 10 min at 4 °C, then washed twice with PBS buffer. The washed cell pellets were resuspended in maltodextrin (15%, *w*/*v*) to approximately 10^7 CFU/mL. A laboratory scale spray dryer (model B-290 Buchi mini spray dryer, Flawil, Switzerland) was used to process samples at a constant air inlet temperature of 180 °C, and the flow rate of the drying air was set at 40 m^3/h, leading to an outlet temperature of around 80–85 °C, in order to obtain powders with less than 5% moisture. Cell viability was tested before and after the spray-drying procedure. The survival percentage was calculated as follows:

$$\text{Survival rate (\%)} = (N/N_0) \times 100 \quad (6)$$

where N represents the number of viable cells per gram of dry matter after drying, and N_0 is the number of viable cells per gram of dry matter in the bacterial suspension before drying [34].

2.13. Statistical Analysis

All the experiments were performed in triplicate, and the results were calculated as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to study significant differences between means with significance level $p < 0.05$ using SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Screening and Selection of Acid and Bile Salt Tolerance LAB as Potential Probiotic

Tolerance to low pH and bile salts are generally considered an essential assessment criteria for probiotic strains to exert their beneficial effects on the gastrointestinal tract environment [35]. The pH of the human stomach rises from 1–2 to 4–5 after the ingestion of food, therefore the majority of in vitro assays are designed to select for strains that can tolerate the extreme low pH range from 2–3 [36]. This assay was designed to exclude some strains that may actually possess probiotic properties but were unable to tolerate extreme low acidity. Therefore, a total of 133 LAB isolates obtained from Miang were investigated for their acid tolerance at pH 2.0. The results determined that only 16 LAB isolates showed the ability to tolerate pH 2.0 for 3 h of incubation with a survival rate of more than 80%, which represented 12% of the total LAB isolates (Table 1). Isolates A9-2 and A14-6 were the most tolerant at pH 2.0, with survival rates of 95%. Acidic conditions have a large effect on the survival rates of *Lactobacillus*. According to Mathara, et al. [37], the percentage of LAB strains isolated from traditional fermented dairy products with a favorable resistance at pH 2.0 was 22.2% of the overall strains. Almost none of the 47 *Lactobacillus* strains isolated from ripened Parmigiano–Reggiano cheese could survive at pH 2.0 [38].

According to Goldin and Gorbach [39], tolerance to bile salt concentration of 0.15–0.3% is recommended for probiotics, since it is in the range of the physiological conditions found in the gastrointestinal tract. Moreover, Jose, et al. [40] reported that the maximum concentration of bile salt that can be found in an average healthy person is 0.3%. Therefore, 0.3% bile was used in this study, and all 16 acid-tolerant LAB isolates demonstrated bile resistance ability, as noted in the results presented in Table 1. The results revealed that 6 of 16 isolates were sensitive to 0.3% bile salt (less than 50%), whereas the others had tolerance in the range of 65 to 92%. Only 5 of 16 isolates showed the ability to tolerate bile salt at concentration of 0.3% with survival rates over 80% during 3 h incubation. On evaluating the combined tolerance to acidic conditions and bile salt, which is an important criterion for effective probiotics selection, as mentioned previously, all five isolates including A9-2, A14-6, A26-8, CMY9, and CMY46 were selected for further identification and probiotic characterization.

Table 1. Viability and survival percentages of sixteen LAB isolates after being incubated at pH 2.0 and 0.3% (*w*/*v*) bile salt at 37 °C for 3 h.

