Selective Thermotolerant Lactic Acid Bacteria Isolated From Fermented Juice of Epiphytic Lactic Acid Bacteria and Their Effects on Fermentation Quality of Stylo Silages

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The aim of the present study was to isolate and identify lactic acid bacteria (LAB) from fermented juice of tropical crops such as Napier grass, Ruzi grass, Purple guinea grass, Stylo legume, and Leucaena and their application to improve the quality of tropical crop silage. Fifteen strains of LAB were isolated. The LAB strains were Gram-positive and catalase-negative bacteria and could be divided into three groups, i.e., *Pediococcus pentosaceus*, *Lactiplantibacillus* (para)plantarum, and *Limosilactobacillus fermentum* according to the biochemical API 50CH test. Based on the analysis of 16S rRNA sequence, the strains isolated in the group *L. (para)plantarum* were distinguished. Two isolates (N3 and G4) were identified as *Lactiplantibacillus plantarum*. Three isolates (St1, St2, and St3) were identified as *L. paraplantarum*. In addition, the identification of other isolates was confirmed in the group *P. pentosaceus* (R1, R4, R5, R8, R11, and L1) and the group *L. fermentum* (N4, G6, G7, and N4). All selected strains were able to grow at 50°C. All LAB strains showed antimicrobial activity against *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931, *Pseudomonas aeruginosa* ATCC 27853, and *Bacillus cereus* ATCC 11778. Four selected LAB strains (St1, St3, N4, and R4) were tested for their capacity to successfully ensile Stylo legume (*Stylosanthes guianensis* CIAT184). Stylo silages treated with LAB were well preserved, the NH₃–N and butyric acid contents were lower, and the lactic acid content was higher than those in the control (p < 0.05). The acetic acid content was the highest in R4-treated silage among the treatments (p < 0.05). The crude protein (CP) content of St1-silage was significantly (p < 0.05)
higher than the others. The inoculation of thermotolerant LAB selected from fermented juice of epiphytic lactic acid bacteria (FJLB) was found to be highly instrumental to obtain well-preserved silage from the Stylo legume.

Keywords: forage crop, identification, inoculants, isolation, silage fermentation

INTRODUCTION

Ensiling is a widely used method of preserving moist forage for livestock in many countries. To achieve stable, nutritious silage, a rapid growth of lactic acid bacteria (LAB) is desired because LAB rapidly convert water-soluble carbohydrates (WSC) into lactic acid, thereby causing a rapid decrease in pH, which prevents, among others, excessive proliferation of clostridia (Ni et al., 2015). Naturally occurring epiphytic LAB populations on plant materials are generally heterofermentative and low in initial numbers (Cai, 1999; Eitan et al., 2006), causing a less successful fermentation as indicated by high pH and NH₃–N content in silage (Liu et al., 2011, 2012). Next to the aforementioned issues, high environmental temperatures may also complicate successful fermentation of forage (Chen et al., 2013; Li et al., 2019). The latter observation may be related to the inability of specific LAB to grow at high temperatures (Weinberg et al., 2001). Indeed, the temperature can reach values up to 50°C (Muck, 2010) when forage is ensiled under tropical conditions. Thus, the inoculation of forage with heat-tolerable LAB prior to ensiling may be instrumental to achieve a well-preserved silage. However, to the best of the author’s knowledge, specific heat-tolerable epiphytic LAB are not yet identified and tested for their potential to successfully ensile tropical forages. In the current study, we isolated and identified LAB from fermented juice of epiphytic LAB (Bureenok et al., 2005) from various tropical forages. Fermented juice of epiphytic LAB (FJLB) contains multiple LAB strains (Wang et al., 2009) and is therefore considered a good source to screen for suitable LAB. Selected LAB were subsequently tested for their capacity to successfully ensile Stylo legume (S. guianensis CIAT184). Stylo was selected because of its practical relevance in the tropics.

