International Journal of Agricultural Technology 2019 Vol. 15(3): 409-424
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Chemical composition and diversity of lactic acid bacteria in guinea grass based silages

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Kongsan, S., Srikijkasemwat, K., Jirajaroenrat, K. and Suphalucksana, W. (2019). Chemical composition and diversity of lactic acid bacteria in guinea grass based silages. International Journal of Agricultural Technology 15(3): 409-424.

Abstract Ensilage is considered that widespread among conserving method of fodder crops. The successes of this process depend on bacteria in lactic acid bacteria (LAB) group as existing in forage crops. The chemical composition and diversity of lactic acid bacteria in silages based on guinea grass was investigated. Result showed that the silages contained 62.55 to 76.47% moisture, 23.53 to 37.45% dry matter (DM) and 6.11 to 15.74% ash. Groundnut silage (T3) showed the lowest crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents. Two hundred and eighty-two isolates of LAB were isolated from five silage formula based on guinea grass supplementation with either lead tree leaves or groundnut. Most of isolates were gram-positive and catalase-negative bacteria. Based on morphological characteristics and PCR-RAPD analysis, the isolates were divided into 21 groups (Guinea Grass Mixed Legume Silage (GMLS) 1 to GMLS21). The phylogenetic relation of the representative strains was analyzed from the data of 16S ribosomal DNA sequencing. Prevalent of LAB found in the silages included *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus hirae*, and *Weissella paramesenteroides*. *L. plantarum* was a dominant population and found distribution in all silage formula. This study revealed that the silage samples, including guinea grass, lead tree and groundnut contain mainly of *L. plantarum*. The information obtained can be used for the anterior design of reasonable inoculants for improvement of silage quality for cattle feed.

Keywords: silage, lactic acid bacteria, PCR-RAPD, 16S rRNA gene sequence

Introduction

Silage is one of ruminant feed supplementations used especially in dry season or feed shortage. It is commonly made by ensiling of grass crops (forage)

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such as grass, maize, sorghum or other cereals. The forage crop preservation occurred by the achievement of a low pH by lactic acid fermentation and the stability anaerobic conditions (McDonald *et al*., 1991). The lactic acid production depended on the spontaneous population of lactic acid bacteria (LAB) in the forage crop. It was found that the LAB population will be different according to the varieties of the plant materials (Yang *et al*., 2010; Pang *et al*., 2011a; Ni *et al*., 2015; Wang *et al*., 2017; Sifeeldein *et al*., 2018). Main successful silage production based on the activity of homofermentative LAB group which were able to produce a high quantity of lactic acid during the fermentation shift (McDonald *et al*., 1991; Ozduven *et al*., 2017). The lactic acid inhibits the activity of undesirable microorganisms resulted in forage crop preservation and improved the silage quality (Lin *et al*., 1992; Cai *et al*., 1998; Ni *et al*., 2015).

In Thailand, guinea grass (*Panicum maximum*) is considered that one of fiber resources generally use for ruminant fodder. However, supplementation by other plants is in need for an improvement of energy and protein, which raise a high cost of the formulated feed. Plants rich in protein e.g. soybean, groundnut or legume and seeds of trees were presented to be indissoluble for ruminants in the tropics (Babayemi *et al*., 2004; Bamikole *et al*., 2004; Babayemi and Bamikole, 2006). Wheat, maize, cassava, agro-industry by products have been used as the energy resource in feed, but they are competitive with food and fuel industry (OECD-FAO, 2009). Pineapple peel from cannery is also used, but the resource is limiting (Sindhu *et al*., 2002). As a result of this, the cost of feed becomes exorbitant. Exploring of an alternative feed resource can reduce the cost of cattle production. Lead tree (*Leucaena leucocephala*) young leaves, pods and seeds comprise high crude protein and crude fat but lower crude fiber and ether extract. Moreover, this plant contains various mineral compositions i.e. calcium, phosphorus, potassium, and iron (Adeneye, 1979; Suphalucksana *et al*., 2017). Groundnut (*Arachis hypogea* L.) is another plant that contains high protein and fat (Tiwari *et al*., 2006). Both plants are easy grown in tropical zone including Thailand (Tudsri, 2004). As a review, ensilage of the guinea grass with lead tree and groundnut would increase a better quality of the silage.