Isolates	Viable Cell (logCFU/mL)		Survival Rate (%)	Viable Cell (logCFU/mL)		Survival Rate (%)
	pH 7.2	pH 2.0		Without Bile	0.3% Bile	
A5-1	8.57 ± 0.12	6.89 ± 0.03	80.4 ± 0.4 ^{i}	8.40 ± 0.05	4.15 ± 0.06	49.4 ± 0.5 ^{h}
A9-2	8.23 ± 0.01	7.82 ± 0.08	95.0 ± 0.6 ^{a}	8.55 ± 0.07	7.85 ± 0.08	91.8 ± 1.4 ^{a}
A10-1	8.44 ± 0.04	6.95 ± 0.12	82.3 ± 1.7 ^{hi}	8.23 ± 0.12	6.32 ± 0.04	76.8 ± 0.6 ^{e}
A13-5	8.24 ± 0.04	7.46 ± 0.10	90.5 ± 0.8 ^{bcd}	8.45 ± 0.04	3.82 ± 0.07	45.2 ± 0.8 ^{ij}
A14-2	8.11 ± 0.12	6.71 ± 0.11	82.7 ± 0.9 ^{ghi}	8.06 ± 0.07	3.87 ± 0.05	48.0 ± 1.3 ^{hi}
A14-6	8.68 ± 0.09	8.26 ± 0.07	95.2 ± 0.5 ^{a}	8.52 ± 0.09	7.75 ± 0.21	91.0 ± 1.8 ^{a}
A21-4	8.67 ± 0.06	7.75 ± 0.02	89.4 ± 1.4 ^{cde}	8.15 ± 0.16	5.28 ± 0.02	64.8 ± 0.9 ^{g}
A26-8	8.45 ± 0.17	7.84 ± 0.04	92.8 ± 0.3 ^{ab}	8.66 ± 0.02	7.45 ± 0.06	86.0 ± 1.5 ^{b}
A27-3	8.16 ± 0.10	7.31 ± 0.06	89.6 ± 0.5 ^{cde}	8.43 ± 0.12	6.61 ± 0.08	78.4 ± 1.6 ^{de}
A29-1	8.71 ± 0.12	7.33 ± 0.03	84.2 ± 0.7 ^{gh}	8.89 ± 0.13	6.33 ± 0.05	71.2 ± 0.7 ^{f}
CMY1	8.10 ± 0.09	7.14 ± 0.04	88.1 ± 0.6 ^{de}	8.77 ± 0.07	3.48 ± 0.17	39.7 ± 0.5 ^{k}
CMY9	8.14 ± 0.07	7.42 ± 0.09	91.2 ± 0.4 ^{bc}	8.45 ± 0.13	7.15 ± 0.03	84.6 ± 1.1 ^{bc}
CMY12	8.27 ± 0.07	7.21 ± 0.15	87.2 ± 0.6 ^{ef}	8.62 ± 0.04	3.75 ± 0.12	43.5 ± 0.9 ^{j}
CMY34	8.62 ± 0.15	7.36 ± 0.08	85.4 ± 1.2 ^{fg}	8.28 ± 0.04	3.46 ± 0.05	41.8 ± 1.6 ^{jk}
CMY46	8.57 ± 0.02	7.85 ± 0.06	91.6 ± 1.5 ^{cd}	8.41 ± 0.06	6.89 ± 0.07	81.9 ± 0.8 ^{cd}
CMY47	8.46 ± 0.08	7.58 ± 0.10	89.6 ± 0.6 ^{de}	8.32 ± 0.08	6.52 ± 0.13	78.4 ± 1.9 ^{de}

Note: Means in column with different superscripts are statistically different at $p < 0.05$.

3.2. Identification of LAB

Five selected isolates that were determined to be Gram-positive by forming a yellow clear zone on MRS agar were presumptively considered LAB. Most of the colonies were round, smooth, convex surface, off-white or yellow colonies. The cell morphology after 24 h incubation of A9-2, A14-6, A26-8, and CMY46 were observed as a rod shape, while the CMY9 was cocci. The nucleotide sequences of 16S rRNA genes of all five selected LAB isolates were also determined, with the purpose of classifying the species of the selected isolates. The full-length 16S rRNA genes were sequenced and compared in similarity with the bacterial 16S rRNA gene in the NCBI database; these results are shown in Table 2. The isolated CMY9 showed more than 99.9% similarity to *Pediococcus pentosaceus*, and the other isolates (A9-2, A14-6, A26-8, and CMY46) showed more than 98% similarity to the *Lactobacillus plantarum* group including *L. pentosus* and *L. plantarum*. Based on high similarity of up to 99% of 16S rRNA gene sequences between *L. pentosus* and *L. plantarum*, it was difficult to differentiate all of selected strains. Confirmation of *recA* gene analysis found that isolate A9-2 was identified to be *L. plantarum*, whereas the isolates A14-6, A26-8, and CMY46 were identified to be *L. pentosus*. The phylogenetic tree of all five LAB isolates is shown in Figure 1. Our results supported the previous findings which reported that the most common species of lactobacilli isolates found in fermented fruits and vegetables including fermented cucumbers, fermented olives, and fermented tea leaves were *L. pentosus*, *L. plantarum*, *L. vaccinostercus*, *L. thaitandensis*, *L. camellia*, and *P. siamensis* [16,17,19,32,41,42].

Table 2. Molecular identification of LAB isolates by 16S rRNA gene sequence analysis.

