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RESEARCH ARTICLE

Characterization of Two Lactic Acid Bacteria and Their Influence on Silage Fermentation of Napiergrass

Gang Guo, Xiao-hui Sun, Xiao-yan Qiu, Masataka Shimojo[1] and Tao Shao*

Institute of Ensiling and Processing of Grass, College of Animal Sciences and Technology, Nanjing Agricultural University, Weigang 1, Nanjing, 210095, China; [1]Laboratory of Animal Feed Science, Department of Animal and Marine Bioresource Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
*Corresponding author: taoshaolan@163.com

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Enterococcus faecium R5-1 (EF) and *Lactobacillus plantarum* N30-6 (LP) isolated from silages were identified and their influence on silage fermentation of napiergrass (*Pennisetum purpureum* Sch.) harvested at various times of sunny day were studied. Strain LP had stronger growth ability, acid tolerating capacity and wider fermentable carbohydrates than strain EF. Napiergrass was cut at 0800, 1300 and 1800 h on a sunny day and inoculated with strains EF and LP at 5 log cfu g^{-1}. The concentrations of dry matter, water soluble carbohydrates and the ratio of lactic acid to acetic acid (LA/AA) were higher ($P<0.05$) and ammonia-N (NH_3-N), acetic acid concentrations and silage pH were lower ($P<0.05$) for uninoculated silages made of napiergrass cut at 1300 and 1800 h compared with that cut at 0800 h. Silage inoculated with LP and EF had lower ($P<0.05$) NH_3-N and acetic acid concentrations and higher ($P<0.05$) LA/AA than uninoculated silage made with napiergrass cut at 1300 and 1800 h. The EF-inoculated could not improve fermentation quality of silage made with napiergrass cut at 0800 h, which had higher ($P<0.05$) NH_3-N and acetic acid concentrations than uninoculated silage. In conclusion, delayed cutting napiergrass at end of a sunny day was associated with better fermentation quality, and the silages inoculated with EF or LP further improved fermentation quality.

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INTRODUCTION

The water-soluble carbohydrates (WSC) concentration and lactic acid bacteria (LAB) counts and composition of forages are important factors for silage making. Ensiling tropical grass may be difficult due to their lower WSC concentration (Zhang *et al*., 2011; Maqsood *et al*., 2012; Atis *et al*., 2012). Nevertheless, grass exhibit diurnal variation in WSC, which reaches a peak level at sundown during photosynthesis (McDonald *et al*., 1991) and can be used as fermentable substrate for LAB during ensiling. On the other hand, most of the epiphytic LAB is heterofermenter, which may not be the most effective organisms for promoting predominant lactic acid fermentation (Cai and Kumai, 1994). Inoculation of silage with homofermentative LAB has been used widely to improve silage quality (Cai 1999; Li and Nishino, 2011). However, the efficiency of LAB inoculants for silages was highly influenced not only by their growing and acid-producing ability, but also by epiphytic microflora and properties of plants to be ensiled. The composition of epiphytic microflora varies with environmental factors during a sunny day (Jacobs and Sundin, 2001).

Napiergrass (*Pennisetum purpureum* Sch.) is widely distributed throughout tropical and subtropical regions of the world and is also one of China's major forage crops used either as fresh green-chop or as hay or silage (Zhang *et al.*, 2011). However, less information is available on the homofermrntative LAB influence on silage fermentation of napiergrass harvested at various times on sunny day. In the present study, the homofermrntative *Enterococcus* and *Lactobacillus* isolated from grass silages were characterized to determine their effects on silage fermentation of napiergrass harvested at various times of sunny day.

MATERIALS AND METHODS

Bacterial isolates and their identification: Two LAB strains (R5-1 and N30-6) were isolated and purified thrice

by MRS agar. Morphology, Gram staining, catalase activity, nitrate reduction, gas production from glucose and growth at different temperatures, salt concentrations and pH of LAB were analyzed by the methods of Cai (1999). Each LAB strain was identified by carbohydrate fermentation of 49 different compounds (API 50 CHL, bioMérieux, France). Bacterial DNA was extracted using the method of Zoetendal *et al.* (1998). The 16S rRNA gene was amplified by a PCR thermal cycler (Takara PCR System TP600, Japan) according to Cai *et al.* (1999) and purified using a commercial DNA purification kit (Axygen, San Francisco, USA). The 16S rRNA sequencing were analysed by 3730xl DNA Analyzer (ABI Applied Biosystems, San Francisco, USA) and the sequence homology searches were performed by BLAST program at GenBank data library. A phylogenetic tree was constructed by the methods of Thompson *et al*. (1994).