The LAB in the forage crop silages (ensiled forage) such as paddy rice silage, alfalfa silage, vegetable residues silage, corn stover and Italian ryegrass silage were identified as *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Weissella*. Among those, *Lactobacillus plantarum* was the dominant specie found in all silages (Ennahar *et al*., 2003; Wang *et al*., 2006; Yang *et al*., 2010; Pang *et al*., 2011b; Tohno *et al*., 2012; Pholsen *et al*., 2016). Several methods of grouping and identification of the LAB were applied, most

based on growth condition, carbohydrate fermentation and DNA-DNA hybridization (Cai *et al*., 1998, 1999; Ennahar *et al*., 2003; Yang *et al*., 2010). However, the methods were time consumable and high cost (Nigatu *et al*., 1998, 2001). Polymerase chain reaction-randomly amplified polymorphic DNA (PCR-RAPD) overcame the previous techniques due to it was accurate, quick and lower cost. This technique has useful proved for LAB grouping (Nigatu *et al*., 1998; 2001; Oneca *et al*., 2003).

Thus, in this finding of the general attributes and the nutritional values of the guinea grass silage and the guinea grass compounded with lead tree and groundnut were inspected. Moreover, the LAB population occurred in the silages mixed of guinea grass, lead tree or groundnut was identified. Plate isolation combination with the PCR-RAPD patterns and the 16S ribosomal RNA gene sequencing were also used for the LAB grouping and identification.

Materials and methods

Silage samples

Plant samples used in this experiment included guinea grass (*Panicum maximum*), lead tree (*Leucaena leucocephala*) and groundnut (*Arachis hypogaea* L.). Guinea grass was collected at 45 days old and cut by 5 cm from the ground level. Lead tree was cut at 90 cm from the shoot. Groundnut was collected at 60 days old and the whole plant without root was used. The samples were chopped to 1-2 cm with chop machine. The chopped materials were mixed according to the silage formulas including T1, 100% guinea grass; T2, 100% lead tree; T3, 100% groundnut; T4, 60% guinea grass plus 40% lead tree and T5, 60% guinea grass plus 40% groundnut. The samples were well mixed with 1% NaCl and filled in black sacs with four replications. The samples were firmly encompassed in 10-L plastic containers, vacuumed and maintained at ambient temperature for 21 days. The experiment was conducted at Lop Buri, Thailand. The location is 14 °48' 0N and 100 °25' 0E at an altitude of 25 m above the sea level. The climate at the experiment site is hot and dry (April) with the minimum temperature of 25^{o}C, the maximum temperature of 37^{o}C and the mean rainfall of about 70 mm.

pH analysis

A total of 25 g sample was thawed in 100 ml sterile water and blended for 10 min as previously explained by Polan *et al*. (1998). The pH values were detected by the pH meter (Schott, Model CG 842, Germany).

Color and aroma scores

Following 21-day fermentation, the color and aroma of the silages were annotated according to the index score of Muhammad *et al*. (2008).

Proximate composition analysis

For each treatment, 1,000 g of fresh material were taken to determine nutrient composition. Sub-samples were oven dried at 60^{o}C for 48 h prior to proximate analysis. Dry matter (DM), Ash, Ether extract (EE), Crude protein (CP) and Crude fiber (CF) were inspected according to the methods of AOAC (1995). Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were decided according to the method of Van Soest and Robertson (1979). All analyses were conducted using Fibertec System M6 (FOSS, USA). Three measurements were performed for each replication.

LAB isolation and phenotypic characterization

The silage samples were collected from a top, a middle and a below portions of silo, 100 g of each, and mixed simultaneously (total samples = 20). LAB isolation was arbitrated by the modified method as previously described of Ennahar *et al*. (2003). The sample 10 g was combined with 90 mL of 0.1% sterilized peptone solution, and serially diluted to 10^{-1} to 10^{-6} in 0.1% sterilized peptone solution. The numbers of colony were measured by the plate count method on the de Man, Rogasa and Sharpe agar (MRS, Merck, Germany) mixed with 1% $CaCO_3$, and incubated at room temperature (30$\pm$4^{o}C) for 48 h with two replications per sample. Colonies were tallied as viable numbers of LAB in colony-forming units (CFU) per gram of fresh matter (FM). Each colony of LAB was purified twice by streaking on MRS agar, and incubated at room temperature for 48 h. The stock cultures of purified isolates were kept by modified method as previously described of Ennahar *et al*. (2003). The purified isolates were cultured in MRS broth at room temperature for 24 h. The stock cultures were mixed with 30% glycerol and stored at -80^{o}C for further analysis.