MATERIALS AND METHODS

Preparation of FJLB and Isolation of LAB Strains

Forage-specific FJLB was prepared from fresh Napier grass (Pennisetum purpureum), Ruzi grass (Brachiaria ruziizensis), Purple guinea grass (Panicum maximum TD58), Stylo legume (S. guianensis), and Leucaena (Leucaena leucocephala) as described by Bureenok et al. (2005). Briefly, 25 g of each forage was macerated in 50 ml of distilled water in a blender. Then, the content of the blender was filtered over a double layer of sterilized cheese cloths into a glass bottle containing 1% glucose solution. The bottles were capped and stored under anaerobic conditions at 30°C for 3 days. Then, each forage-specific FJLB was spread on lactobacilli de Man, Rogosa, Sharpe (MRS) agar and incubated at 35°C for 48 h under anaerobic conditions. Thereafter, the predominant LAB colony was isolated and purified twice by streaking on MRS agar plates.

Morphological and Physiological Tests of the Selected Lactic Acid Bacterial Strains

Gram stain, morphology, catalase activity, and gas production from glucose were determined according to the methods for LAB identification as described by Kozaki et al. (1992). Growth at different pH values was observed in MRS broth (adjusting pH with 0.5 N HCl or NaOH) after incubation at 37°C. Growth at different temperatures was observed in MRS broth after incubation at 35°C and 45°C for 5 days. The turbidity of each tube was also noted as an indication of growth or no growth. Each treatment was tested with triplicate tubes. Growth curves for the isolates at 50°C were constructed by plotting the optical density at 600 nm against time. Carbohydrate fermentation was performed by API 50 CHI assay (BioMérieux, Marcy-l’Étoile, France). LAB isolates were cultivated in 5 ml of MRS broth overnight at 30°C. The turbidity of the cultured broth was examined by the McFarland method. Cell suspension was transferred into each of the wells on the API 50 CH strips. All wells were coated with sterile liquid paraffin oil and incubated at 30°C. The results were read after 24 h and verified after 48 h. Fermentation of the carbohydrate medium was indicated by a yellow color, except for esculine (dark brown). Color reactions were scored against a chart provided by the manufacturer.

Identification of LAB Strains by 16S rRNA Sequence Analysis

The DNA of LAB isolates was extracted and purified using a Genomic DNA mini kit (Blood/culture cell) (Geneaid Biotech Ltd., Taiwan) according to the instructions of the manufacturer. Partial fragments of the 16s rRNA genes of each bacterial isolate were amplified using the forward primer 20F (5’-GAG TTT ACC TTG TTA CGA CTT-3’) and the reverse primer 1500R (5’-GTG ACC TTA CGA CTT-3’) (Brosius et al., 1981). The polymerase chain reaction (PCR) mixtures contained the extracted DNA as a template, 2.0 mM MgCl₂, 0.2 mM dNTP, and 10 µl of 10X Taq buffer, 2.5 units of Taq polymerase, and the total volume was brought up to 100 µl. The PCR cycle of reactions consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and primer extension at 72°C for 1 min with a final extension at 72°C for 3 min. The amplicons of LAB were analyzed by means of gel electrophoresis using 0.8% (w/v) agarose and purified with a GeneHelp Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). The purified PCR products were sequenced by The Macrogen Laboratory (Seoul, South Korea). The resulting 16S rRNA gene sequence of the isolate was analyzed.
The isolated LAB strains were inoculated in MRS broth and Lactic Acid Bacteria Inhibition Activity Determination of (Kim et al., 2012).

The isolated LAB strains were inoculated in MRS broth and statically incubated at 30°C for 48 h. Cell-free supernatants were collected by centrifugation (10,000 × g, 4°C for 15 min) of LAB cultures and filtered through a 0.22-µm-diameter filter to remove residual cells. The agar well diffusion method (Li et al., 2015) was used to evaluate the antimicrobial activity of the selected LAB strains against the following indicator strains of bacteria: Escherichia coli ATCC 25922, Shigella sonnei ATCC 25931, Pseudomonas aeruginosa ATCC 27853 (Gram-negative bacteria), and Bacillus cereus ATCC 11778 (Gram-positive bacteria). Cell-free supernatants (100 µl) were added into wells (7.80 mm in diameter) on nutrient agar plates inoculated with the indicator strains. All plates were incubated for 16–18 h at 30°C. The diameters of inhibition zones were recorded.

