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Characterization and application of lactic acid bacteria for tropical silage preparation

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ABSTRACT

Strains TH 14, TH 21 and TH 64 were isolated from tropical silages viz. corn stover, sugar cane top and rice straw, respectively prepared in Thailand. These strains were selected by low pH growth range and high lactic acid-producing ability; similar to some commercial inoculants. Based on the analysis of 16S rRNA gene sequence and DNA-DNA relatedness, strain TH 14 was identified as *Lactobacillus casei*, and strains TH 21 and TH 64 were identified as *L. plantarum*. Strains TH 14, TH 21, TH 64, and two commercial inoculants, CH (*L. plantarum*) and SN (*L. rhamnosus*), were used as additives to fresh and wilted purple Guinea and sorghum silages prepared using a small-scale fermentation method. The number of epiphytic LAB in the forages before ensilage was relatively low but the numbers of coliform and aerobic bacteria were higher. Sorghum silages at 30 d of fermentation were all well preserved with low pH (3.56) and high lactic acid production (72.86 g/kg DM). Purple Guinea silage inoculated with LAB exhibited reduced count levels of aerobic and coliform bacteria, lower pH, butyric acid and ammonia nitrogen, increased lactic acid concentration compared with the control. Strain TH 14 more effectively improved lactic acid production cf. inoculants and other strains.

Keywords: *Guinea grass, lactic acid bacteria, sorghum, tropical silage.*

INTRODUCTION

In tropical developing countries including Thailand, ruminant husbandry must be supported by forage

crops which are not available in the dry season (Hare *et al.* 2009). Silage has become an increasingly important source of animal feed in the tropics in this season. Suitable plants for silage making include perennial and annual grasses. Purple Guinea grass (*Panicum maximum* cv. TD 58) and sorghum (*Sorghum bicolor*) are forage crops that are widely used to make silage. Both crops are high in DM yield and drought tolerant (Black *et al.* 1980; Hare *et al.* 2009; Williams & Shinners 2012; Xing *et al*. 2009).

Lactic acid bacteria (LAB) are a major component of the microbial flora that is usually present on the surface of many forage crops (Pang *et al*. 2011). Some forage-associated LAB have been characterized by phenotypic features and the analysis of 16 S rRNA sequence and DNA-DNA relatedness, and they have been identified as species of the genera *Enterococcus, Weissella, Lactococcus, Pedicoccus, Leuconostoc* and *Lactobacillus* (Cai *et al*. 1999; Pang *et al*. 2011). It is well established that LAB play an important role in silage fermentation (Cai *et al*. 1999). The number and characteristics of LAB have become a significant factor in predicting the adequacy of silage fermentation and determining whether to apply bacterial inoculants to silage. In order to improve silage quality, many LAB-containing biological additives have been developed and are currently available (Cai *et al*. 1999). These inoculants by increasing lactic acid concentrations inhibit the growth of harmful bacteria. However, while an increasing number of studies have reported positive benefits from using bacterial inoculants as silage additives in Japan, United States and Europe, relatively few have reported the effect of LAB inoculants on silage fermentation in the tropics. Meeske and Basson (1998) evaluated the effect of inoculants containing *Lactobacillus acidophilus, L. delbruekii ssp. bulgaricus* and *L. plantarum* on corn silage and found no effect on pH values and lactic acid production. This is because of the high LAB concentrations present in the plant before ensiling, but the characteristics of epiphytic LAB and their true function in silage making in the tropics were unclear. Therefore, further study of the characteristics of LAB species including commercial inoculants and selected strains in tropical silage making is required.

The objectives of the present study were to screen, isolate and identify LAB from tropical silages, with particular reference to species that are most likely to play an important role in fermentation quality improvement. Isolates were identified at the molecular level using 16S rDNA sequence and DNA-DNA relatedness analysis. The effects of selected LAB and inoculants on chemical composition and silage fermentation characteristics of purple Guinea grass and sorghum were also studied.

METHERIALS AND METHODS

Silage Preparation and Experiments

Purple Guinea grass, cv. TD 58 fertilized with cattle manure at a rate of 6,250 kg/ha and sorghum, cv. IS 23585 with urea and potassium at 600 and 100 kg/ha, respectively were grown in the experimental farm, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. Purple Guinea was harvested

at 60 d of regrowth on 12 October 2013 and sorghum 77 d after emergence on 7 November 2013. In order to study the effect of moisture adjustment on silage fermentation quality, 50% of the purple Guinea was wilted for 6 h in the shade. Fresh and wilted purple Guinea grass and fresh sorghum were ensiled using small-scale plastic bag fermentation (Cai *et al*. 1999). 100 g of 2 cm chopped herbage was packed into plastic film bags (Hiryu KN type, 180 by 260 cm, Asahikasei Co. Ltd., Tokyo, Japan) and the bags sealed with a vacuum sealer (SQ-303W; Sharp Co. Ltd., Tokyo, Japan). Fresh samples were ensiled within 3 h of harvesting. The wilt samples were sealed immediately after wilting.

Eighty-two strains of LAB isolated from tropical forages and their silages were identified and characterized. Three selected strains TH 12, TH 14 and TH 64 isolated from silage prepared with sweet corn (*Zea mays* L.) stover, sugar cane (*Saccharum officinarum* L.) top and rice (*Oryza sativa* L.) straw, and two commercial inoculant strains CH (Chikuso-1, *L. plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan) and SN (Snow Lact L, *L. rhamnosus*, Snow Brand Seed Co., Ltd) were used as additives for silage making. Strains TH 12, TH 14 and TH 64 were selected because of their lower pH growth range and higher lactic acid production compared with other isolates. The silage treatments were: untreated (control), strains TH 12, TH 14 and TH 64, and commercial inoculant strains CH and SN. These strains were used as additives at 1.0×10^5 colony forming unit (cfu) g^{-1} of fresh matter (FM). The MRS broth (Difco Laboratories, Detroit, Mich.) was inoculated with these strains and incubated overnight. After incubation, the optical density at 620 nm of the suspension was adjusted with sterile 0.85% NaCl solution to 0.42. The LAB inoculum was 1ml of suspension/kg of FM in all cases. There were five replicates (bags) in each treatment and all were stored at room temperature together in the same store-room (21.0 to 37.0 °C); three bags from each treatment were opened for evaluations of silage fermentation 30 d after ensiling.