The purified isolates were cultured on MRS agar at room temperature for 24 to 48 h prior to phenotypic characterization. Each purified isolate was determined for morphological characteristics, gram staining, catalase activity and gas production according to the methods described by Kandler and Wiess (1986) and Holt *et al*. (1994).

PCR-RAPD analysis and grouping

Amplification of OPA-3 fragments was performed using the OPA-3 primer (5'-AGTCAGCCAC-3') (Quere *et al*., 1997). A single colony was picked with a sterile toothpick and transferred to PCR tubes (Oneca *et al*., 2003). A total volume of 25 µl reaction contained 1X PCR buffer (75 mM Tris-HCl (pH 8.8 at 25^{o}C), 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2 mM $MgCl_2$, 400 µM dNTP, 0.4 µM OPA-3 primer, 0.1 unit *Taq* DNA polymerase (Fermentas, USA) and were made up to 25 µl with sterilized distilled water. PCR-RAPD amplification was performed by T-Personal thermocycler (Biometra, T1 thermocycler, Germany) using the conditions as followed: an initial denaturation at 94^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 36^{o}C for 1 min, elongation at 72^{o}C for 1 min, and final extension at 72^{o}C for 10 min. The PCR products were visualized on a 1.5% agarose gel in 0.5x TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) with running at 50 V for 120 min. The 1 kb DNA ladder (Fermentas, USA) was used as a standard maker.

Grouping of the PCR-RAPD profiles were indicated with scored bands, which 1 meaned a presented band and 0 meaned an absent band of appearance at the same plane. All score bands were analyzed by NTsys® program version 2.20E (Rohlf, 2000). The LAB grouping was analyzed by the Pearson product-moment correlation coefficient (*r*) and the dendrogram and clustering were created by unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973). The results obtained from the PCR-RAPD profiles were used in combination with the phenotypic characterizations in order to select the representative strains for further sequencing.

16S rRNA gene amplification

The genomic DNA was extracted from the representative strain of LAB and purified by the method described by Sambrook and Russell (2001). The 16S rRNA gene fragments were amplified using the bacteria-universal primers as reported by Kanokratana *et al*. (2004): BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and REVB (5'-GGTTACCTTGTTACGACTT-3'). The 25-µl PCR reaction contained with 1X PCR buffer (75 mM Tris-HCl pH 8.8 at 25^{o}C, 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM each of primer, 0.1 unit *Taq* DNA polymerase (Vivantis, USA), 100 ng of DNA template and made up to 25 µl with sterilized distilled water. The conditions of PCR amplification are as following: an initial denaturation at 94^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 45 s, annealing at 50^{o}C for 1 min, elongation at 72^{o}C

for 2 min. The final extension was performed at 72^{o}C for 10 min. The PCR products were visualized on a 1.0% agarose gel in 0.5x TAE buffer with running at 100 V for 50 min. The bands of PCR products were excised from the gel and purified by using Gel/PCR purification mini kit (Favorgen, Taiwan).

Analysis of 16S rRNA gene

The purified PCR products were directly ligated with the pTZ57R/T vector using InsTAclone^{TM} PCR cloning kit (Fermentas, Canada) according to the manufacturer's protocol. The recombinant clones were transformed into *Escherichia coli* strain DH5α using heat shock method as described by Sambrook and Russell (2001). Positive colonies were identified by blue/white colony screening. The positive colonies were confirmed by colony PCR technique using M13F primer (5'-GTAAAACGACGGCCAGT-3') and M13R primer (5'-GGAAACAGCTATGACCATG-3'). The 10-µl PCR reaction contained with 1X PCR buffer (75 mM Tris-HCl pH 8.8 at 25^{o}C, 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.16 µM each of primer, 0.1 unit *Taq* DNA polymerase (Vivantis, USA), 1 needle of a single colony as a DNA template and made up to 10 µl with sterilized distilled water. The conditions of PCR amplification are as following: an initial denaturation at 94^{o}C for 3 min, 30 cycles of denaturation at 94^{o}C for 30 s, annealing at 50^{o}C for 40 s, elongation at 72^{o}C for 1 min., 50 s. The final extension was performed at 72^{o}C for 10 min. The PCR products were visualized on a 1.0% agarose gel in 0.5x TAE buffer with running at 100 V for 40 min. The 1 kb DNA ladder was used as a standard maker. Plasmids were extracted by FavorPrep^{TM} Plasmid DNA Extraction Mini Kit (Favorgen, Taiwan) after cultured in Luria Bertani (LB) broth at 220 rpm, 30^{o}C for 14 to 16 h. DNA sequencing was analyzed (1st Base, Malaysia).