Preparation of the Experimental Silages

Stylo (S. guianensis CIAT184) was harvested 60 days after regrowth and chopped with a forage cutter to 2–4 cm and then sampled immediately to determine its macronutrient composition. Four selected LAB strains (Lactiplantibacillus paraplantarum St1, L. paraplantarum St3, Limosilactobacillus fermentum N4, and Pediococcus pentosaceus R4) were applied as silage additives. Next to the selected LAB strains, also forage-specific FJLB was prepared from fresh Stylo as silage additives. For the first step of the screening process, 15 strains were isolated from various forage-specific FJLBs: two strains from Napier grass (N1 and N4), five strains from Ruzi grass (R1, R4, R5, R8, and R11), four strains from Purple guinea grass (G3, G4, G6, and G7), three strains from Stylo legume (St1, St2, and St3), and one strain from Leucaena (L1). All strains were typified as Gram-positive and catalase-negative (Table 1). Among them, R1, R4, R5, R8, R11, and L1 were cocci; others were rod shaped. Based on the end products of glucose fermentation, strains N4, G3, G4, G6, and G7 were classified as heterofermenters, while the remaining strains (N3, G4, St1, St2, St3, R1, R4, R5, R8, R11, and L1) were classified as homofermenters. Except for strain L1 at pH 8, all other strains grew well at various pH levels (3.5, 4.0, 4.5, and 8.0). Moreover, all strains grew well at 35 and 45°C. The ability to grow at 50°C was tested with all the strains (Figure 1) by measuring the density of cell populations in liquid culture over time, and it appeared that the strains St1, St2, St3, N3, and G4 exhibited slight growth, while the other strains grew well.

Based on the API 50CH results, the 15 isolates could be classified into three groups (Supplementary Table 1). Group

Inhibition Activity Determination of Lactic Acid Bacteria

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and edited with the use of the Chromas 2.33 and BioEdit program (Hall, 1999). A comparative analysis of 16S rRNA gene sequences from the LAB isolates and all type strains related to the isolate was performed using CLUSTAL W version 1.83 (Thompson et al., 1994). The phylogenetic tree construction based on the 16S rRNA gene was performed using the neighbor-joining approach (Saitou and Nei, 1987) listed in the MEGA version 7 software (Kumar et al., 2016). The phylogenetic distances between the sequences were calculated according to Kimura’s two-parameter model (Kimura, 1980). The robustness of individual branches of the tree was estimated by using bootstrap based on 1,000 replicates (Felsenstein, 1985). The 16S rRNA gene sequence similarities of the isolate were determined using the database of EZBioCloud1 (Kim et al., 2012).

Chemical Analyses

The dry matter (DM) content of fresh Stylo and the experimental forages was determined after oven-drying at 60°C for 48 h. The nitrogen (N) contents were determined by the macro Kjeldahl method (AOAC, 1995). A factor of 6.25 was used to convert N into crude protein (CP). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to the method of Van Soest et al. (1991), and values are expressed inclusive of residual ash. Buffering capacity and WSC content were determined according to the method as described by Playne and McDonald (1966) and Dubois et al. (1956), respectively.

Lactic acid and volatile fatty acids (VFAs) in silage extracts were measured by HPLC (Aminex HPX-87H, 300 mm × 7.8 mm i.d.; column temperature, 40°C flow rate, 0.60 ml/min, Shimadzu Ltd., Kyoto, Japan). LAB in fresh Stylo legume and the experimental silages were enumerated on MRS agar, and plates were incubated at 35°C for 48 h. The NH3–N content of the silage extract was determined using a steam distillation technique (Cai, 2004).

Statistical Analyses

Data were subjected to one-way analysis of variance (ANOVA); the differences between treatment means were compared using Tukey’s t-test using SPSS for Windows version 16.0 SPSS (2007). Statistical Package for the Social Science. SPSS Inc., Chicago, United States. The level of statistical significance was declared at p < 0.05.