Microbiological Analysis of Purple Guinea and Sorghum before Ensiling and Their Silages

Samples from before ensiling and their silages with 3 replications at 30 d of ensiling were used for microbiological analysis. The microorganism composition was analyzed using plate count method as described by Kozaki *et al.* (1992). 10 g of silage with 90 ml of sterilized distilled water was shaken well by hand, and 10^{-1} to 10^{-5} serial dilutions were made in 0.85% sodium chloride solution. From each dilution, 0.05 ml of suspension was spread on agar plates. LAB were counted on Lactobacilli MRS agar (Difco Laboratories, Detroit, Mich.) after incubation in an anaerobic box (Sugiyamagen Ltd., Tokyo, Japan) at 30°C for 2 d. LAB were detected and counted after morphological observation and determination of Gram staining, catalase reaction, spore formation, nitrate reduction, and fermentation type (Kozaki *et al.*, 1992).

To assess the percentage of inoculated strains to total LAB in silages at 30 d of ensiling, 20 colonies were isolated at random from the agar plates. Each colony of LAB was purified twice by streaking on MRS agar. The pure cultures were grown on MRS agar at 30°C for 24 h. The inoculated strains were confirmed by carbohydrate fermentation tests of Analytical Profile Index (API 50 CH) strips (bioMerieux, Tokyo, Japan) and 16S rRNA gene sequence analysis. Colonies were counted as viable numbers of microorganisms (cfu per g of FM). The purified colonies of LAB were collected with nutrient broth (Difco) containing 10% dimethyl sulfoxide and stored as stock cultures at -80°C for further examination. The type strains of LAB were obtained from the Japan Collection of Microorganisms (JCM), The Institute of Physical and Chemical Research, Wako, Saitama, Japan. Aerobic bacteria were counted on nutrient agar (Difco), and molds and yeast were counted on potato dextrose agar (Nissui-seiyaku). The agar plates were incubated at 30°C for 2 to 7 d, however, for 3 to 7 d of incubation, some colonies were too enlarged and they could not be counted. In this experiment, mold colony was counted on 2 d of incubation. Yeasts were distinguished from molds or bacteria by colony appearance and microscopic observation of cell morphology after determination of Gram staining.

Gram stain, morphology, catalase activity, spore formation, motility, nitrate reduction, and gas production from glucose, growth at OD 620 nm and lactic acid production in MRS broth were determined according to methods for LAB described by Kozaki *et al.* (1992). Growth of LAB at pH 3.5, 4.0, 4.5 and growth at temperatures 15°C, 45°C were determined in MRS broth after incubation at 30°C for 5 d. The isomers of lactate formed from glucose were determined enzymatically with reagents obtained from Boehringer GmbH, Mannheim, Germany.

16S rRNA Gene Sequence Analysis of Selected Strains

For 16S rRNA gene sequence analysis of selected strains, cells grown for 8 h in MRS broth at 30°C were used for DNA extraction and purification as described by Suzuki et al. (1996). Amplification of the 16S rRNA gene was carried out in a Thermal Cycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, California, USA) by using the PCR and reagents from Takara *Taq* PCR Kit (Takara Shuzo Co., Ltd., Otsu, Japan). The sequences of the PCR products were determined directly with a sequence kit (ALFexpress AutoCycle, Pharmacia Biotech, Piscataway, NJ, USA) with the prokaryotic 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) in combination with Applied Biosystems model 310A (Applied Biosystems, Foster City, CA, USA) automated sequencing system. More than 1500 bases of 16S rDNA were determined for species identification. The sequence information was imported into the DNASTAR software program (DNASTAR, Inc., Madison, WI, USA) for assembly and the 16S rRNA gene sequences of strains TH 14, TH 21 and TH 64 were compared with sequences of type strains published in

DDBJ, GenBank and EMBL by BLAST program, then, the sequence was imported into the CLUSTAL W software program (Hitachi Software Engineering Co. Ltd., Tokyo, Japan) for alignment. The topologies of trees were evaluated by bootstrap analysis of the sequence data with the software package MEGA version 5.0 (Tamura *et al.* 2011) based on 1000 random re-samplings (Eitan *et al*., 2006). Nucleotide substitution rates (*K*nuc values) were calculated (Kimura & Ohta 1972), and phylogenetic trees were constructed by the neighbor-joining (Saitou & Nei 1987) phylogenetic trees were inferred using MEGA 5.0 software according to the Kimura 2-parameter model. *Bacillus subtilis* NCDO 1769^T was used as an outgroup organism.

DNA-DNA Relatedness Analysis of Selected Strains

For DNA base composition and DNA-DNA hybridization test, the DNA was extracted from cells harvested from MRS broth culture which had been incubated for 8 h at 30^{o}C. It was purified by the procedure of Saitou and Miura (1963). DNA base composition was determined by the method of Tamaoka and Komagata (1984) by using high-performance liquid chromatography following enzymic digestion of DNA to deoxyribonucleosides. The equimolar mixture of four deoxyribonucleotides in a GC kit (Yamasa Shoyu Co. Ltd., Choshi, Japan) was used as the quantitative standard. DNA-DNA relatedness was determined by the method of Ezaki *et al*. (1989) using photobiotin and microplates.

Chemical Analysis of Purple Guinea and Sorghum before Ensiling and Their Silages

Dry matter (DM), crude protein (CP), ether extract (EE), and organic matter (OM) were analyzed by the AOAC (1990) Methods 934.01, 976.05, 920.39, and 942.05, respectively. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were analyzed by the methods of Van Soest *et al.* (1991). Acid detergent lignin (ADL) was analyzed by the standard methods of Faichney and White (1983).

