Effects of *Lactobacillus plantarum* BCC65951 inoculation in Napier Pakchong 1 silage on *in vitro* rumen degradability and growth performance of Brahman cattle

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Research

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Abstract

Background: Napier grass Pakchong 1 is used as a fodder crop in Southeast Asia. Unfortunately, its fermentation for silage production is challenging owing to the low dry mass content and epiphytic lactic acid bacteria. Here, *Lactobacillus plantarum* BCC65951 (LAB) inoculation was tested for impact on silage fermentation and use. Silage was prepared with or without inoculation of LAB and stored for 180 days prior to analysis.

Results: Gas production at 4-24 h was significantly higher in LAB group (P=0.001). Therefore, the result implied the faster ruminal degradation of LAB, especially during the first 24 h. LAB-inoculated silage exhibited greater levels of lactic acid and *in vitro* rumen degradability compared to control silage, and significantly lower pH and acetic acid content. To investigate the effect of LAB inoculation on animal performance, ten Brahman bulls were fed on control or LAB-inoculated silage in a crossover experimental design. Animals fed with LAB-inoculated silage had significantly higher voluntary roughage intake and greater average daily body weight gain than those fed with control silage.

Conclusions: Results from this study revealed the beneficial effect of Napier Pakchong 1 ensiled with *L. plantarum* BCC65951 inoculation, both on the fermentative quality of the silage and also the performance of animals fed with the inoculated silage.

Introduction

Napier Pakchong 1 grass, a hybrid between *Pennisetum purpureum x P. glaucum*, developed at the Nakhon-Ratchasima Animal Nutrition Research and Development Center, Thailand, is used as a fodder crop [1]. It is notable for its high yield (about 6200 kg. fresh grass/hectare per cutting at 45-60 days of maturity) [1]. Therefore, it is grown throughout Thailand and neighboring countries in Southeast Asia for both ruminant feed and also biomass for energy production (Figure 1).

In the view of animal feed, the forage is produced in excess of animal consumption during the rainy season, but the yield of fresh grass is inadequate in dry season since most of the grass is grown in areas without irrigation. Preserving the surplus forage in form of silage during the rainy season is the most appropriate way to meet the roughage requirement all year round. However, making Napier grass silage is challenging because of the grass has low dry matter contents (approximately 16-20%). Similar to other tropical grass, Napier grass is also low in epiphytic lactic acid bacteria which are essential for ensiling to produce good quality silage [2, 3]. Napier grass silage always has a low and inconsistent fermentative quality and more importantly the quality of silage often decline rapidly led to the short storage lifetime i.e. less than 90 days. The low density of epiphytic lactic acid bacteria in tropical forage compared with other microorganisms means that production of lactic acid is insufficient to maintain the low pH necessary for fermentation during the ensiling process [3].

Previous studies showed that inoculation of lactic acid bacterial (LAB) improved fermentative quality of silage when compared with grass silage fermented without inoculation(Driehuis, Oude Elferink, & Van

Wikselaar, 2001). Furthermore dry matter loss and densities of undesirable bacteria in the inoculated silage were also lower [4]. However, commercial starter cultures are isolated from epiphytic bacteria of fodder crop grown in the temperate zone which are poorly adapted to the tropical climate with higher temperature and humidity [5]. For this reason, the use of starter culture isolated from forage grown in the tropics could be an appropriate approach to increase fermentative quality, rumen degradability, extend storage time and also improve growth performance of cattle.

Lactobacillus plantarum (*L. plantarum*) is a lactic acid bacterium that is commonly found in forage. It is a facultative heterofermentative bacterium that ferments sugars to produce lactic acid, ethanol or acetic acid, and carbon dioxide. Moreover, *L. plantarum* fermentation is associated with reduced deamination which preserves protein integrity [6]. The consistent increases of microbial biomass shown by previous *in vitro* study may explain its positive effects on milk production and animal growth performance [6].

Lactobacillus plantarum BCC65951 was isolated from sugarcane silage; therefore, it could be well-suited as a silage starter for the fermentation of the silage under typical conditions of tropical countries. The objectives of this study were to determine fermentative quality, rumen degradability of Napier Pakchong 1 ensiled with or without an inoculation of *Lactobacillus plantarum* BCC 65951. In addition, growth performance of Brahman cattle fed Napier Pakchong 1 ensiled with or without an inoculation and stored for 180 days was also investigated.