RESULTS

The Morphological and Physiological Properties of LAB Strains Isolated From FJLB

The isolation of bacteria using the MRS medium under anaerobic conditions allowed the identification of different LAB with similar or identical morphology from each FJLB. Based on the first step of the screening process, 15 strains were isolated from various forage-specific FJLBs: two strains from Napier grass (N1 and N4), five strains from Ruzi grass (R1, R4, R5, R8, and R11), four strains from Purple guinea grass (G3, G4, G6, and G7), three strains from Stylo legume (St1, St2, and St3), and one strain from Leucaena (L1). All strains were typified as Gram-positive and catalase-negative (Table 1). Among them, R1, R4, R5, R8, R11, and L1 were cocci; others were rod shaped. Based on the end products of glucose fermentation, strains N4, G3, G4, G6, and G7 were classified as heterofermenters, while the remaining strains (N3, G4, St1, St2, St3, R1, R4, R5, R8, R11, and L1) were classified as homofermenters. Except for strain L1 at pH 8, all other strains grew well at various pH levels (3.5, 4.0, 4.5, and 8.0). Moreover, all strains grew well at 35 and 45°C. The ability to grow at 50°C was tested with all the strains (Figure 1) by measuring the density of cell populations in liquid culture over time, and it appeared that the strains St1, St2, St3, N3, and G4 exhibited slight growth, while the other strains grew well.

Based on the API 50CH results, the 15 isolates could be classified into three groups (Supplementary Table 1). Group

1http://eztaxon-e.ezbiocloud.net/
### TABLE 1 | The characteristics of the selected lactic acid bacteria (LAB) isolates.

| Source of isolated strain of LAB | Napier grass | Ruzi grass | Purple guinea grass | Stylo legume | Leucaena |
|----------------------------------|-------------|------------|---------------------|--------------|----------|
| LAB strain                       | N3 | N4 | R1 | R4 | R5 | R8 | R11 | G3 | G4 | G6 | G7 | St1 | St2 | St3 | L1 |
| Shape                            | Rod | Rod | Coccus | Coccus | Coccus | Coccus | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Coccus | |
| Gram stain                       | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| Gas from glucose                 | − | + | − | − | − | − | − | + | − | + | + | − | − | − | − | |
| Fermentation type                | Homo | Hetero | Homo | Homo | Homo | Homo | Hetero | Homo | Hetero | Hetero | Homo | Homo | Homo | Homo | Homo | |
| Catalase activity                | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Growth at pH                     | 3.5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| 4.0                              | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| 4.5                              | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| 8.5                              | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | w |
| Growth at temperature            | 35°C | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| 45°C                             | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |

Identified as (16S rRNA)

- *Lactiplantibacillus plantarum*
- *Limosilactobacillus fermentum*
- *Pediococcus pentosaceus*
- *Limosilactobacillus fermentum*
- *Lactiplantibacillus paraplantarum*
- *Lactiplantibacillus fermentum*
- *Limosilactobacillus fermentum*
- *Lactiplantibacillus paraplantarum*
- *Lactiplantibacillus paraplantarum*
- *Pediococcus pentosaceus*

+, positive; −, negative; w, weakly positive.
P. pentosaceus consisted of strains R1, R4, R5, R8, and R11 isolated from FJLB of Ruzi grass and strain L1 isolated from FJLB of Leucaena. LAB strains within this group were able to ferment xylose but not D-lactose, D-saccharose, and D-melibiose. Group L. (para)plantarum consisted of strain N3 (FJLB of Napier grass), strain G4 (FJLB of Purple guinea grass), and strains St1, St2, and St3 (FJLB of Stylo legume). The LAB strains within the group L. (para)plantarum were able to ferment α-methyl-D-mannopyranoside and D-lactose. Group L. fermentum contained the strains N4 (FJLB of Napier grass) and G3, G6, and G7 isolated from FJLB of Purple guinea grass. LAB within the group L. fermentum produced acid from D-raffinose but not N-acetyl glucosamine.

16S rRNA Gene Sequencing Analysis
In a phylogenetic tree based on 16S rRNA gene sequences, all 15 strains isolated from FJLB were divided into three groups similar to the API analysis (Figure 2). Six strains (R8, R5, R1, L1, R11, and R4) were grouped with P. pentosaceus on the phylogenetic tree with a bootstrap value of 100% and showing more than 99% similarity in their 16S rRNA gene sequences. Thus, these strains were identified as P. pentosaceus. Considering the phylogenetic positions observed, the type strain of L. paraplantarum DSM 10667T was distinguished from L. plantarum CIP 103151T. Strains N3 and G4 were close to the L. plantarum CIP 103151T, with 100 and 99.91% similarity in their 16S rRNA gene sequences, respectively. Strains St1, St2, and St3 were categorized in the L. paraplantarum cluster and showed a similarity of 16S rRNA of 100% with L. paraplantarum DSM 10667T. Four strains (N4, G3, G6, and G7) were most closely related to L. fermentum JCM 1173T. Strains N4, G3, and G6 exhibited 100% 16S rRNA gene sequence pairwise similarities with the closely related species, L. fermentum JCM 1173T. Only strain G7 showed 99.91% similarity of 16S rRNA gene sequences with the type strain L. fermentum JCM 1173T.