Fermentation products of the silages were determined from cold water extracts as described by Cai (2004). Silage (10 g) was homogenized with 90 ml of sterilized distilled water, the pH was measured with a glass electrode pH meter (MP230; Mettler Toledo, Greifensee, Switzerland) and the ammonia-N concentration was determined by steam distillation of the filtrates. Lactic acid buffer capacity (LBC) was determined by titrating with NaOH from pH 4.0 to 6.0 (mmol kg^{-1} DM) after first reducing pH to below 4.0 using HCl as described by Muck *et al*. (1991). The organic acid contents and water-soluble carbohydrate (WSC) including glucose, sucrose, and fructose were measured by HPLC methods as described by Cai (2004). Gross energy (GE) was determined using an automatic adiabatic bomb calorimeter (AC 500; LECO, Michigan, USA).

Statistical Analysis of Purple Guinea and Sorghum before Ensiling and Their Silages

Data on the chemical composition of the purple Guinea and sorghum and their silages at 30 d of ensiling were analyzed by analysis of variance, and the significance of differences among means was tested by the multiple range test (SAS 1998).

RESULTS

Counts of Microorganisms in Purple Guinea and Sorghum before Ensiling and in Silages

The counts of microorganisms in purple Guinea and sorghum before ensiling are shown in Table 1. Overall, fresh or wilted purple Guinea and sorghum before ensiling were 10^3 to 10^5 LAB in cfu/g FM, 10^6 to 10^7 coliform bacteria and aerobic bacteria, 10^3 to 10^5 yeasts, and 10^3 to 10^4 molds. During the wilting process in purple Guinea, the numbers of coliform bacteria, aerobic bacteria and yeasts increased, LAB decreased, and the molds show similar levels. The counts of microorganisms in sorghum were higher than purple Guinea grass.

The counts of microorganisms in purple Guinea and sorghum silages at 30 d of ensiling are shown in Table 2. The numbers of viable LAB in sorghum silages were lower than in both fresh and wilted purple Guinea silages; 10^7 to 10^9 for fresh and wilted purple Guinea and 10^5-10^6 for sorghum. The percentages of inoculated to total LAB in all three silages at 30 d of ensiling are: strain TH 14 (98.5 to 100%), TH 21 (73.6 to 92.5%), TH 64 (90.3 to 94.3%), CH (95.6 to 97.2%) and SN (87.3 to 94.6%).

Purple Guinea grass and sorghum silages inoculated with LAB had lower counts of aerobic bacteria cf. controls. Coliform bacteria in control silages of fresh and wilted purple Guinea ranged from 10^5 to 10^7 while they were below the detectable level (10^1 cfu/g FM) in LAB-inoculated silages. Yeasts were 10^4 to 10^6, but molds were below the detectable level (10^1 cfu/g FM) in all silages.

Characterization of Selected Strains and Inoculant Strains

Physiological and biochemical properties of isolates are shown in Table 3. All LAB strains were Gram-positive, short rod-forming, catalase-negative and facultative anaerobic lactobacilli that did not produce gas from glucose and were able to grow at temperatures from 15°C to 45°C. All strains can grow well under aerobic and anaerobic conditions in MRS broth. Strains TH 14 and SN formed optical isomers of lactic acid as L(+) form while strains TH 21, TH 64 and CH formed racemic mixtures of lactic acid as DL. Strains TH 14, TH 21 and TH 64 were selected by their excellent characteristics with a lower range of growth pH and higher productivity of lactic acid than other isolates in silage environment.

Identification of Selected Strains

Based on the phylogenetic analysis, selected strains TH 14, TH 21 and TH 64 were placed in the cluster making up the genus *Lactobacillus* (Fig. 1). Type strain of *L. casei* ATCC15820^T was the species most

closely related to the strains TH 14, and type strain of *L. plantarum* JCM 1149^T and *L. pentosus* JCM 1558^T were the species most closely related to the strains TH 21 and TH 64. Strain TH 14 and *L. casei* JCM ATCC15820^T showed a high sequence similarity value at 99.5%, and strains TH 21, TH 64 and *L. plantarum* JCM 1149^T showed their sequence similarity from 99.5 to 99.7% with each other.

Following DNA-DNA hybridization analysis, strain TH 14 had the highest level of DNA relatedness (84.6%) to the type strain of *L. casei*. Strains TH 21 and TH 64 showed 88.2 to 91.4% DNA relatedness to the type strains of *L. plantarum*. Based on the analysis of 16S rDNA sequence and DNA-DNA relatedness, strain TH 14 was identified as *L. casei, and* strains TH 21 and TH 64 were *L. plantarum*.

Chemical Composition of Purple Guinea Grass and Sorghum Before and After Ensiling

The DM of Purple Guinea increased by 10% during wilting (Table 4). The DM in fresh purple Guinea grass was lower ($P < 0.05$), but in wilted one was higher ($P < 0.05$) than sorghum. CP, NDF, ADF and ADL in sorghum were lower ($P < 0.05$), but OM was higher than fresh or wilted purple Guinea grass. GE of the three herbages was similar (P = 0.052). LBC of sorghum was much higher ($P < 0.05$) than purple Guinea grass. The WSC was high in sorghum (33.47 g/kg DM) while it was very low (0.30 to 0.38 g/kg DM) in purple Guinea grass.

At 30 d of ensiling, in silage inoculated with TH 14, the OM and CP were significantly ($P < 0.05$) higher and the NDF, ADF and ADL were significantly ($P < 0.05$) lower than the control (Table 5). TH 14 and control treatments had similar GEs, and they are also significantly ($P < 0.05$) higher than other treatments. Forages (F), additives (A) and their interaction (F x A) influenced ($P < 0.001$) NDF and GE, but did not influence ($P < 0.001$) CP. The OM, CP, EE, ADF and ADL did not differ ($P = 0.006$ to 0.887) among the LAB additive treatments.