Similarity search of the 16S rRNA gene sequences were made using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/Blast/). The sequence information was imported into the CLUSTALW software program for assembly and alignment. The 16S rRNA gene sequences of the representative strains were compared to the sequences reported in the GenBank database. Nucleotide substitution rates were calculated by Kimura-2-parameter model (K_{nuc}) (Kimura, 1980). The phylogenetic trees were constructed by the neighbor-joining (NJ) method and visualized with using the MEGA4 program. The topologies of trees were tested by bootstrap analysis based on 1,000 random re-samplings (Tamura *et al*., 2007). *Bacillus subtilis* NCDO 1796 (accession number; X60646) was used as an outgroup organism.

Nucleotide sequence accession numbers

The nucleotide sequences for the 16S rRNA genes of the representative strains T1R2C3, T1R4C24, T2R2C12, T2R1C4, T5R3C15, T5R3C24, T1R1C12, T2R4C3, T5R3C19, T3R2C13, T3R2C12, T1R3C2, T4R2C14, T5R1C10, T1R1C23, T5R2C10, T5R4C21, T4R2C13, T2R4C19, T4R3C18 and T3R1C1 were put in the GenBank data library under the accession numbers JX193617 to JX193637, respectively.

Statistical analysis

Completely random design (CRD) was used in the experiment. Data were analyzed by Statistical Analysis System (SPSS, 2008) using Duncan's Multiple Range Test (DMRT). Significantly difference of the means were determined at $p<0.05$ or $p<0.01$.

Results

Characteristics and chemical composition of silages

After ensilage, the examined silages had characteristic of good color, aromatic and acidic conditions. The silages consisted about 6-10% moisture content. The pH values of fresh green samples were approximately 6.2-6.7 and were reduced to about pH 4.50 at 21 days of ensiling. The chemical composition of the silages showed significantly difference in all parameters (Table 1). Dry matter (DM) was about 90-94%. The guinea grass silage (T1) contained the significant lowest values ($P<0.01$) of organic matter (OM) and crude protein (CP) contents, whereas the leucaena silage (T2) gave the highest OM and CP values ($P<0.01$) compared to other formulas. The groundnut silage showed the lowest crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents ($P<0.01$). When mixing of the guinea grass with 40% of leucaena (T4) or groundnut (T5), the protein and fiber contents were significantly increased ($P<0.01$).

Phenotypic characterization

Total count of viable LAB in each silage was approximately 6-7 log CFU/g of FM. A total 282 isolates were randomly selected including 57, 48, 70, 40 and 67 isolates obtained from the T1 to T5 silages, respectively. Overall, all isolates were gram-positive and catalase-negative bacteria, except one isolate

was catalase-positive (Table 2). The cell shapes were cocci to bacilli. Most of isolates produced gas.

Grouping of LAB

The isolates were divided into 16 different patterns (A to P) according to a cut off at 85% of similarity coefficient with using UPGMA cluster analysis of the dendrogram (data not show). Combination of the phenotypic characteristics with the PCR-RAPD patterns, the LAB were divided into 21 groups, designated as Guinea Grass Mixed Legume Silage (GMLS) 1 to GMLS21 (Table 2). The group GMLS1 contained the highest number of isolates (135). This group had a RAPD pattern (pattern A) similar to the GMLS2 and GMLS3, but different in gas production. Most of the isolates were defined as homofermentative LAB except those in the GMLS3, GMLS13, GMLS18 and GMLS19.

16S rRNA gene analysis of silage LAB

Sequences of 16 rRNA gene were analyzed by BLAST search program in the representative strains of each group are shown in Table 2. In this study, most of the representative strains showed the high sequence homology value more than 99% with each type strains, except one the representative strains (GMLS10) showed value equal to 100%. Base on 16S rRNA gene sequence analysis, these the representative strains could be assigned to *Lactobacillus plantarum*, *Pediococcus acidilactici*, *P. pentosaceus*, *Enterococcus hirae*, and *Weissella paramenseteroides*.