Antibacterial Activity Against Pathogenic Bacteria
A total of 15 isolated LAB were tested for antagonistic activity against E. coli, S. sonnei, P. aeruginosa, and B. cereus by means of an agar diffusion test. Most of the strains showed antimicrobial activity against E. coli and P. aeruginosa (Supplementary Table 2). Except for strains G6 and G7, all other LAB strains showed zones of inhibition against S. sonnei and B. cereus.

Selected Indices of Fermentation and Chemical Composition of the Experimental Silages
The silage treated with St1 had a higher CP content compared with others (Table 2). N4-silages had lower WSC than the other silages. Lactic acid content was lower in the control silages (p < 0.05). The higher amount of acetic acid was higher in silage treated with L. fermentum N4. Except for the control silage, butyric acid could not be detected in all the silages treated with either FJLB or selected LAB. Stylo silage inoculated with all LAB strains had lower (p < 0.05) NH₃–N content than the control silage. Control silage pH was higher than the St1-treated silages (p < 0.05).
### TABLE 2 | Chemical composition of fresh and ensiled Stylo, and selected indices of fermentation after 45 days of ensiling with or without additional LAB.

| Chemical composition | Fresh Stylo | Control | Stylo ensiled with additional LAB | SEM | Sig. |
|----------------------|-------------|---------|-----------------------------------|-----|------|
| Dry matter (g kg⁻¹ fresh) | 280 | 275<sup>ab</sup> | 265<sup>ab</sup> | 279<sup>a</sup> | 258<sup>b</sup> | 266<sup>ab</sup> | 269<sup>ab</sup> | 1.44 | 0.017 |
| Crude protein | 102 | 90<sup>b</sup> | 101<sup>b</sup> | 109<sup>a</sup> | 98<sup>c</sup> | 101<sup>b</sup> | 91<sup>cd</sup> | 0.69 | <0.001 |
| Neutral detergent fiber | 689 | 673 | 680 | 686 | 649 | 669 | 693 | 6.99 | 0.487 |
| Acid detergent fiber | 499 | 510<sup>ab</sup> | 517<sup>a</sup> | 485<sup>ab</sup> | 472<sup>b</sup> | 497<sup>ab</sup> | 514<sup>ab</sup> | 3.70 | 0.026 |
| Hemicellulose | 190 | 163 | 163 | 163 | 177 | 162 | 179 | 7.32 | 0.631 |
| Water-soluble carbohydrates | 45.0 | 3.0<sup>b</sup> | 4.0<sup>ab</sup> | 4.8<sup>a</sup> | 4.5<sup>b</sup> | 1.4<sup>c</sup> | 4.1<sup>ab</sup> | 0.15 | <0.001 |
| Buffer capacity (meq kg⁻¹ fresh) | 250 | na | na | na | na | na | na | na | na |
| LAB (log CFU g⁻¹ fresh) | 4.88 | 6.70<sup>ab</sup> | 7.03<sup>a</sup> | 6.20<sup>b</sup> | 7.15<sup>a</sup> | 6.82<sup>a</sup> | 7.06<sup>a</sup> | 0.05 | 0.002 |
| Lactic acid | na | 33.91<sup>b</sup> | 55.54<sup>ab</sup> | 75.50<sup>a</sup> | 67.60<sup>ab</sup> | 86.75<sup>a</sup> | 81.72<sup>a</sup> | 2.93 | 0.002 |
| TFA | na | 52.91<sup>b</sup> | 64.34<sup>a</sup> | 85.16<sup>b</sup> | 84.43<sup>ab</sup> | 148.47<sup>a</sup> | 89.18<sup>b</sup> | 4.12 | <0.001 |
| Profile of individual Fa (fraction of TFA) | | | | | | | | |
| LVA/TFA | na | 0.62<sup>bc</sup> | 0.86<sup>a</sup> | 0.89<sup>a</sup> | 0.83<sup>ab</sup> | 0.58<sup>c</sup> | 0.91<sup>a</sup> | 0.18 | <0.001 |
| HAc/TFA | na | 0.29<sup>b</sup> | 0.14<sup>bc</sup> | 0.11<sup>bc</sup> | 0.17<sup>bc</sup> | 0.37<sup>a</sup> | 0.08<sup>b</sup> | 0.02 | 0.001 |
| HBut/TFA | na | 0.06<sup>a</sup> | bdl | bdl | bdl | bdl | bdl | bdl | 0.005 | <0.001 |
| HProp/TFA | na | 0.02 | 0 | 0 | 0 | 0 | 0 | 0.04 | 0.003 | 0.093 |
| NH₃-N (g kg⁻¹ total N) | na | 121.92<sup>a</sup> | 103.64<sup>ab</sup> | 70.12<sup>c</sup> | 80.64<sup>bc</sup> | 84.07<sup>bc</sup> | 88.76<sup>bc</sup> | 2.82 | 0.003 |
| pH | 5.64 | 4.71<sup>a</sup> | 4.40<sup>a</sup> | 4.32<sup>ab</sup> | 4.37<sup>ab</sup> | 4.41<sup>ab</sup> | 4.51<sup>b</sup> | 0.03 | 0.047 |