Isolates	Closest Species	Similarity (%)	Length (bp)	Accession Number	Note
A9-2	*Lactobacillus plantarum* ATCC 14917	99.8	1486	MW564014	*recA* gene confirmed
A14-6	*Lactobacillus pentosus* DSM 20314	99.9	1467	MW564015	*recA* gene confirmed
A26-8	*Lactobacillus pentosus* DSM 20314	99.9	1476	MW564016	*recA* gene confirmed
CMY9	*Pediococcus pentosaceus* DSM 20336	99.9	1476	MW564017	-
CMY46	*Lactobacillus pentosus* DSM 20314	99.5	1472	MW564018	*recA* gene confirmed

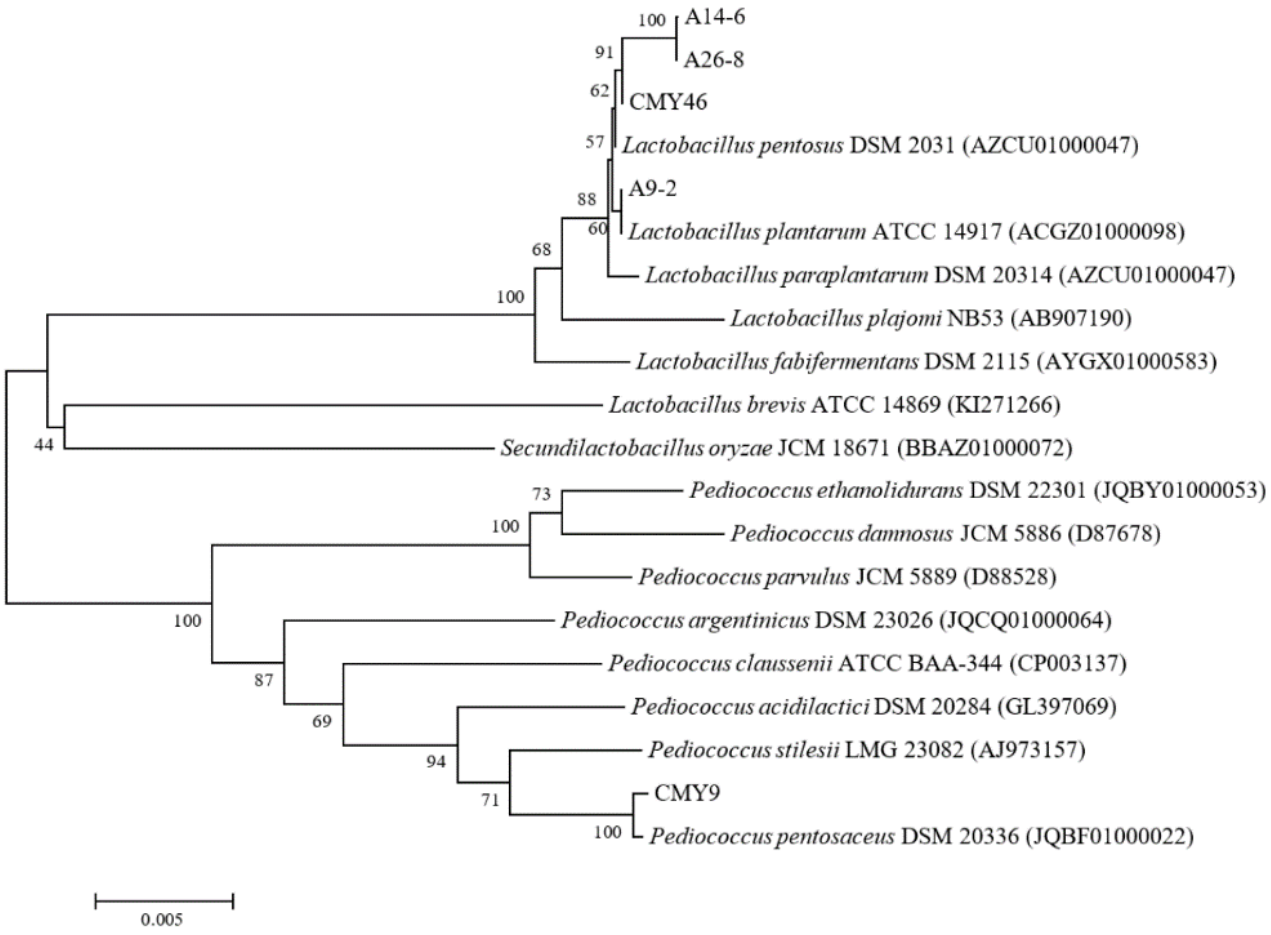

Figure 1. Phylogenetic tree of five selected LAB isolates based on 16S rRNA gene sequence analysis and other related species.