Discussion

The increasing trend ($P<0.01$) of CP from supplementation with either lead tree leaves or groundnut was consistent with other reports (Azim *et al*., 2000; Mustafa *et al*., 2001; Muhammad *et al*., 2008). Our result suggesting that supplementation of leucaena or groundnut in grass silage could improve the silage quality, which also recommended by Titterton and Maasdorp (1997) and Suphalucksana *et al*. (2017).

The results as obtained from the PCR-RAPD analyzed combined the phenotypic characterizations for groupings were similar as previously report (Oneca *et al*., 2003; Pérez-Pulido *et al*., 2005). Oneca *et al*. (2003) reported that combining methods as mentioned for grouping of LAB occurred in milk and

Table 1. Chemical composition of pre-ensilages and silages

Parameters[1/]	T1		T2		T3		T4		T5		C.V.(%)	P-value
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post		
pH[2/]	6.73	5.53^{a}	6.22	4.97^{d}	6.51	4.80^{e}	6.43	5.37^{b}	6.53	5.11^{c}	0.95	0.0001
Moisture (%)[2/]	79.01	76.47^{a}	62.30	62.55^{e}	68.81	67.20^{d}	72.24	70.04^{c}	74.45	72.66^{b}	2.28	0.0001
DM (%)[2/]	20.99	23.53^{e}	37.71	37.45^{a}	31.20	32.80^{b}	27.76	29.96^{c}	25.56	27.34^{d}	5.27	0.0001
Ash (% DM)[2/]	11.15	15.74^{a}	7.14	6.11^{d}	12.45	10.99^{c}	11.35	10.82^{c}	12.70	12.77^{b}	4.87	0.0001
CP (% DM)[2/]	7.94	7.91^{d}	13.93	14.00^{a}	10.91	11.20^{b}	12.34	11.18^{b}	10.49	9.61^{c}	5.95	0.0001
CF (% DM)[2/]	33.98	42.69^{b}	45.34	49.48^{a}	25.93	25.46^{d}	41.11	44.36^{b}	34.59	34.91^{c}	5.51	0.0001
EE (% DM)[2/]	5.68	4.24^{bc}	3.89	4.07^{c}	4.89	5.58^{a}	3.55	5.32^{a}	3.75	5.10^{ab}	13.09	0.0143
OM (% DM)[2/]	88.86	84.26^{d}	92.87	93.90^{a}	87.56	89.01^{b}	88.65	89.18^{b}	87.31	87.24^{c}	0.62	0.0001
NFE (% DM)[2/]	35.99	22.60^{c}	24.76	19.25^{d}	39.89	37.14^{a}	26.80	22.05^{c}	33.76	30.83^{b}	5.87	0.0001
NDF (% DM)[2/]	58.66	56.55^{a}	44.64	49.61^{bc}	30.63	28.54^{d}	58.67	53.41^{ab}	52.48	45.30^{c}	6.15	0.0001
ADF (% DM)[2/]	31.07	37.88^{b}	38.83	45.47^{a}	23.23	29.48^{c}	40.59	45.54^{a}	35.82	38.54^{b}	5.36	0.0001

[1]/DM, dry matter; CP, crude protein; CF, crude fiber; EE, ether extract; OM, organic matter; NFE, nitrogen free extract; NDF, neutral detergent fiber; ADF, acid detergent; T1, 100% guinea grass silage; T2, 100% lead tree silage; T3, 100% groundnut silage; T4, 60% guinea grass plus 40% lead tree silage; T5, 60% guinea grass plus 40% groundnut silage.
[2]/Average of four replications. Means followed by a different superscript letter in each row are significantly different at $P<0.05$ and $P<0.01$

Table 2. Phenotypic characteristics of lactic acid bacteria strains isolated from silages and grouping with RAPD pattern