Unless indicated otherwise, values are expressed as g kg⁻¹ dry matter. CFU, colony-forming units; Con, Stylo ensiled without additional lactic acid bacteria; FJLB, fermented juice of epiphytic lactic acid bacteria; LPL-1, Lactiplantibacillus paraplantarum St1; LPL-3, Lactiplantibacillus paraplantarum St3; LF, Limosilactobacillus fermentum N4; PP, Pediococcus pentosaceus R4; TFA = total fermentation acids (i.e., lactic acid (LA) + acetic acid (HAc) + propionic acid (HProp) + butyric acid (HBut)); na, not analyzed; bdl, below detection limit (zero value was used when data were statistically analyzed). Means with different superscripts within rows differ significantly (p < 0.05).
FIGURE 2 | Phylogenetic relationships of lactic acid bacteria isolated from forage-specific fermented juice of lactic acid bacteria. The phylogenetic tree based on 16S rRNA gene sequences of 831 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. *Bacillus subtilis NCDO1769* and *Bifidobacterium bifidum DSM 20456* were used as the outgroup.

**DISCUSSION**

**LAB Strains Isolated From FJLB**

Fermented juice of epiphytic LAB has been successfully used as an additive to ensile tropical grasses (Bureenok et al., 2005, 2011). In the current study, homolactic and heterolactic bacteria were isolated from the FJLB of different forage crops, i.e., Napier grass, Ruzi grass, Purple guinea grass, Stylo legume, and Leucaena. Homofermentative LAB produce two molecules of lactic acid from the fermentation of hexoses, whereas heterofermentative LAB produce one molecule of lactic acid, one molecule of other products (acetic acid, propionic acid, or ethanol), and CO₂ (Kung et al., 2003). In the case of facultative heterofermentation, LAB not only produce mainly lactic acid from hexose but also degrade pentose polymers, such as xylose, to lactic acid and acetic acid or ethanol (Oude Elferink et al., 2000). Based on the results of 16S rRNA analysis, the dominant LAB strains isolated from FJLB were identified as *L. plantarum*, *L. paraplantarum*, *L. fermentum*, from the fermentation of hexoses, whereas heterofermentative LAB produce one molecule of lactic acid, one molecule of other products (acetic acid, propionic acid, or ethanol), and CO₂ (Kung et al., 2003). In the case of facultative heterofermentation, LAB not only produce mainly lactic acid from hexose but also degrade pentose polymers, such as xylose, to lactic acid and acetic acid or ethanol (Oude Elferink et al., 2000). Based on the results of 16S rRNA analysis, the dominant LAB strains isolated from FJLB were identified as *L. plantarum*, *L. paraplantarum*, *L. fermentum*,
and *P. pentosaceus*. Khota et al. (2016) reported that the natural dominant strains of LAB species from Guinea grass and Napier grass were identified as *L. plantarum* and *Lactococcus lactis* casei that could grow at lower pH and produce more lactic acid compared to the other isolates. Moreover, *L. plantarum* has been isolated from many kinds of grass such as king grass, vetch, tall fescue, and perennial ryegrass (Wang et al., 2017; Shah et al., 2018). The *Pediococcus* spp. have been observed as the prevalent species in forage plants or silages such as corn, Alfalfa, Guinea, and Triticale grass (Cai et al., 1999; Kongsan et al., 2019; Soundharajan et al., 2019). The predominance of LAB species that were found in the silage may be due to the prevalence of Mn²⁺ in plant materials (Daeschel et al., 1987; Boyaval, 1989). Epiphytic LAB such as *L. plantarum*, *L. fermentum*, and *P. pentosaceus* can accumulate Mn²⁺ from plants into their cells, thereby acting as a defense mechanism against oxygen toxicity (Daeschel et al., 1987; Kongsan et al., 2019). In this study, we screened thermotolerant LAB for developing a silage inoculant to be applied under tropical conditions. All LAB strains isolated from FJLB were able to grow at 30°C. Guo et al. (2020) reported that the *L. plantarum* strain isolated from the feces of dairy cows was able to grow at 50°C. Normally, the maximum temperature for optimum LAB growth and reproduction should not exceed 45°C (McDonald et al., 1991). Matsushita et al. (2016) reported that the genomic analysis of therotolerant strains indicated a large number of mutations that are related to cell surface functions, ion and amino transporters, some transcription factor, and ROS (reactive oxygen species) in cells. There are many reports about LAB strains that have a limited capacity to adapt to high environmental temperatures and therefore have no positive effect on the process of fermentation during ensiling (Chen et al., 2013; Gulfam et al., 2017). Indeed, Guan et al. (2020) reported that LAB could not be detected after 60 days when corn was ensiled at 45°C instead of 30°C. Thus, it seems that thermotolerant LAB strains are potentially of interest to serve as an inoculant to achieve well-preserved silages in (sub)tropical regions. Furthermore, it was observed that most of the isolates in this study were able to inhibit the growth of pathogenic bacteria including *E. coli*, *S. sonnei*, and *B. cereus*. Antimicrobial compounds produced by LAB were classified as organic acids, hydrogen peroxide, and bacteriocin-like compound (Heredia-Castro et al., 2015). Li et al. (2015) reported that 39 LAB strains isolated from corn stover silage had inhibitory effect against *Salmonella enterica* ATCC 43971T, *E. coli* ATCC 11775T, and *Micrococcus luteus* ATCC4698T. The current LAB strains have the potential to inhibit the proliferation of undesirable and detrimental microorganisms, which also warrants the use of these LAB in silage making.