Material And Methods

Strain and cultivation condition

L. plantarum BCC 65951 were grown for 16-18 h at 37 °C in MRS (Difco, Fisher Scientific, Pittsburgh, PA, USA) broth harvested by centrifugation (Beckman Coulter Avanti J-E, Beckman Coulter Life Sciences, Indianapolis, IN, USA) at 10,000 x G for 10 minutes, washed and resuspended with 0.85% saline solution. The cultures were diluted to supply a starting concentration of approximately 10^7cfu/g fresh forage in the silage fermentations.

Ensiling process

Napier Pakchong 1 grass was harvested at 60 days of maturity and ensiled into two treatments: no inoculants (control) and inoculated with *L. plantarum* BCC65951 at 10^7cfu/g fresh weight. Silages were prepared using a small scale system, approximately 20 kg portions of forage material chopped into 1-3 cm. length, which was packed tightly in layered plastic bags and vacuum seal. The plastic bags were stored at ambient temperature for 180 days. Thereafter approximately 1 kg of each replicate was collected as the representative of the experimental silage, the samples were kept at minus 20^0C before subjected to chemical and rumen degradability analyses.

Analysis of fermentation quality

The silage samples were collected at different positions including at the top, in the middle and in the bottom of the stack and finally mixed together. For chemical analysis, 12.5 g of the shredded Napier grass samples were homogenized in a 0.02 ml N H_2SO_4 in a stomacher (Seward,West Sussex, UK) for 4 min at 200 rpm. The extract was centrifuged (10,000 rpm, 4^0C, 10 min) and filtered through 0.45 µm membranes (Minisart RC4) prior to be analyzed for fermentation quality [7]. The extract was measured for pH value immediately with a pH meter (Sartorius PB-20, Goettingen, Germany). The volatile fatty acids (VFAs) were analyzed by gas chromatography (GC) [7] . The lactic acid contents were measured by HPLC-UV using a Bio-Rad Aminex HPX-87H column (Bio-Rad Lab., Hercules, CA, USA) with a 0.6 ml/min flow rate of 0.02 N H_2SO_4 at 60^0C [8] .

Chemical Composition Analysis

The fresh samples were dried at 60^0C for 48 h. After that, the sample was ground through a 1 mm of sieve size with a Wiley Mill. Dry matter (DM; Method 934.01, [9]), crude protein (CP; Method 968.06, [9]), ether extract (EE; Method 920.39, [9]) and crude fiber (CF; Method 962.06, [9]) were analyzed. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed using Detergent methods [10].

Determination of rumen degradability by *in vitro* gas-production technique

Rumen *fluid was collected* from four fistulated Thai native bulls. About 230 mg of feed was weighed into 100 ml calibrated glass syringes. Rumen fluid was added to the buffered mineral solution (9.8 g $NaHCO_3$ + 2.77 g Na_2HPO_4 + 0.57g KCl + 0.47g NaCl + 0.12g $MgSO_4$. $7H_20$ + 0.16 g $CaCl_2.2H_20$), and maintained in a water bath at 39°C under continuous flushing with CO_2. About 30 ml of buffered rumen fluid was dispensed into syringes containing the samples and all syringes were incubated in a water bath maintained at 39°C. Gas production was determined at 4, 8, 12, 24, 48, 72 and 96 h of incubation.

Net gas productions (ml/200mg, DM) after 24 h incubation was calculated using the equations as follows [11]:

(ml /200 mg DM)=

Where

GP_t = Net gas production (ml/200mg, DM) after 24 h incubation

V_t = Gas volume at time *t*

V_t = Gas volume at *t* = 0

GP_0 = Blank value at time *t*

W = Weight in mg DM of sample in each syringe

FH = Correction factor of hay

FC = Correction factor of concentrate

The incubation residue was distilled by neutral detergent solution to remove the microbial biomass from undegraded substrate [12]. The microbial biomass yield (MBY) was calculated after 24 h incubation according to the following formula:

MBY = truly digested sample − apparently digested sample

truly digested sample

Measurement of growth performance

Ten *Brahman bull (*10.3±0.8 month) with average205 ± 12.8 kg of body weight were housed in individual pens. Vaccination against epidemic diseases and anthelmintic were applied for parasites elimination 10 days before the experiment started and all the experimental animals were allowed to have the adaptation period with the experimental diet for 15 days. The experiment was conducted with a cross-over design. The cattle were fed 1.81 kg. DM/head/d of concentrate and *ad libitum* silage. Clean drinking water was available at all time. To assure that the animals received feed *ad libitum*, silage was offered until at least 5% refusal was obtained. Feed offered and refused were weighted every day to calculate voluntary feed intake. To weight the animal, all feed was taken away from feed bunk at 8.00 PM the day before, and all animals were weighted at 8.00 AM on the following day and fresh feed was provided. This weighting procedure was repeated for 2 consecutive days at the beginning of the experiment after adaptation period and also at the end of each particular period. Average live weights of each individual animal were recorded for statistical analysis.