Fermentation Quality of Purple Guinea Grass and Sorghum Silages

Forages, additives, and their interaction (F x A) influenced ($P < 0.001$) DM, pH, and all five fermentation products (Table 6). Sorghum silages were all well preserved with a low ($P < 0.05$) pH (< 3.7). Forage means for sorghum silage showed higher ($P < 0.05$) lactate than both fresh and wilted purple Guinea. The highest ($P < 0.05$) lactic acid concentration and the lowest ($P < 0.05$) pH were found in sorghum silages. Compared with the control, LAB-inoculation in all three silages showed lower ($P < 0.05$) pH, acetic, propionic and butyric acids, and ammonia-N, but higher ($P < 0.05$) lactic acid. The additive mean of TH 14 silages showed the highest ($P < 0.05$) lactic acid concentration, the lowest ($P < 0.05$) pH and ammonia-N.

DISCUSSION

LAB play an important role in silage fermentation and silage is now the most common preserved feed for cattle production in many countries (McEniry *et al*. 2011; Pang *et al*. 2011). When the epiphytic LAB reaches at least 10^5 cfu/g FM, silage is usually well preserved (Cai *et al*. 1999). Table 1 shows LAB values in sorghum above 10^5, however, it was lower in fresh and wilted purple Guinea grass. Aerobic and coliform bacteria were relatively high (> 10^6) in all three herbages. This suggests that silage fermentation may need to be improved using LAB inoculants (Cai *et al*. 1999).

The selected and inoculant strains used in this study were *L. plantarum, L. rhamnosus* and *L. casei*. They can grow well in low pH conditions, promote lactic acid fermentation and inhibit the growth of aerobic and coliform bacteria (Cai *et al*. 1999).

Lactobacilli are often found living in association with silage, and some isolated from forage crops and silages have been identified as *L. plantarum,* and *L. casei* (Cai *et al*. 1998). However, available phenotypic procedures to assign isolates to known species are difficult because it is not easy to differentiate clearly between species of lactobacilli, for example, the *L. pentosus* and *L. plantarum* species have very similar 16S rRNA gene sequences, differing only by 2 bp (Hammes & Vogel, 1995). This finding is in agreement with Pang *et al.* (2010) who found carbohydrate fermentation patterns showed ambiguity. Although the pattern of strains isolated from silage and two type strains (*L. pentosus* and *L. plantarum*) were quite similar they could not be identified at the species level based on the 16S rRNA gene sequence and API 50 CHL analysis. Therefore, other phylogenetic analysis methods were required to distinguish these strains accurately.

In the present study, the selected strains were Gram-positive, catalase-negative rods that produced major metabolic product as lactate from glucose. Following phylogenetic analysis of 16S rRNA gene sequences, selected strains TH 14, TH 21 and TH 64 were placed in the cluster making up the genus *Lactobacillus*. However, they could not be identified to the species level on the basis of phenotypic characteristics.

There have been several reports of lactobacilli composing the major microbial population of forage crops and silage, where they may contribute to silage fermentation. Some silage-associated lactobacilli have been characterized by phenotypic features and 16S rRNA gene sequences and have been described as novel species: for example, *L. paraplantarum*, *L. brevis*, *L. buchneri*, *L.acidophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. pentosus* (Cai *et al*. 1998, 1999; Ennahar *et al*. 2003; Moon 1984; Pang *et al*. 2011; Tannock 1999). In recent years, the phylogenetic relationships of LAB have been studied extensively in 16S rDNA sequence ribotyping and DNA-DNA hybridization experiments, and a new species *L. nasuensis* isolated from silage has been added (Cai *et al*. 2012). In the present study, the strains TH14, TH21 and TH64 had a high similarity of 16S rDNA sequences to their type strains (> 99.5), confirming that these strains belong to the genus *Lactobacillus,* and that they are most closely related to

L.plantarum and *L. casei*. The DNA-DNA hybridization results demonstrated that strain TH 14 was identified as *L. casei*, and strains TH 21 and TH 64 were identified as *L. plantarum*.

The addition of LAB at ensiling is intended to ensure rapid and vigorous fermentation that results in faster production of lactic acid, lower pH values at earlier stages of silage fermentation, and inhibition of growth of some harmful bacteria (Cai *et al*. 1999). Many studies (Cai *et al*. 1998, 1999; Ennahar *et al*. 2003; Moon 1984; Pang *et al*. 2011) have reported the advantage of both LAB screening and the use of commercial inoculants. Generally, farm silage is based on natural lactic acid fermentation. The epiphytic LAB transform the WSC into organic acids in the ensiling process. As a result, the pH is reduced and the forage is preserved. However, LAB, especially *Lactobacilli*, are present in forage in very low numbers (Cai *et al*. 1998). When LAB fail to produce sufficient lactic acid during fermentation to reduce the pH and inhibit the growth of clostridia and coliform bacteria, the resulting silage will be poor quality.

Purple Guinea grass and sorghum are popular forage crops that are widely used for silage making in many countries, including Thailand. In the present study, compared to sorghum, lower numbers of LAB and low WSC were present in purple Guinea grass resulting in poor quality of control silage. The factors involved in assessing fermentation quality include the chemical composition of the herbages and the physiological properties of epiphytic LAB. Since the purple Guinea had relatively lower WSC and lower numbers of LAB than sorghum, during silage fermentation, the LAB could not produce sufficient lactic acid to inhibit the growth of harmful bacteria. In our study, silages inoculated with LAB were well preserved, with lower pH and higher lactic acid concentration compared with their controls. Strain TH14 was more effective in improving silage quality than inoculants and other strains. The most plausible explanation lies in the physiological properties of LAB. The strains TH14, TH 21 TH 64, CH and SN used in this study were homofermentative types of LAB which grew well under low pH conditions; strain TH 14 have a high lactic acid production capacity and could produce more lactic acid than other strains. Therefore, inoculation with these LAB, especially strain TH 14 should result in beneficial effects by promoting the propagation of LAB and inhibiting the growth of aerobic bacteria, as well as improving silage quality. It is considered that strain TH 14 have the ability to produce more lactic acid with less WSC condition than other strain. For this, it will be deemed necessary in the future experiment.