Bacterial inoculants and ensiling: Primary growth of napiergrass (vegetative stage) was harvested at 0800 (AM), 1300 (M) and 1800 h (PM) on 19 July 2012 from experimental field at Nanjing Agriculture University. Weather at harvest was clear. The harvested napiergrass was immediately chopped into approximately 1-2 cm length with a fodder chopper, thoroughly mixed and collected. The silage treatments were designed as follow: uninoculated control (C), *Enterococcus faecium* R5-1 (EF) and *Lactobacillus plantarum* N30-6 (LP). The levels of each LAB inoculation were determined at 5 log cfu g^{-1} FW. About 710 grams of chopped grass with or without LAB were packed into triplicate plastic laboratory silos (1 L capacity, Yuan *et al*., 2013) and stored at ambient temperature for 30 days.

Chemical analyses and microbial enumeration: The dry matter (DM) content of fresh and post-ensiled crops was determined by drying in an oven at 65°C for 48 h. The buffering capacity of pre-ensiled crops was determined according to Yu *et al.* (2011). The total nitrogen (TN) was determined according to method 976.05 of AOAC (1990). Silage pH, NH_3-N, lactic acid and volatile fatty acids (VFAs) were determined using their water extracts (Shao *et al*., 2007) by the method of Rong *et al.* (2013). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van-Soest *et al.* (1991). The microorganism numbers were determined by the plate count method. LAB, aerobic bacteria, yeasts and moulds were counted on GYP-$CaCO_3$ (Cai, 1999), nutrient and Rose Bengal agar, respectively.

Statistical analyses: Data on chemical and microbial composition of per-ensiled forages of three cutting was analyzed by 1-way analysis of variance, and data on chemical composition of 30-d silages was analyzed by 2-way analysis of variance with cutting times and inoculate treatments as main factors and Fisher's least significant difference test using the GLM procedure of SAS. Effects were considered significant at $P<0.05$.

RESULTS

Phenotypic and genotypic characterization of LAB strains: Strains R5-1 and N30-6 were gram-positive, catalase-negative and homofermentative cocci and rods, respectively (Table 1). The two strains grew normally at 15°C and in 3% NaCl. Strain R5-1 grew normally at 45°C and in 6.5% NaCl, while strain N30-6 grew weakly at 45°C and did not grow in 6.5% NaCl. Strain N30-6 had stronger acid-tolerating capacity than strain R5-1, which did not grow at the pH 4.0. Strain R5-1 did not ferment amygdalin, α-methyl-D-mannoside, gluconate, L-arabinose, mannitol, melezitose, raffinose rhamnose and sorbitol, which made them different from strain N30-6.

Table 1: Phenotypic characteristics of two lactic acid bacteria strains

Characteristics‡	R5-1	N30-6
Shape	Cocci	Rod
Fermentation type	Homofermentative	Homofermentative
Gram stain	+	+
Catalase	-	-
Gas form glucose	-	-
Growth at		
15℃	+	+
45℃	+	w
Growth in NaCl		
3%	+	+
6.5%	+	-
Growth at pH		
3.0	-	-
3.5	-	w
4.0	-	+
4.5	+	+
5.0	+	+
6.0	+	+
Amygdalin	-	+
α-Methyl-D-Mannoside	-	+
Gluconate	-	+
L-Arabinose	-	+
Mannitol	-	+
Melezitose	-	+
Raffinose	-	+
Sorbitol	-	+
Trehalose	w	+

‡ +, positive; −, negative; w, weakly positive; Two strains produced acid from arbutin, β-Gentiobiose, cellbiose, esculin, fructose, galactose, glucosamine, glucose, lactose, maltose, melibiose, *N*-acetyl mannose, ribose, salicin and sucrose, but failed to produce acid from adonitol, α-methyl-D-glucoside, β-methyl-xyloside, D-arabinose, D-arabitol, D-fucose, D-tagatose, dulctiol, D-xylose, erythritol, glycerol, glycogen, inositol, inulin, 2-K-gluconate, 5-K-gluconate, L-arabitol, L-fucose, L-xylose, lyxose, rhamnose, sorbose, starch, turanose and xylitol.