Statistical analysis

The fermentation *quality of silage,* chemical composition and *in vitro* gas production technique was tested for the equality of variance then statistically analyzed by t-test while, growth performance was statistically analyzed according to the cross-over design using SPSS program. Differences among means were tested using the adjusted Duncan's test, the significance declared at $P<0.05$[13].

Results

Fermentative quality of silage

The organic acid production and pH at the end of the ensiling study period are summarized in table 1. Lactic acid was the predominant organic acid detected in both control and starter culture inoculated fermentation followed by acetic acid whereas butyric acid was presented in low amount. The results obtained from the statistical analysis showed that ensilaging with *L. plantarum* starter culture significantly influenced pH, lactic and acetic production ($P< 0.05$) but did not affect the productions of

butyric acid. At the end of the studied period, the pH value of silage fermented with *L. plantarum* inoculants was significantly lower than that of control fermentation. Although the total acids production of the control and LAB group was not significantly different, but silage fermented with added starter culture had significantly higher concentrations of lactic acid as well as significantly lower acetic acid concentration when compared with control fermentation (Table 1). Addition of *L. plantarum* starter culture contributed to significantly different in the organic acid ratio of silage. Silage fermented with added starter culture has higher lactic acid and lower acetic ratio than that of the control fermented silage.

Table 1 Organic acids profile of silage with or without LAB inoculation

Item	pH	gram/100grams Dry Weight				Ratio of organic acid (%)		
		Lactic	Acetic	Butyric	Total acid[a]	Lactic	Acetic	Butyric
C	4.36[a]	5.94[b]	3.06[a]	0.85	11.06	54.04[b]	27.55[a]	7.64
LAB	3.92[b]	8.37[a]	1.14[b]	0.47	10.85	77.16[a]	10.88[b]	4.06
SEM	0.08	0.49	0.44	0.22	0.82	3.27	3.50	1.82
P-value	0.002	0.002	0.005	0.135	0.805	<0.001	0.003	0.097

C, Control; LAB, *L. plantarum* BCC 65951.

[a]Total acids = lactic acid + acetic acid + butyric acid + propionic acid + pyruvic acid +formic acid

[a,b]*Means within* a column with *different superscripts differ* significantly ($P < 0.05$).

Chemical composition of silage

Dry matter and organic matter percentage of silage in control group were higher than that of in LAB ($P<0.05$) (Table 2). Crude protein, ether extract, water soluble carbohydrate, neutral detergent fiber, acid detergent fiber, acid detergent lignin, cellulose and hemi-cellulose of Napier silage in both control and LAB groups did not differ significantly.

Table 2 Nutrient compositions of silage fermented with or without *L. plantarum* BCC65951 inoculation

Nutrient composition (%)	Treatment		SEM	*P*-value
	Control	*L. plantarum* BCC 65951		
Dry matter	21.31[a]	20.10[b]	0.238	0.005
Organic matter	89.80[a]	88.50[b]	0.384	0.022
Crud fiber	33.31	33.74	0.226	0.457
Crud protein	6.71	6.80	0.130	0.789
Ether extract	3.16	3.75	0.225	0.244
Water soluble carbohydrate	17.17	14.10	1.040	0.147
Ash free neutral detergent fiber	62.77	63.85	0.395	0.208
Ash free acid detergent fiber	46.51	47.40	0.678	0.621
Acid detergent lignin	7.99	7.61	0.225	0.513
Cellulose	38.53	39.80	0.575	0.363
Hemi-cellulose	16.26	16.47	0.389	0.852

[a, b] Means within a row with different superscripts are significantly different ($P<0.05$)

Rumen degradability

Table 3 showed that gas production during 4-24 h incubation was significantly higher in LAB than that of the control group. Thus, the result revealed that grass silage ensiled with *L. plantarum* BCC65951 has digested faster by bacteria in the rumen, especially during the first 24 h of the degradation.