3.3. Antimicrobial Activity against Pathogens

The selected LAB isolates were evaluated for antimicrobial potential against the indicator microorganisms *B. cereus*, *S. aureus*, and *S.* Typhimurium, by the disc diffusion method. An unneutralized CFCS from LAB isolates and a reference strain (*L. acidophilus*) showed a clear zone of inhibition against all indicator microorganisms tested. The results revealed that all LAB isolates exhibited average inhibition zone of 10.4–14.4 mm. *Lactobacillus pentosus* A14-6 was the most effective in inhibiting target pathogens, with 14.4 ± 0.5 and 13.3 ± 0.3 mm clear zones against *S. aureus* and *S.* Typhimurium, respectively, and showed an inhibition zone better than *L. acidophilus* against both pathogens (13.8 ± 0.3 and 12.0 ± 0.4 mm), as shown in Figure 2A–C. In contrast, neutralized CFCS of all selected isolates did not show clear zone formation (Figure 2D–F). Generally, an unneutralized CFCS always shows an inhibitory effect because of the acidic pH, which is mostly not favorable for the growth of most pathogenic bacteria, whereas the inhibitory properties found in neutralized CFCS are commonly caused by bacteriocin or bacteriocin-like metabolites [27,43]. The study by Sankar, et al. [44] found that *L. plantarum* showed antibacterial

activity against *S. aureus*, *Enterococcus fecalis*, *Escherichia coli*, and *Listeria monocytogenes*. Among these, the highest growth inhibition recorded was against *S. aureus*, and minimum activity was observed against *L. monocytogenes*.

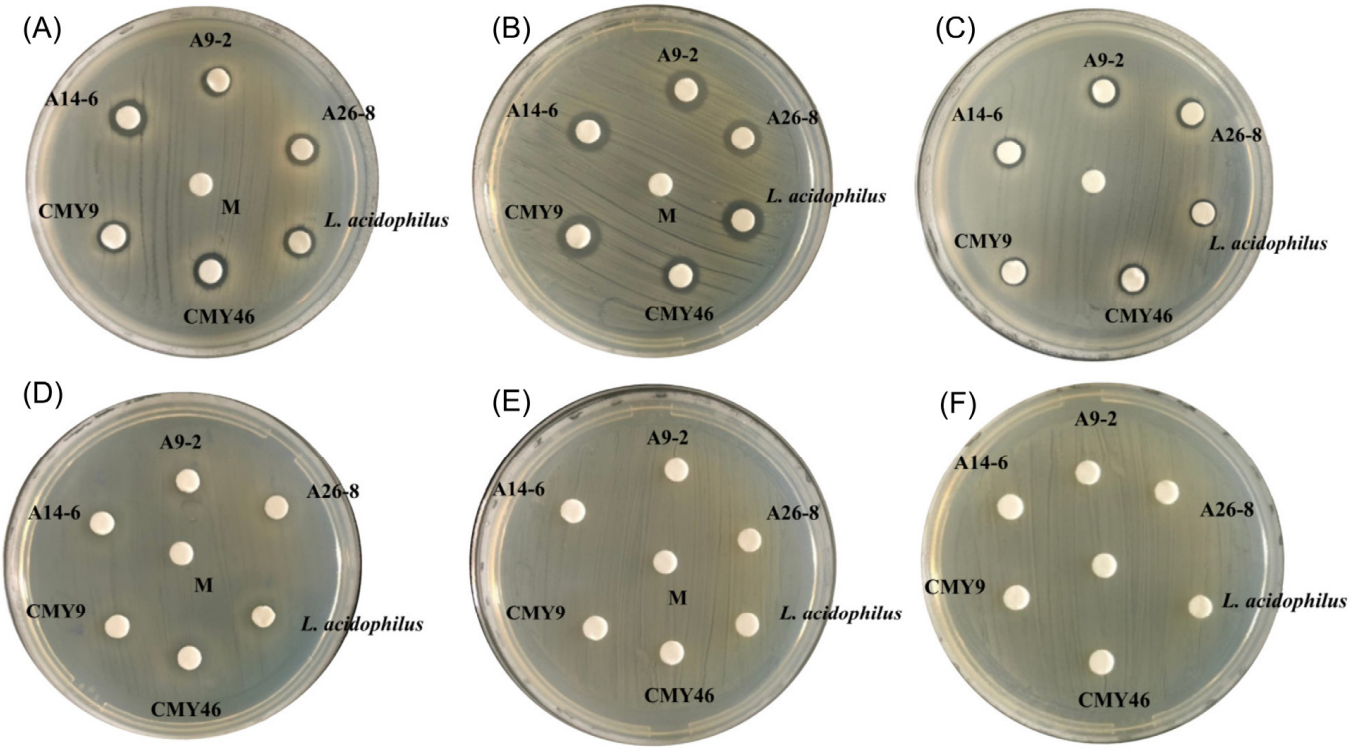

Figure 2. Disc diffusion assay of cell-free culture supernatants (CFCS), obtained from *L. plantarum* A9-2, *L. pentosus* A14-6, *L. pentosus* A26-8, *P. pentosaceus* CMY9, *L. pentosus* CMY46, and *L. acidophilus* culture in MRS broth (M) uninoculated MRS broth, against *Bacillus cereus* (**A**,**C**), *Staphylococcus aureus* (**B**,**E**), *Salmonella* Typhimurium (**C**,**F**). (**A**–**C**): unneutralized CSCF, (**D**–**F**): neutralized CFCS (pH 7).