Table 2: Chemical and microbial compositions of fresh napiergrass harvested at various times of sunny days.

Item	Harvested time‡				
	0800 h	1300 h	1800 h	SEM	Signifi-cance
Dry matter (g kg^{-1} FW)	155.86	163.56	165.15	1.91	
Crude protein (g kg^{-1} DM)	79.76^{a}	75.20^{ab}	72.44^{b}	1.28	*
Buffering capacity (meq kg^{-1} DM)	65.44^{b}	74.29^{a}	73.33^{a}	1.68	*
Neutral detergent fiber (g kg^{-1} DM)	663.02	657.30	652.49	3.30	
Acid detergent fiber (g kg^{-1} DM)	399.32	402.44	398.85	3.63	
Water soluble carbohydrates (g kg^{-1} DM)	60.88^{b}	72.24^{ab}	84.36^{a}	3.83	**
Lactic acid bacteria (log cfu g^{-1} FW)	5.03^{a}	4.76^{b}	4.83^{b}	0.04	*
Aerobic bacteria (log cfu g^{-1} FW)	7.82^{a}	7.67^{ab}	7.60^{b}	0.04	*
Mold and yeast (log cfu g^{-1} FW)	4.48	4.60	4.27	0.09	

‡ The harvested day (19 July 2012) was sunny. The temperature and relative humidity were 28 and 74%, 33 and 50%, and 28.5 and 73% at the three cuts on this day; Values with different lower case letters show significant differences $P<0.05$. *$P<0.05$, **$P<0.01$.

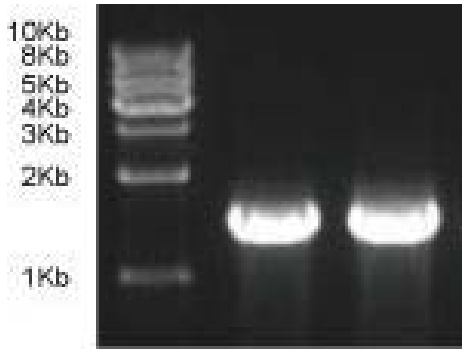

Fig. 1: PCR products of strains R5-1and N30-6.

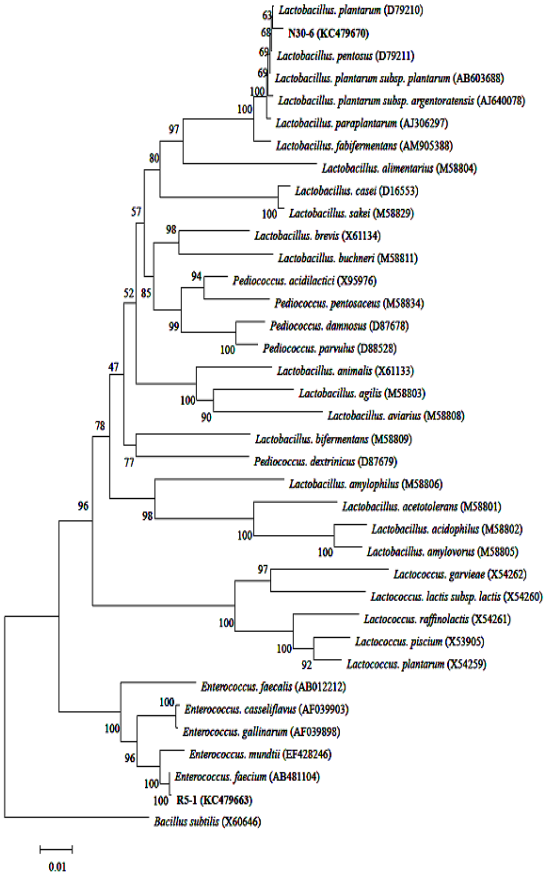

Fig. 2: Phylogenetic tree showing the relationship of strains R5-1, N30-6 and related species on the 16S rRNA gene sequences by the neighbor-joining bootstrap analysis. *Bacillus subtilis* is used as an outgroup. The bar shows 1% sequence divergence.

About 1,500 bases of 16S rRNA gene of strains R5-1 and N30-6 were determined (Fig. 1). Phylogenetic tree is shown in Figure 2. The sequence of strains R5-1 and N30-6 were in line with *E. faecium* and *L. plantarum*, respectively. Strain R5-1 belongs to *E. faecium* and strain N30-6 belongs to *L. plantarum*.