The results confirmed that *Lactobacillus casei* TH 14 was suitable as a potential silage inoculant and that this strain was more effective in improving silage quality than inoculants or other strains.

CONCLUSIONS

Selected strain TH 14 isolated from tropical silage was identified as species *L. casei* based on the analysis of 16 S rRNA gene sequence and DNA-DNA relatedness. This strain was able to grow at low pH and the inoculation of herbage with TH 14 resulted in the highest accumulation of lactic acid during ensilage

compared to all other inoculants used in this study. Therefore, *L. casei* TH14 is considered suitable as a potential inoculant for tropical silage preparation.

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406 **Table 1** Microbiological analysis of purple Guinea grass and sorghum at before ensiling.

	Microorganism (cfu/g FM)				
	Lactic acid bacteria	Coliform bacteria	Aerobic bacteria	Yeast	Mold
Purple Guinea					
Fresh	1.5×10^4	3.4×10^6	3.0×10^6	2.6×10^3	3.5×10^3
Wilted	4.2×10^3	6.9×10^7	1.3×10^7	4.2×10^4	1.2×10^3
Sorghum	3.8×10^5	5.2×10^7	1.4×10^7	2.0×10^5	1.5×10^4

407 cfu: colony forming unit; FM: fresh matter.

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409 **Table 2** Microbiological analysis of purple Guinea grass and sorghum silages at 30 d of ensiling.

		Microorganism (cfu/g FM)				
		LAB (% inoculated strain to total LAB)	Coliform bacteria	Aerobic bacteria	Yeast	Mold
Purple Guinea						
Fresh	Control	1.9×10^8 (0)	4.1×10^7	3.5×10^7	1.4×10^5	ND
	TH14	5.9×10^9 (98.5)	ND	4.2×10^5	3.7×10^6	ND
	TH21	8.2×10^9 (82.4)	ND	2.0×10^4	2.8×10^6	ND
	TH64	3.3×10^9 (90.3)	ND	5.8×10^4	3.0×10^5	ND
	CH	3.5×10^9 (95.6)	ND	2.0×10^5	3.0×10^5	ND
	SN	5.9×10^9 (87.3)	ND	5.2×10^5	4.4×10^5	ND
Wilted	Control	1.1×10^8 (0)	3.0×10^5	2.5×10^6	5.8×10^6	ND
	TH14	6.7×10^9 (100)	ND	6.4×10^4	8.0×10^5	ND
	TH21	5.2×10^8 (92.5)	ND	5.3×10^4	2.5×10^5	ND
	TH64	2.8×10^9 (94.3)	ND	3.3×10^4	3.2×10^5	ND
	CH	4.5×10^8 (97.2)	ND	2.5×10^5	2.3×10^6	ND
	SN	3.0×10^7 (90.6)	ND	4.0×10^5	1.8×10^6	ND
Sorghum	Control	6.4×10^6 (0)	ND	4.2×10^5	5.0×10^5	ND
	TH14	2.0×10^6 (99.5)	ND	4.8×10^4	3.5×10^5	ND
	TH21	8.7×10^5 (73.6)	ND	3.2×10^3	3.5×10^4	ND
	TH64	3.8×10^5 (92.8)	ND	5.0×10^4	3.2×10^4	ND
	CH	6.5×10^5 (96.9)	ND	2.1×10^3	6.3×10^5	ND
	SN	2.0×10^6 (94.6)	ND	2.0×10^4	4.8×10^4	ND

410 cfu: colony forming unit; FM: fresh matter; ND: Not detected.

411 TH 14: *Lactobacillus casei*; TH 21 and TH 64: *L. plantarum*; CH: commercial inoculant Chikuso-1, *L.*
412 *plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan; and SN: commercial inoculant Snow Lact L, *L.*
413 *rhamnosus*, Snow Brand Seed Co., Ltd.

414 **Table 3** Characteristics of lactic acid bacteria from tropical silages and inoculants used in this study.

Character	Inoculant CH	Inoculant SN	*Lactobacillus casei* TH 14	*Lactobacillus plantarum* TH 21	*Lactobacillus plantarum* TH 64
Source	Inoculant	Inoculant	Sweet corn Sotover silage	Sugar cane stalk silage	Rice straw silage
Cell form	Rod	Rod	Rod	Rod	Rod
Fermentation type	Homo	Homo	Homo	Homo	Homo
Lactate isomer	DL	L(+)	L(+)	DL	DL
Gas produced from glucose	-	-	-	-	-
Growth in MRS at					
aerobic condition	+	+	+	+	+
anaerobic condition	+	+	+	+	+
Growth at temperature					
15 ℃	+	+	+	+	+
45 ℃	+	+	+	+	+
Growth at pH					
3.5	+	+	+	+	+
4.0	+	+	+	+	+
4.5	+	+	+	+	+
Growth at OD 620 nm in MRS broth	2.1	2.0	2.3	2.3	2.2
Lactate production in MRS broth (%)	1.3	1.2	1.5	1.3	1.5
Final pH in MRS broth	3.7	3.8	3.6	3.7	3.6
Similarity of 16S rDNA sequence (%)*	-	-	99.9	99.7	99.5
DNA-DNA Homology (%)	-	-	84.6	91.4	88.2

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416 All strains were Gram-positive and catalase-negative bacteria.

417 +: positive; -: negative; CH: Chikuso-1, *Lactobacillus plantarum*, Snow Brand Seed Co., Ltd, Sapporo,
418 Japan; SN: Snow Lact L, *L. rhamnosus*, Snow Brand Seed Co., Ltd; MRS: Lactobacilli MRS broth
419 (Difco).

420 *Similarity of 16S rDNA sequence was analyzed between selected strain and their type strain.