Characteristics	Groups of lactic acid bacteria strains isolated from the Guinea grass Mixed Legume Silage (GMLS).										
	1	2	3	4	5	6	7	8	9	10	11
No. of isolates	135	5	21	3	11	2	5	3	2	9	25
Representative strain	T1R2C3	T1R4C24	T2R2C12	T2R1C4	T5R3C15	T5R3C24	T1R1C12	T2R4C3	T5R3C19	T3R2C13	T3R2C12
Shape[1/]	R	C	CB	R	R	R	R	R	R	R	R
Gram stain	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gas production	-	-	+	-	-	-	-	-	-	-	-
Fermentation type[2/]	HM	HM	HT	HM	HM	HM	HM	HM	HM	HM	HM
RAPD pattern	A	A	A	B	C	D	E	F	G	H	I
Similarity of 16S rRNA[3/]	99	99	99	99	99	99	99	99	99	100	99
Assigned species[4/]	*Lp*	*Pa*	*Lp*	*Lp*	*Lp*	*Lp*	*Lp*	*Lp*	*Lp*	*Lp*	*Lp*

[1]/R, Rod; C, Cocci; CB, Coccobacilli

[2]/HM, homofermentative; HT, heterofermentative.

[3]/16S rRNA sequence similarity (%) between isolate and each type strain was identified by BLAST search program.

[4]/*Lp*, *Lactobacillus plantarum*; *Pa*, *Pediococcus acidilactici*; *Pp*, *Pediococcus pentosaceus*; *Eh*, *Enterococcus hirae*; *Wp*, *Weissella paramenseteroides*.

Table 2. (Continue) Phenotypic characteristics of lactic acid bacteria strains isolated from silages and grouping with RAPD pattern

Characteristics	Groups of lactic acid bacteria strains isolated from the Guinea grass Mixed Legume Silage (GMLS).									
	12	13	14	15	16	17	18	19	20	21
No. of isolates	25	2	13	4	2	1	1	1	1	11
Representative strain	T1R3C2	T4R2C14	T5R1C10	T1R1C23	T5R2C10	T5R4C21	T4R2C13	T2R4C19	T4R3C18	T3R1C1
Shape[1/]	R	CB	R	C	C	R	R	C	R	R
Gram stain	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	+	-	-	-
Gas production	-	+	-	-	-	-	+	+	-	-
Fermentation type[2/]	HM	HT	HM	HM	HM	HM	HT	HT	HM	HM
RAPD pattern	J	J	K	L	M	N	N	N	O	P
Similarity of 16S rRNA[3/]	99	99	99	99	99	99	99	99	99	99
Assigned species[4/]	*Lp*	*Lp*	*Lp*	*Pp*	*Eh*	*Lp*	*Wp*	*Wp*	*Lp*	*Lp*

[1]/R, Rod; C, Cocci; CB, Coccobacilli
[2]/HM, homofermentative; HT, heterofermentative.
[3]/16S rRNA sequence similarity (%) between isolate and each type strain was identified by BLAST search program.
[4]/*Lp*, *Lactobacillus plantarum*; *Pa*, *Pediococcus acidilactici*; *Pp*, *Pediococcus pentosaceus*; *Eh*, *Enterococcus hirae*; *Wp*, *Weissella paramenseteroides*.

cheese samples were could be reduced LAB numbers from 1026 stains to 7 main clusters as difference. As well, Pérez-Pulido *et al*. (2005) also showed that 133 isolates of LAB obtained from collected during the fermentation of capers were could be reduced to 75 isolates after RAPD analysis. Likewise, results from this study showed that LAB obtained from selected LAB in five formula silages, which equal to 282 isolates were could be reduced to 21 groups as difference. Based on results of grouping, these LAB isolates were leaded to presumption that comprised members of the genera *Lactobacillus*, *Pediococcus*, *Enterococus*, and *Weissella*. However, as available phenotypic procedures were difficult to define isolates to know species, because it is tough to differentiate simply and clearly between species of LAB (Cai *et al*., 1998; Ennahar *et al*., 2003). Therefore, the representative strains of each groups were analyzed by 16S rRNA gene sequence for identify species to future.