Herbage composition: The DM content of fresh forages was about 160 g kg^{-1} (Table 2). The WSC concentrations were lowest in the morning and increased (P<0.05) through the mid to late afternoon, contrarily, the CP concentration decreased (P<0.05) and NDF and ADF concentrations tended to slightly decrease (P<0.05). The buffering capacity was lower (P<0.05) and epiphytic LAB, aerobic bacteria counts were higher (P<0.05) for fresh napiergrass cut in AM as compared to those of PM- and M-cut forages.

Fermentation quality of silages: The cutting time significantly (P<0.05) influenced pH, ratio of lactic acid to acetic acid (LA/AA) and DM, NH_3-N, WSC, lactic acid and acetic acid concentrations, but it did not affect the concentrations of both NDF and ADF (Table 3). In silage made with AM-cut forages, pH and NH_3-N concentrations were lower (P<0.05) for LP-inoculated silage as compared to those of EF-inoculated and uninoculated silages, and the NH_3-N and acetic acid concentrations were higher (P<0.05) for EF-inoculated silage as compared to those of uninoculated silages. In silage made with M-cut forages, the NH_3-N and acetic acid concentrations were lower (P<0.05) and WSC concentration and LA/AA were higher (P<0.05) for EF and LP-inoculated silage as compared to those of uninoculated silage. In silage made with PM-cut forages, LP-inoculated silage had lower (P<0.05) NH_3-N and acetic acid concentrations and higher (P<0.05) WSC concentration and LA/AA, and EF-inoculated silage had a lower (P<0.05) acetic acid concentrations and a higher (P<0.05) LA/AA than those of uninoculated silage. Butyric acid was absent or detected in small amounts in all silages.

DISCUSSION

E. faecium and *L. plantarum* are often found in association with fresh grass or silages and usually used as inoculants to improve silage quality (McDonald *et al*., 1991; Cai 1999; Li and Nishino, 2011). In the present study, the phenotypic characteristics of strains R5-1 and N30-6 showed that they were homofermentative LAB and were considered to be enterococci and lactobacilli, respectively, by comparison with some reports (McDonald *et al*., 1991; Cai, 1999; Pang *et al*., 2011). The two strains could not be identified down to the species level on the basis of phenotypic characteristics. The 16S rRNA sequence analysis method is usually used to identify the organisms by genus and species (Bjorkroth *et al*., 2002). Following 16S rRNA sequence analysis, strain R5-1 and N30-6 were clearly assigned to *Enterococcus* and *Lactobacillus*, respectively. Strain R5-1 was most closely related to the *E. faecium*, because it showed 99.1% similarity in 16S rRNA sequences and 100% supported by bootstrap analysis on the phylogenetic tree. Strain N30-6 was clearly related to *L. plantarum,* showing 99.9% similarity in 16S rRNA sequences.

The WSC concentration of forages is one of the main factors for the speedy establishment and growth of LAB during ensiling. McDonald *et al.* (1991) considered that photosynthesis causes a net increase in WSC concentration of forages during the day. The increased WSC concentration is associated with a decrease in NDF and CP concentrations in forages during photosynthesis (Griggs *et al*., 2005). In the present study, forage WSC concentration increased, contrarily, CP concentration decreased and NDF concentration tended to slightly decline from sunup to sundown. On the other hand,

Table 3: The effect of LAB additives on nutritive quality of 30-d fermented silages made of napiergrass cut at various times of sunny day