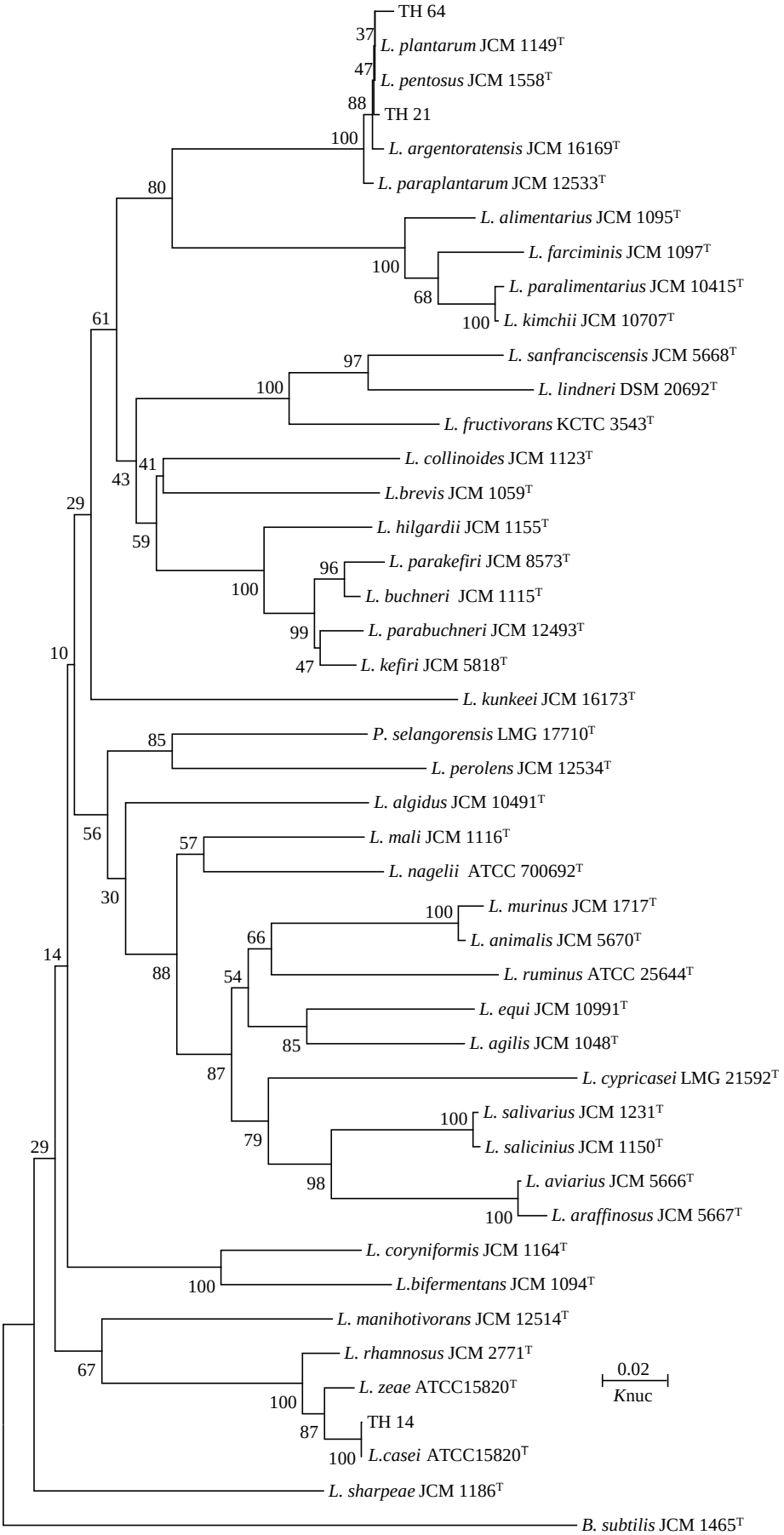
TH 64
L. plantarum JCM 1149T
L. pentosus JCM 1558T
TH 21
L. argentoratensis JCM 16169T
L. paraplantarum JCM 12533T
L. alimentarius JCM 1095T
L. farciminis JCM 1097T
L. paralimentarius JCM 10415T
L. kimchii JCM 10707T
L. sanfranciscensis JCM 5668T
L. lindneri DSM 20692T
L. fructivorans KCTC 3543T
L. collinoides JCM 1123T
L.brevis JCM 1059T
L. hilgardii JCM 1155T
L. parakefiri JCM 8573T
L. buchneri JCM 1115T
L. parabuchneri JCM 12493T
L. kefiri JCM 5818T
L. kunkeei JCM 16173T
P. selangorensis LMG 17710T
L. perolens JCM 12534T
L. algidus JCM 10491T
L. mali JCM 1116T
L. nagelii ATCC 700692T
L. murinus JCM 1717T
L. animalis JCM 5670T
L. ruminus ATCC 25644T
L. equi JCM 10991T
L. agilis JCM 1048T
L. cypricasei LMG 21592T
L. salivarius JCM 1231T
L. salicinius JCM 1150T
L. aviarius JCM 5666T
L. araffinosus JCM 5667T
L. coryniformis JCM 1164T
L.bifermentans JCM 1094T
L. manihotivorans JCM 12514T
L. rhamnosus JCM 2771T
L. zeae ATCC15820T
TH 14
L.casei ATCC15820T
L. sharpeae JCM 1186T
B. subtilis JCM 1465T
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Figure 1 Phylogenetic tree showing the relative positions of strains TH 14, TH21, TH 64 isolated from tropical silages and related *Lactobacillus* species as inferred by the neighbor-joining method of complete 16S rRNA gene sequences. Bootstrap values for a total of 1,000 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an out group. The bar indicates 2% sequence divergence. *L.*: *Lactobacillus*; *B.*: *Bacillus*; *K*nuc: nucleotide substitution rates.

Table 4 Chemical composition, gross energy (GE), lactate buffer capacity (LBC) and water-soluble carbohydrate (WSC) content of purple Guinea and sorghum at before ensiling.