3.4. Survival in the Simulated Gastrointestinal Conditions

An in vitro model for the evaluation of survival in simulated gastrointestinal conditions was used for the investigation of five LAB isolates along with the probiotic reference strain, *L. acidophilus*. The effects of artificial saliva, gastric, and duodenal juices on the viability of LAB isolates are presented in Figure 3. The statistical comparison of the viability for each species at the end of the treatments revealed that the surviving capability of LAB isolates against the artificial gastrointestinal conditions was clearly comparable to the *L. acidophilus* probiotic strain [45]. All LAB isolates showed tolerance and a good survival rate of more than 90% after being tested under the stress of simulated gastrointestinal conditions. The LAB candidates showed higher surviving capability against in vitro gastrointestinal conditions than the *L. acidophilus* probiotic strain. Another consideration is that tea leaves contain phenolic compounds, particularly tannins and other tea phenolics, which are considered to inhibit microbial growth [14,16,46] and the microorganisms living and compatible in Miang substrate might have mechanisms allowing them to tolerate the tea tannins. In addition, the cell wall of *L. plantarum* and *L. pentosus* contain the meso-diaminopimelic acid (mDAP) peptidoglycan, which means they can survive in the tannin-rich substrate [14,16,18] and therefore potentially have greater survival capability against in vitro gastrointestinal conditions than the *L. acidophilus* probiotic strain.

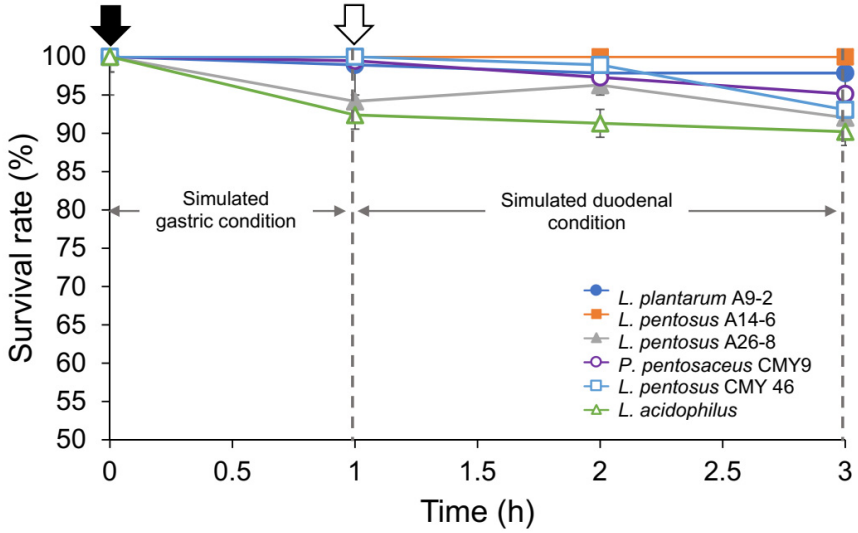

Figure 3. Survival of *L. plantarum* A9-2, *L. pentosus* A14-6, *L. pentosus* A26-8, *P. pentosaceus* CMY9, *L. pentosus* CMY46, and *L. acidophilus* under simulated gastrointestinal conditions. Black and white arrows indicate addition of simulated gastric juice and simulated duodenal juice, respectively.

3.5. Resistance to Antibiotics

Antibiotics are major antimicrobial agents utilized to fight bacterial pathogens. However, antibiotic resistance and its transfer to pathogens in the natural environment can cause significant danger and suffering for many people with pathogen infections [47]. Therefore, it is desirable that probiotics are sensitive to commonly prescribed antibiotics at low concentrations. The results of antibiotic susceptibilities (Table 3) showed all five LAB candidates were sensitive to erythromycin and tetracycline, and were moderately susceptible to gentamycin. On the other hand, all strains were resistant to kanamycin, vancomycin, and polymycin. The vancomycin resistance of the selected probiotic LAB in this experiment supports the fact that the majority of the lactobacilli were intrinsically resistant to glycopeptide [2]. The susceptibility and resistance of LAB against various antibiotics is variable depending on the species; for example, LAB isolated from infant feces are resistant to kanamycin and streptomycin [48]. Thus, the resistance mechanisms observed among these strains are probably inherent or intrinsic to their species and could therefore not be attributed to the acquisition of resistance genes [49]. It has been reported that probiotic strains should be susceptible to at least two clinically relevant antibiotics [50]. All selected LAB strains in this experiment were susceptible to erythromycin and tetracycline, and this meets the good probiotics criteria in term of antibiotic-resistant properties. Furthermore, all LAB strains showed negative hemolytic activity, which is the supporting property for their in vivo safety.