**Fermentation and Chemical Composition of the Stylo Silages**

Factors such as a low DM and WSC content and a high buffering capacity of material crop indicate poor conditions for lactic acid fermentation (Zhang et al., 2016). Moreover, initial LAB numbers in tropical forages are commonly too low for successful ensiling (Auerbach and Theobald, 2020). The DM content decreased by 0.28% in St1-silage after ensiling. The reduction of the CP content during the fermentation process was because of plant and microbial proteolytic processes in the ensiled material, which change the nitrogenous compounds in silages and result in an increase in soluble N and NH₃–N (Kung et al., 2018). The low pH values in all LAB-treated silages inhibited the growth of clostridia, which most likely prevented excessive CP loss (Tian et al., 2014). The ADF content of silages treated with St1 and St3 strains was reduced by 2.4–5.8% from the material crop, which is inconsistent with Liu et al. (2012). The decrease in the ADF content indicates a beneficial effect of the treatment in the improvement of the silage nutritive value and probably leads to an increase in silage digestibility in the rumen. It is generally accepted that well-preserved silages should contain pH values less than 4.5 and NH₃–N content not exceeding 100 g kg⁻¹ total N (Kung et al., 2018). In this experiment, four strains of LAB, St1, St3, N4, and R4, were selected to serve as inoculants due to their ability to produce high lactic acid, to grow at a high temperature, to produce antimicrobial activity including the isolation source. In this study, the pH values in all LAB-treated silages were low enough to prevent protein degradation to NH₃–N. Compared with the homolactic bacteria, heterolactic *L. fermentum* N4 produced greater amounts of acetic acid, but lactic acid was still the predominant end product of fermentation. The current observation is in line with that of Lau and Liong (2014) who also reported that lactic acid was the main acid produced by *L. fermentum*. Despite the greater proportion of acetic acid in the silage treated with *L. fermentum* N4, both the low pH and NH₃–N values in this silage indicate that Stylo was successfully ensiled when *L. fermentum* N4 was used as an inoculant. The lactic acid content was lower in the control silages. Compared with the homolactic bacteria, heterolactic *L. fermentum* N4 also produced high lactic acid content. *L. fermentum* produced lactic acid in a more predominant amount than acetic acid in MRS broth (Lau and Liong, 2014). This may explain the high production of lactic acid in N4-silage. The higher amount of acetic acid was found when heterolactic *L. fermentum* N4 was added in the silages. Acetic acid is a main fermentation end product when silages are inoculated with heterolactic bacteria (e.g., *Lentilactobacillus buchneri*) with a content approximately 4% DM (Kleinschmit and Kung, 2006). Adding these strains would increase the acetic acid content to inhibit yeast, which could result in better aerobic stability of silages (Paradhipta et al., 2020). The intake of acetic acid (5% as DM basis) did not negatively affect the composition and sensory quality of milk (Daniel et al., 