	DM	OM	CP	EE	NDF	ADF	ADL	GE	LBC	Fructose	Glucose	Total WSC
	g/kg DM							(Mcal/kg)	(meq/kg DM)	g/kg DM		
Purple Guinea												
Fresh	255.20^{c}	921.70^{b}	65.10^{a}	21.70^{a}	827.70^{b}	517.00^{a}	84.60^{a}	4.29	1,391.07^{b}	0.07^{b}	0.31^{b}	0.38^{b}
Wilted	359.40^{a}	919.20^{b}	66.20^{a}	17.20^{b}	847.80^{a}	504.00^{b}	83.50^{a}	4.35	1,293.82^{b}	0.05^{b}	0.25^{b}	0.30^{b}
Sorghum	259.70^{b}	971.80^{a}	52.10^{b}	15.60^{b}	604.20^{c}	383.60^{c}	46.10^{b}	4.31	2,425.88^{a}	7.82^{a}	24.78^{a}	33.47^{a}
SEM	0.008	0.039	0.015	0.010	0.004	0.031	0.039	0.014	104.708	0.081	0.153	0.076
P-value	<0.001	<0.001	0.004	0.016	<0.001	<0.001	0.004	0.052	0.003	<0.001	<0.001	<0.001

*: Sucrose,maltose and lactose in all samples were at below the detectable level (0.001 g/kg DM).

$^{a\ to\ c}$: Means within columns with different superscript letters differ ($P < 0.05$).

DM: dry matter; OM: organic natter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.

437 **Table 5** Chemical composition of purple Guinea and sorghum silages at 30 d of ensiling.

		OM	CP	EE	NDF	ADF	ADL	GE
		g/kg DM						(Mcal/kg)
Purple Guinea								
Fresh	Control	916.50^{f}	56.50	22.50^{a}	760.60^{ab}	550.70^{a}	64.20^{de}	4.26^{e}
	TH 14	926.80^{b}	60.20	21.50^{ab}	738.60^{cd}	524.60^{bcd}	59.80^{def}	4.17^{hij}
	TH 21	924.50^{bcd}	54.90	19.50^{bc}	754.40^{abcd}	526.10^{bcd}	65.60^{de}	4.22^{f}
	TH 64	922.80^{cde}	54.20	21.60^{ab}	752.10^{abcd}	534.20^{abcd}	66.70^{de}	4.20^{fgh}
	CH	925.10^{bcd}	56.00	19.60^{bc}	753.90^{abcd}	535.10^{abcd}	70.00^{cd}	4.20^{fghi}
	SN	925.40^{bc}	55.20	19.80^{bc}	760.90^{ab}	544.80^{ab}	68.50^{cde}	4.22^{f}
Wilted	Control	923.80^{bcd}	55.70	16.50^{d}	764.30^{a}	539.30^{abc}	63.80^{de}	4.18^{ghij}
	TH 14	923.80^{bcd}	61.60	18.40^{cd}	739.60^{bcd}	520.10^{cd}	55.00^{ef}	4.21^{fg}
	TH 21	925.30^{bc}	56.40	16.90^{d}	769.60^{a}	539.90^{abc}	66.30^{de}	4.16^{j}
	TH 64	926.20^{bc}	55.60	16.80^{d}	757.10^{abc}	534.50^{abcd}	64.70^{de}	4.16^{j}
	CH	921.70^{de}	57.90	18.20^{cd}	749.60^{abcd}	521.50^{cd}	66.00^{de}	4.15^{j}
	SN	919.60^{ef}	63.00	19.40^{bc}	733.30^{d}	516.20^{d}	50.20^{f}	4.16^{ij}
Sorghum	Control	970.50^{a}	55.40	18.50^{cd}	606.50^{g}	345.70^{fg}	100.70^{a}	4.38^{bc}
	TH 14	970.00^{a}	57.60	18.70^{cd}	605.50^{g}	338.40^{g}	80.60^{bc}	4.45^{a}
	TH 21	968.30^{a}	60.60	20.40^{abc}	681.00^{ef}	370.30^{e}	100.20^{a}	4.38^{bc}
	TH 64	968.30^{a}	57.80	20.00^{bc}	688.40^{e}	379.50^{e}	72.80^{bcd}	4.40^{b}
	CH	967.00^{a}	59.70	20.70^{abc}	673.10^{ef}	367.00^{e}	84.40^{b}	4.36^{cd}
	SN	967.20^{a}	61.90	19.60^{bc}	663.40^{f}	362.30^{ef}	100.50^{a}	4.34^{d}
	SEM	0.013	0.019	0.009	0.076	0.075	0.048	0.013
Forage means	Fresh guinea	923.50^{b}	56.20^{b}	20.70^{a}	753.40^{a}	535.90^{a}	65.80^{b}	4.21^{b}
	Wilted guinea	923.40^{b}	58.40^{a}	17.70^{c}	752.20^{a}	528.60^{a}	61.00^{b}	4.17^{c}
	Sorghum	968.60^{a}	58.80^{a}	19.60^{b}	653.00^{b}	360.50^{b}	89.90^{a}	4.38^{a}
Additive means	Control	936.90^{c}	55.90^{b}	19.20	710.50^{c}	478.60^{a}	76.20^{a}	4.27^{a}
	TH 14	940.20^{a}	59.80^{a}	19.50	694.50^{d}	461.00^{b}	65.10^{c}	4.28^{a}
	TH 21	939.40^{ab}	57.30^{ab}	18.90	735.00^{a}	478.80^{a}	77.40^{a}	4.25^{bc}
	TH 64	939.10^{ab}	55.80^{b}	19.40	732.50^{a}	482.70^{a}	68.10^{bc}	4.25^{b}
	CH	937.90^{bc}	57.90^{ab}	19.50	725.50^{ab}	474.50^{a}	73.50^{ab}	4.23^{c}
	SN	937.40^{bc}	60.00^{a}	19.60	719.20^{bc}	474.40^{a}	73.00^{ab}	4.24^{bc}
	Forages (F)	<0.001	0.018	<0.001	<0.001	<0.001	<0.001	<0.001
	Additives (A)	0.006	0.008	0.887	<0.001	0.006	0.007	<0.001
	F x A	<0.001	0.085	0.005	<0.001	0.003	<0.001	<0.001

438 $^{a\ to\ f}$: Means within columns with difference superscript letters differ ($P < 0.05$).