Table 3. Susceptibility of LAB isolates to antibiotics.

Isolates	Antibiotics						
	VA	**K**	**CN**	**S**	**TE**	**E**	**PB**
L. plantarum A9-2	R	I	S	R	S	S	R
L. pentosus A14-6	R	R	I	R	S	S	R
L. pentosus A26-8	R	R	I	R	S	S	R
P. pentosaceus CMY9	R	R	I	R	S	S	R
L. pentosus CMY46	R	R	I	R	S	S	R

Note: VA: vancomycin (30 µg/disc), K: kanamycin (30 µg/disc), CN: gentamycin (10 µg/disc), S: streptomycin (10 µg/disc), TE: tetracycline (30 µg/disc), E: erythromycin (15 µg/disc) PB: polymycin (30 µg/disc). Zone of clearing: various antibiotic resistant (R), moderately susceptible (I), and susceptible (S).

3.6. Hydrophobicity and Auto-Aggregation

Hydrophobicity and auto-aggregation assays were employed as indirect screening tools to test and select the adhesion potentiality of probiotic bacteria to host intestinal mucosa [51]. The cell surface hydrophobicities of the selected LAB strains are presented in Figure 4A. All isolates showed a high percentage of hydrophobicity towards chloroform which ranged from 58.3 to 92.9% after a 1 h incubation period. The highest hydrophobicity percentage was found with *L. pentosus* A14-6. Generally, hydrophobicity above 40% is desired for a probiotic strain [52], and all selected probiotic LAB in this study therefore qualified in terms of cell hydrophobicity properties. Previous studies have reported on the high degree of variation of the hydrophobicity property among probiotic LAB [31,53,54]. *L. plantarum* and *L. casei* originally isolated from Ladakh fermented foods have shown cell surface hydrophobicities ranging from 5–74% with *n*-hexadecane [31]. The high hydrophobicity property of probiotic strains represents higher interaction with the epithelium cells of gastrointestinal tract, which indicate the better exclusion of pathogens [55]. Differences in cell surface hydrophobicity are caused from variations in the levels of cell surface protein expression of the species [56].

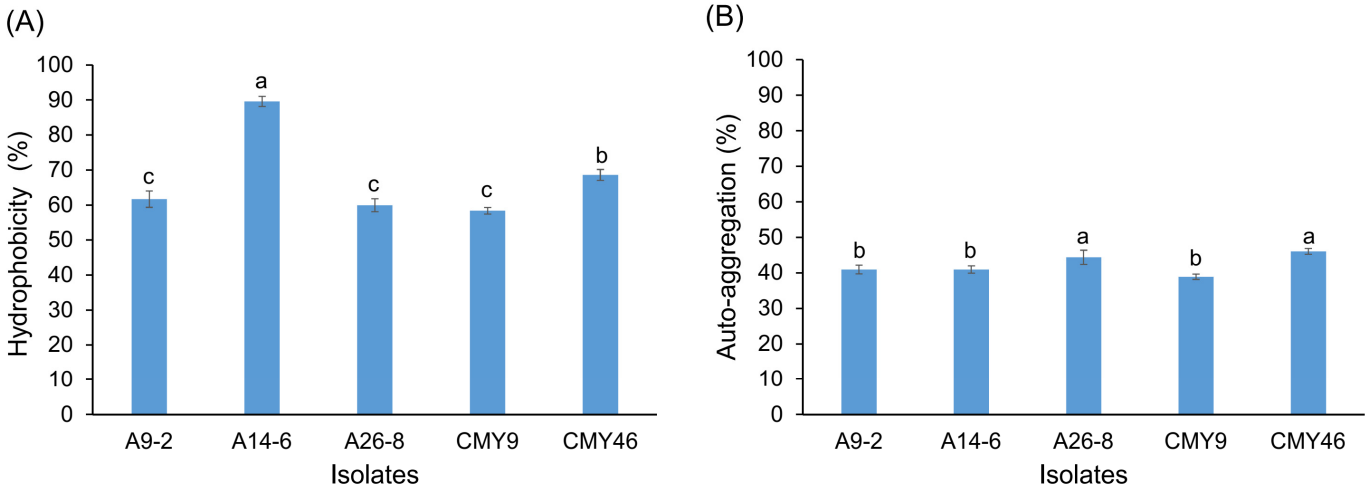

Figure 4. Cell surface hydrophobicity (**A**), and auto-aggregation (**B**) of *L. plantarum* A9-2, *L. pentosus* A14-6, *L. pentosus* A26-8, *P. pentosaceus* CMY9, and *L. pentosus* CMY46. Different letters (a–c) indicate significant differences of the values ($p < 0.05$).