2013); hence, the level of acetic acid in this study will not affect the feed intake and animal performance. Propionic acid is an aerobic microbial inhibitor that can inhibit the activity of molds and yeasts (Chen et al., 2017). The strain of *L. fermentum* under application can increase the aerobic stability of the treated silage. The butyric acid content was higher (*p < 0.05*) in the control silages and not detected in all treated silages. This may be caused by LAB inoculation that reduced the growth of saccharolytic clostridia, which can ferment sugar, lactic acid, and acetic acid to butyric acid (Borreani et al., 2018). N4-silages had lower WSC than the other silages. This may be caused by a greater utilization of WSC to produce a high amount of lactic and acetic acid in this strain. However, low residual WSC content is an important factor for aerobic stability of silage because yeast and molds can utilize WSC resulting in...
the rapid deterioration of silage after air exposure (Weinberg and Muck, 1996). The fermentation quality of the silages in the current study indicates that the microbial inoculants favorably affected the fermentation of Stylo legume.

CONCLUSION

Four selected strains, *L. paraplantarum* St1 and St3, *L. fermentum* N4, and *P. pentosaceus* R4, were shown to improve the fermentation quality and nutritive values of Stylo silage. The silage treated with St1 showed relatively high protein content than control and the other inoculants. The current results suggested that thermotolerant LAB strains isolated from FJLB could be used as a silage inoculant under tropical conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/Genbank/, MW673719, https://www.ncbi.nlm.nih.gov/Genbank/, MW673710, https://www.ncbi.nlm.nih.gov/Genbank/, MW673711, https://www.ncbi.nlm.nih.gov/Genbank/, MW673712, https://www.ncbi.nlm.nih.gov/Genbank/, MW673713, https://www.ncbi.nlm.nih.gov/Genbank/, MW673714, https://www.ncbi.nlm.nih.gov/Genbank/, MW673715, https://www.ncbi.nlm.nih.gov/Genbank/, MW673716, https://www.ncbi.nlm.nih.gov/Genbank/, MW673717, https://www.ncbi.nlm.nih.gov/Genbank/, MW673718, https://www.ncbi.nlm.nih.gov/Genbank/, MW673719, https://www.ncbi.nlm.nih.gov/Genbank/, MW673720, https://www.ncbi.nlm.nih.gov/Genbank/, MW673721, https://www.ncbi.nlm.nih.gov/Genbank/, MW673722, and https://www.ncbi.nlm.nih.gov/Genbank/, MW673723.

AUTHOR CONTRIBUTIONS

SB, NP, and JS contributed to the conception and design of the study, and wrote sections of the manuscript. NP organized the LAB identifications. SB performed the silage experiment and the statistical analysis. SB and NP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.673946/full#supplementary-material

SUPPLEMENTARY TABLE 1 | API 50 CH fermentation patterns of isolated LAB from each FJLB.

SUPPLEMENTARY TABLE 2 | Diameter of inhibition zones caused by the selected strains of LAB which were added to agar plates inoculated with either *Escherichia coli* ATCC 25922 (*E. coli*), Shigella sonnei ATCC 25931 (*S. sonnei*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), or *Bacillus cereus* ATCC 11778 (*B. cereus*). All values are expressed as mm, mean ± SD.

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