	Harvest time[1]												
	0800 h			1300 h			1800 h				Significance[2]		
Item	C	EF	LP	C	EF	LP	C	EF	LP	SEM	T	I	T × I
Dry matter (g kg^{-1} FW)	158^{c}	149^{d}	163^{bc}	167^{ab}	165ab	166ab	165ab	171a	168ab	1.31	**		**
pH	4.21a	4.21a	3.92b	3.84bc	3.73c	3.71c	3.80bc	3.74c	3.68c	0.041	**	**	
Ammonia-N (g kg^{-1} TN)	126b	172a	87.53c	80.02cd	53.0de	43.5e	80.1cd	69.9d	51.6e	7.99	**	**	**
Water soluble carbohydrates (g kg^{-1} DM)	7.23de	6.52e	7.62de	8.56cd	11.4ab	11.9ab	10.2bc	10.4bc	13.0a	0.462	**	**	
Lactic acid (g kg^{-1} DM)	38.5d	35.2d	40.9cd	40.0cd	49.2a	41.9bc	43.8ab	43.4ab	41.0bc	1.02	*		
Acetic acid (g kg^{-1} DM)	15.5b	22.9a	12.5b	6.89c	3.90de	3.63e	6.48d	3.77e	2.72e	1.35	**	*	**
Butyric acid (g kg^{-1} DM)	0.281	ND	ND	ND	ND	ND	ND	ND	ND				
Lactic acid /acetic acid	2.64c	1.58c	3.48c	5.96bc	12.9a	13.0a	6.79b	11.9a	15.4a	1.04	**	**	
Neutral detergent fiber (g kg^{-1} DW)	640	638	649	645	647	655	637	634	640	2.79			
Acid detergent fiber (g kg^{-1} DW)	420	416	433	421	426	433	414	408	418	2.78			
lactic acid bacteria (log CFU g^{-1} FW)	6.95a	6.83a	6.74a	6.85a	6.52a	5.78b	6.73a	6.56a	5.83b	0.090	**	**	**

[1]C: uninoculated control, EF: *Enterococcus faecium* R5-1, LP: *Lactobacillus plantarum* N30-6. ND means not detected; [2]T: cutting time, I: inoculant. Values with different lower case letters show significant differences P<0.05. *P<0.05, **P<0.01.

epiphytic LAB is another important factor influencing the fermentation quality of silages with or without inoculants. Epiphytic microorganisms are located primarily on the leaves and their population is lower under high temperature and low relative humidity conditions, because leaves provide a few of nutrient such as sugars and moisture to microorganisms (Jacobs and Sundin, 2001; Lindow and Brandl, 2003). Furthermore, some unpigmented bacterial strains might be killed by solar ultraviolet radiation (Jacobs and Sundin, 2001). In the present study, AM-cut napiergrass had higher counts of epiphytic aerobic bacteria and LAB than other cutting times. The concentrations of WSC, DM and LA/AA were higher and silage pH and concentrations of NH_3-N and acetic acid were lower for uninoculated silages made of M- and PM- cut forages as compared to AM-cut forage. This is in agreement with Owens *et al*. (2002), who observed that alfalfa and red clover harvested at afternoon gave a better silage quality.

It is generally held that the efficiency of LAB inoculants for silages depends on many factors, including the type and chemical composition of forages, epiphytic microflora and the characteristics of LAB. Silage inoculated with homofermentative LAB results in fast decrease in pH, high LA/AA and low NH_3-N concentration (Weinberg and Muck, 1996). In this study, silages inoculated with *L. plantarum* N30-6 or *E. faecium* R5-1 improved fermentation quality than uninoculated silage made with M- and PM-cut napiergrass. Silages inoculated with *L. plantarum* N30-6 also could be improved, but *E. faecium* failed to improve fermentation quality of silage made with AM-cut forages. This is in agreement with some studies (Weinberg *et al.,* 1993, Cai, 1999), who have reported that the inoculation of forage with *L. plantarum* was more beneficial in improving silage quality than *E. faecium*. The most plausible explanation lies in the chemical and epiphytic bacteria composition of the forages and physiological properties of inoculants. There were lowest WSC concentrations and the highest epiphytic LAB and aerobic bacteria counts for AM-cut forages compared with other cutting times. Therefore, we hypothesized that in AM-cut forages, the epiphytic LAB (generally heterofermentative cocci, Cai and Kumai, 1994) or aerobic bacteria may not be overwhelmed by the inoculant LAB and consumed more WSC during the initial stage of ensiling, which resulted in increased concentrations of NH_3-N and acetic acid, then the less fermentable substrate failed to promote fermentation by the strong acid-producing LAB. Furthermore, compare with *E. faecium*, *L. plantarum* can easily and successfully colonise in fresh forages, because it can ferment a wide variety of substrates, is highly competitive and produces large amounts of acid quickly (McDonald *et al*., 1991). The *L. plantarum* had stronger acid-tolerating capacity than *E. faecium*, which could not grow at low pH (<4.5).

Conclusion: Delayed cutting napiergrass at the end of a sunny day was associated with better silage fermentation quality. Silages inoculated with *L. plantarum* N30-6 could improve fermentation quality whenever napiergrass harvest time. *E. faecium* R5-1 could improve fermentation quality of napiergrass cut at 1300 and 1800, but failed to improve fermentation quality of napiergrass cut at 0800.

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