The relation of LAB in silage formulas are shown in Table 3. Generally, LAB is often found existence in relationships with plant material, dairy product and includes forage crops and silage (Daeshel *et al*., 1987; Stiles and Holzapfel, 1997; Cai *et al*., 1999, 2010; Wang *et al*., 2017; Sifeeldein *et al*., 2018). Several researches have reported LAB as the prominent microbial inhabitant on silage, especially the genus of *Lactobacillus* was reported as most of population occurring in among the other genus LAB (Ennahar *et al*., 2003; Pang *et al*., 2011b; Pholsen *et al*., 2016). Ennahar *et al*. (2003) reported that *Lb. plantarum* was the most of LAB occurring in paddy rice silage. Yang *et al*. (2010) also found that homofermentative lactobacilli which is *Lb. plantarum* was the most of population occurring in vegetable residues. In contrast, Pang *et al*. (2011a) reported that *W. cibaria* was the most of LAB occurring in corn silage. In this study, the species of LAB as identified to *Lb. plantarum* was found occurred in all silage formulas (T1 to T5). It was shown that *Lb. plantarum* is the most dominant of LAB species occurring in this study. Many researches established that *Lb. plantarum* had helpful effects to improvement the silage quality with high produced the lactic acid content (Cai, 1999; Zhang *et al*., 2000; Pang *et al*., 2011b). The spreading of *Lb. plantarum* in the silage formulas may be due to the prevalent of Mn^{2+} (manganese) in plant material, because the Mn^{2+} content was accumulated into the cell of *Lb. plantarum* (including genus of *Pediococcus* and *Leuconostoc*) for defense mechanism in against oxygen toxicity (Daeshel *et al*., 1987). *P. acidilactici* were found both silages in guinea grass and guinea grass mixed with groundnut, whereas *P. pentosaceus* was only found in 100% guinea grass silage. Cai *et al*. (1999) reported that the prevalent both *P. acidilactici* and *P. pentosaceus* could be using inoculation to improve the quality of alfalfa and Italian ryegrass silages. Other LABs, so as *Enterococcus*, *Leuconostoc* and *Weissella* strains have been dissociated at low

frequencies in fodder crops and their silages (Lin *et al*., 1992; Cai *et al*., 1998; Cai, 1999). *E. hirae* were found guinea grass and guinea grass mixed with groundnut. *W. paramesenteroides* were found in lead tree based silages.

Table 3. The relation of LAB occurring in silage formula

Species of LAB	Silage formulas[1/]				
	T1	T2	T3	T4	T5
E. hirae	+	-	-	-	+
L. plantarum	+	+	+	+	+
P. acidilactici	+	-	-	-	+
P. pentosaceus	+	-	-	-	-
W. paramesenteroides	-	+	-	+	-

[1/] T1, 100% guinea grass silage; T2, 100% lead tree silage; T3, 100% groundnut silage; T4, 60% guinea grass plus 40% lead tree silage; T5, 60% guinea grass plus 40% groundnut silage. +, found; -, not found. *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *P.*, *Pediococcus*, and *W.*, *Weissella*.

The distribution of both *E. hirae* and *W. paramesenteroides* in silage was reported no affect in improvement of the silage quality (Cai *et al*., 1998; Cai, 1999). In addition, *W. paramesenteroides* was capable to build high acetic acid content, which may cause some fermentation wastage (Cai *et al*., 1998; Zhang *et al*., 2000). However, it was found that the lactic acid cocci, e.g. genus of *Lactococcus*, *Leuconostoc*, *Weissella*, *Streptococcus*, *Pediococcus*, and *Enterococcus*, starting the lactate fermentation in ensilage and establish an anaerobic environment appropriate for the development of *Lactobacillus* only in the preliminary stage of ensiling processes (Lin *et al*., 1992; Cai, 1999) and display a more important part in promotion of lactic acid fermentation for a longer time (Cai *et al*., 1998; 2010).

In conclusion, guinea grass could be slightly improved to crude protein value by addition of groundnut, whereas addition of lead tree to guinea grass brings to clearly improve the nutritional value of the silage with highest crude protein and organic matter. *Lactobacillus plantarum* was the prominent species of lactic acid bacteria in the formula-five silages.

Acknowledgement

The author would like to thanks Assoc. Prof. Dr. Yanin Opatpatanakit (Faculty of Animal Science and Technology, Maejoe University, Thailand) and Assist. Prof. Dr. Aphacha Jindaprasert (Faculty of Agro-Industry, King's Mongkut Insitute of Technology Ladkrabang, Thailand) for recommendation to the discussion, and Dr. On-uma Rungnoi (Faculty of Agricultural Technology, King's Mongkut Insitute of Technology Ladkrabang, Thailand) for supporting the NTsys® program. This research was partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education

and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

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(Received: 27 August 2018, accepted: 5 April 2019)