Auto-aggregation of probiotic LAB is believed to align with adhesion of the LAB to the intestinal epithelium. The percentages of auto-aggregation of all selected LABs were found to be in the range of 38.9 to 46.0% after 2 h incubation (Figure 4B), which indicated moderate capability to colonize host intestinal cells, while the auto-aggregative values of *S.* Typhimurium and *S. aureus* were 33.7% and 15.8%, respectively, after incubation at 37 °C for 2 h [28]. The higher auto-aggregation properties are reported to increase in relation to LAB colonization in the gastrointestinal tract. Auto-aggregation values are strain-specific and the wide range of auto-aggregation abilities (1.63–80.50%) have been reported for *L. brevis*, *L. plantarum*, *L. acidophilus*, *L. curvatus*, *L. sake*, *L. fermentum*, and *P. pentosaceus* isolated from fermented foods [31].

3.7. Radical Scavenging Activities of LAB

Antioxidant activities of CFCS and intact cells of the five selected LAB isolates were evaluated by the DPPH free radical scavenging assay method and their antioxidant capacities are presented in Figure 5A. CFCS of all probiotic LAB strains showed higher radical scavenging activities compared to their intact cells. However, the intact cells of *L. pantarum* A9-2, *L. pentosus* A14-6, and *L. pentosus* A26-8 showed higher DPPH scavenging activity than *P. pentosaceus* CMY9 and *L. pentosus* CMY46. Recently, LAB such as *Lactobacillus* sp. and *Bifidobacterium* sp. revealed significant antioxidant characteristics, which a study has

suggested means that the antioxidant properties mainly depend on the type of probiotic bacteria used [57]. Moreover, the antioxidant activity of *L. plantarum* derived from pickles, tea, sauerkraut, fermented dairy products, fermented beverages, and the feces of healthy infants have also been reported [32,58,59]. The mechanisms of the antioxidant activity of LAB intact cells were suggested to be responsible due to metal ion chelation, enzymes such as antioxidases from probiotics, antioxidant compounds produced by probiotic cells, or countering radicals generated in the intestinal tract [60,61]. However, the oxidation-resistant ability of probiotics and their mechanisms are not completely clear.