439 DM: dry matter; OM: organic natter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid
440 detergent fiber; ADL: acid detergent lignin; GE: gross energy.

441 TH 14: *Lactobacillus casei;* TH 21 and TH 64: *L. plantarum*; CH: commercial inoculant Chikuso-1,
442 *L.plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan; and SN: commercial inoculant Snow Lact L, *L.*
443 *rhamnosus*, Snow Brand Seed Co., Ltd.

Table 6 DM, pH and five fermentation products of purple Guinea and sorghum silages at 30 d of ensiling.

		DM	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Ammonia-N
		g/kg		g/kg DM				
Purple Guinea								
Fresh	Control	227.50^{ef}	6.58^{a}	26.81^{fg}	12.17^{d}	1.03^{c}	3.20^{a}	1.42^{b}
	TH 14	246.00^{e}	4.55^{def}	61.86^{cd}	11.78^{d}	0.40^{efg}	0.18^{de}	0.37^{de}
	TH 21	237.50^{ef}	4.62^{cde}	48.35^{de}	13.64^{d}	0.41^{efg}	0.71^{d}	0.50^{ef}
	TH 64	238.60^{ef}	4.68^{efg}	49.00^{de}	12.57^{cd}	0.48^{def}	0.19^{b}	0.25^{d}
	CH	233.70^{ef}	4.72^{cd}	36.94^{ef}	15.04^{c}	0.43^{defg}	0.30^{de}	0.49^{d}
	SN	225.80^{f}	4.84^{c}	32.16^{fg}	17.53^{b}	0.43^{defg}	0.76^{cd}	0.50^{d}
Wilted	Control	342.50^{c}	5.93^{b}	18.18^{g}	3.97^{g}	0.57^{de}	0.33^{de}	0.50^{d}
	TH 14	380.60^{ab}	4.40^{g}	69.64^{bc}	5.67^{fg}	0.25^{g}	0.00^{e}	0.15^{f}
	TH 21	338.30^{d}	4.48^{efg}	62.05^{cd}	9.14^{e}	0.28^{fg}	0.00^{e}	0.30^{ef}
	TH 64	322.30^{cd}	4.54^{g}	56.37^{cd}	9.11^{e}	0.28^{fg}	0.12^{de}	0.29^{ef}
	CH	366.20^{b}	4.49^{fg}	64.26^{c}	9.18^{e}	0.27^{g}	0.00^{e}	0.25^{ef}
	SN	393.10^{a}	4.42^{g}	64.56^{c}	7.80^{ef}	0.24^{g}	0.00^{e}	0.29^{ef}
Sorghum	Control	238.80^{ef}	3.70^{h}	59.51^{cd}	22.51^{a}	1.61^{a}	2.07^{b}	1.64^{a}
	TH 14	245.20^{ef}	3.38^{j}	108.30^{a}	15.34^{bc}	0.61^{d}	0.49^{de}	0.58^{d}
	TH 21	228.40^{ef}	3.58^{hi}	64.79^{c}	3.49^{g}	1.29^{b}	1.16^{b}	0.92^{c}
	TH 64	233.30^{ef}	3.64^{hi}	82.64^{b}	3.89^{g}	1.40^{b}	1.44^{de}	0.86^{c}
	CH	227.20^{ef}	3.55^{hi}	60.39^{cd}	4.43^{g}	1.27^{b}	1.43^{b}	0.87^{c}
	SN	235.00^{ef}	3.52^{ij}	65.48^{c}	5.47^{fg}	1.25^{b}	1.38^{b}	0.88^{c}
	SEM	0.067	0.063	4.242	0.895	0.065	0.094	0.060
Forage means	Fresh guinea	234.90^{b}	5.00^{a}	43.64^{c}	13.71^{a}	0.54^{b}	1.02^{a}	0.59^{b}
	Wilted guinea	357.20^{a}	4.71^{b}	55.48^{b}	7.48^{c}	0.31^{c}	0.08^{b}	0.30^{c}
	Fresh sorghum	234.70^{b}	3.56^{c}	72.86^{a}	9.52^{b}	1.24^{a}	1.24^{a}	0.96^{a}
Additive means	Control	269.60^{cd}	5.40^{a}	27.78^{c}	12.88^{a}	1.13^{a}	1.87^{a}	1.13^{a}
	TH14	290.60^{a}	4.14^{c}	71.83^{a}	10.93^{b}	0.44^{c}	0.23^{c}	0.37^{d}
	TH21	268.10^{cd}	4.19^{bc}	59.31^{b}	9.42^{cd}	0.66^{b}	0.40^{bc}	0.65^{b}
	TH64	264.70^{d}	4.29^{b}	62.67^{b}	8.53^{d}	0.72^{b}	0.52^{bc}	0.47^{cd}
	CH	275.70^{bc}	4.25^{b}	58.02^{b}	8.86^{d}	0.74^{b}	0.49^{bc}	0.54^{bc}
	SN	284.60^{ab}	4.26^{b}	55.57^{b}	10.27^{bc}	0.73^{b}	0.71^{b}	0.56^{bc}
Significance of main effects and interactions								
	Forages (F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Additives (A)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	F x A	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a\ to\ j}$, Means within columns with different superscript letters differ ($P < 0.05$). Values are means of three silage samples.TH 14: *Lactobacillus casei;* TH 21 and TH 64: *L. plantarum*; CH: commercial inoculant Chikuso-1, *L. plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan; and SN: commercial inoculant Snow Lact L, *L. rhamnosus*, Snow Brand Seed Co., Ltd.