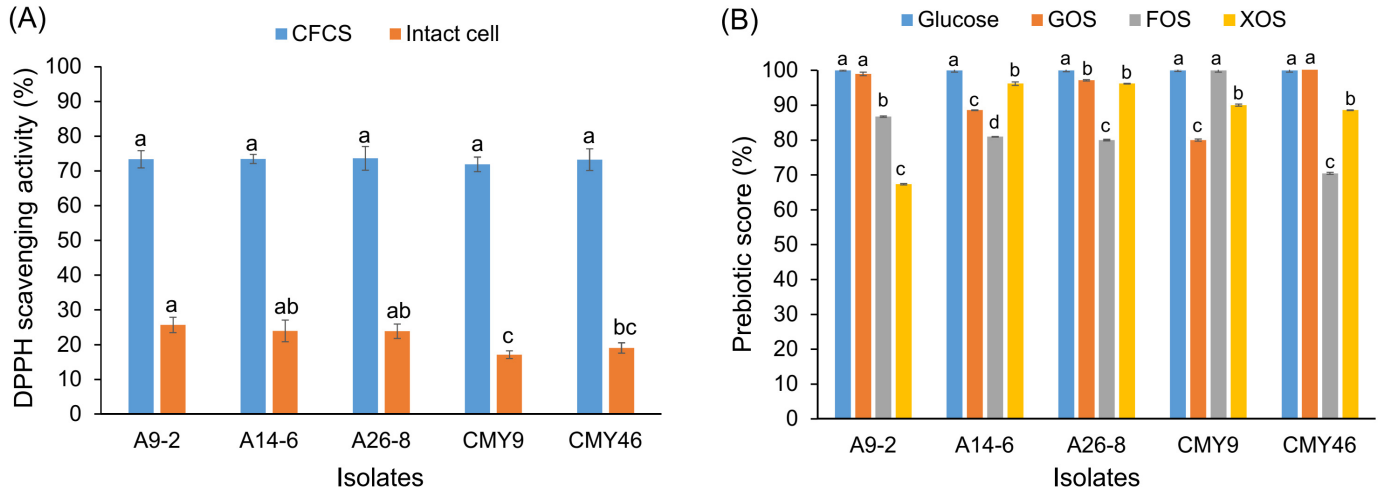

Figure 5. DPPH scavenging activity (**A**) and prebiotic scores (**B**) of *L. plantarum* A9-2, *L. pentosus* A14-6, *L. pentosus* A26-8, *P. pentosaceus* CMY9, and *L. pentosus* CMY46. Different letters (a–c) indicate significant differences of the values ($p < 0.05$).

3.8. Prebiotic Utilization and Prebiotic Scores

The synbiotic concept was created to overcome the difficulties of growing of probiotics in the gastrointestinal tract. Synbiotics have beneficial synergistic effects, greater than those observed for the individual administration of only prebiotics or probiotics [62]. Therefore, the study of prebiotic utilization by the selected LAB was explored. The growths of all LAB strains in the presence of various prebiotics, including GOS, XOS, and FOS, in comparison to glucose (positive control) are presented in Figure 5B. *L. plantarum* A9-2, *L. pentosus* A26-8, and *L. pentosus* CMY46 showed the maximum growth with GOS, meanwhile, the strains *L. pentosus* A14-6 and *P. pentosaceus* CMY9 showed their maximum growth with XOS and FOS, respectively. The results from this study indicate that all selected probiotic LAB had potential for application in the design of formulation of synbiotics with various types of commercial prebiotics. However, the improved specificity of probiotic LAB and prebiotic types may lead to the highest efficiency of formulated synbiotic products. Therefore, a determination of specific properties between prebiotics and probiotics is the most appropriate approach to be further fine tuned for commercial applications.

3.9. Viability of Probiotic Strains during Spray-Drying

The effect of spray-drying on the viability of the LAB strains is shown in Table 4. The viability of *L. pantarum* A9-2, *L. pentosus* A14-6, *L. pentosus* A26-8, *P. pentosaceus* CMY9, and *L. pentosus* CMY46 decreased after spray-drying by 2.01, 2.03, 2.10, 1.69 and 1.88 log, respectively. The survival rates of all strains were up to 75% after spray-drying. Even with reduction of microbial count, this preliminary study of spray-drying indicated the feasibility of applying these LAB strains at industrial levels and can be starting point for improving final microbial counts. The improved viability and efficacy of probiotics after exposure to the spray-drying process may be improved by the use of other thermoprotectants such as different sugars, skim milk, and whey protein. Previous reports mentioned that the conditions of the spray-drying process influence the quality and efficacy of spray-dried

probiotic powder [63–66]. Therefore, the optimization of the spray-drying process is required before applying these selected LAB strains in a commercial scale.

Table 4. Survival of five selected LAB isolates after spray-drying with maltodextrin as the thermoprotectant.

Isolates	Viable Cell (logCFU/g)		Survival Rate (%)
	Before Spray-Drying	After Spray-Drying	
L. plantarum A9-2	8.42 ± 0.02	6.41 ± 0.07	76.1 ± 0.65 ^{bc}
L. pentosus A14-6	8.61 ± 0.06	6.58 ± 0.11	76.4 ± 0.74 ^{bc}
L. pentosus A26-8	8.49 ± 0.11	6.39 ± 0.05	75.3 ± 0.38 ^{c}
P. pentosaceus CMY9	8.55 ± 0.07	6.86 ± 0.04	80.2 ± 0.18 ^{a}
L. pentosus CMY46	8.36 ± 0.09	6.48 ± 0.05	77.5 ± 0.23 ^{b}

Note: Means in column with different superscripts are statistically different at $p < 0.05$.

4. Conclusions

The results obtained from this study indicate that Miang, traditional fermented tea leaves, can serve as a beneficial source of potential probiotic candidates. Five selected LAB strains originally isolated from Miang samples clearly demonstrated survival under simulated gastrointestinal conditions, which indicates their beneficial capabilities could be applied as probiotics. These selected LAB strains also showed specificity for being utilized in combinations of commercial prebiotics and tolerance against spray-drying process. Among the five strains, *L. pentosus* A14-6 in particular had the most promising probiotic potential, with cellular antioxidative characteristics that support its feasibility for application in various food products or as targeted towards the development of synbiotic nutraceuticals. However, the safety and functional properties of these strains should be further evaluated to confirm their health-beneficial properties in in vivo models.

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