Table 3 *In vitro* rumen degradability and microbial biomass yield of silage with or without *L. plantarum* BCC 65951 inoculations

Item	Treatment		*P*-value
	Control	*L. plantarum* BCC 65951	
Gas production (milliliter)			
at 4 h of incubation	$7.20^{b} \pm 0.75$	$7.99^{a} \pm 0.48$	0.033
at 8 h of incubation	$14.91^{b} \pm 1.37$	$16.75^{a} \pm 1.03$	0.013
at 12 h of incubation	$16.45^{b} \pm 2.10$	$18.67^{a} \pm 1.08$	0.018
at 24 h of incubation	$22.42^{b} \pm 1.90$	$26.27^{a} \pm 1.83$	0.001
at 48 h of incubation	41.51 ± 2.77	43.54 ± 6.75	0.454
at 72 h of incubation	53.01 ± 3.28	53.89 ± 8.04	0.775
at 96 h of incubation	58.15 ± 4.64	58.23 ± 8.86	0.980
Microbial biomass yield			
(gram)	0.0788 ±0.09	0.0789 ± 0.08	0.936
(% of true digested substance)	41.12 ± 7.08	42.60±2.31	0.181

[a, b] Means within a row with different superscripts are significantly different ($P<0.05$).

Animal performance

Feed ingredients and chemical composition of the concentrate that offered to the animals in this experiment was presented in Table 4, whereas table 2 showed chemical composition of experimental silage. Each individual animal received 1.81 kg.DM of concentrate feed per day and *ad libitum* silage. Voluntary feed intake of inoculated silage was significantly greater than the intake in control group (Table 5). However, the chemical composition of concentrate and Napier Pakchong 1 silage with or without inoculation was not significantly different. The average daily gain of the bulls receiving inoculated silage was significantly greater when compared with the animal received control fermented silage (Table 5).

Table 4 Feed ingredients and Chemical composition of the concentrate

Ingredient	(%)	Chemical composition	(%)
Rice bran	50.8	Dry matter	90.5
Cassava chip	10.0	Crud protein	18.0
Soybean meal	35.2	Total Digestible Nutrients	77.9
Dicalcium phosphate	2.0	Ether extract	2.4
Vitamin and mineral premix	2.0	Ash free acid detergent fiber	7.9
Ash free neutral detergent fiber	13.7		

Table 5 Feed intake and growth performance of Brahman cattle fed on Napier silage without or with inoculation

Item	Control	*L. plantarum* BCC 65951
Number of the bulls (N)	10	10
Feed intake (DMI) kg/head/day		
Concentrate	1.81	1.81
Roughage	3.12±0.1[b]	3.28±0.1[a](+5.21%)
Growth performance		
Initial weight (kg±SEM)	204.8±16.8	205.2±4.6
Average Daily Gain (gram±SEM	557.1±88.4[b]	792.7±108.3[a] (+42.29%)

[a, b] Means within a row with different superscripts are significantly different (P<0.05)

Discussion

Fermentative quality of silage

The pH, organic acids concentration and proportion of the organic acids namely lactic, acetic, and butyric acid with total acid founded in silage fermentation can be used as an indicator of silage quality [14]. Several studies have shown the benefit of adding LAB inoculants in silage fermentation to ensure rapid accumulation of organic acids during the ensiling period, which will be leading to the lower pH values [15-18]. In this study, inoculation of Napier silage with *L. plantarum* improved fermentation as reflected in reductions in pH value and acetic acid, concentrations, together with an increase of lactic acid concentrations when compared with the control fermented silage. Furthermore, fermentation with added *L. plantarum* starter culture contributes to significantly higher lactic and lower acetic ratio. Silage treated

with *L. plantarum* remained in good quality for up to 6 months of storage based on the recommendation by Department of Agriculture and Rural Development, Government of Alberta [14]. On the other hand, after 6 months of storage, the quality of naturally fermented silage was less stable and remained only in moderate quality of silage. This result indicates that using *L. plantarum* inoculants for ensilaging can contribute to better quality of silage with extended storage time.

Chemical composition of silage

Almost all of nutrient compositions measured did not differ significantly when compared inoculated silage with control fermentation. However, significantly greater dry matter and organic matter content of silage in control group were observed (Table 2).The reduction in DM concentration was greater in LAB compared to that of control group, which may due to the inclusion of LAB that increased water production because of greater fermentation activity, resulting in an increase of metabolic water, then the lower DM content in the inoculated silage was observed [19].

Rumen degradability

The result from this experiment are in consistent with previous studies, several *L. plantarum* strains have been shown to increase rumen *in vitro* fermentation [6] and lead to *faster degradation* rates in the rumen [20]. The faster degradation of roughage in rumen would be advantageous to the animal by increasing the rate of rumen passage, leading to increased feed intake and consequently increase animal production. Table 3 showed that inoculated silage was degraded faster during the first 24 hours. The first 24 h of silage degradation is critical, since rumen fluid is retained for about 27 h [21]. We infer from the data that the rate of rumen passage is greater when animal received inoculated silage, which could affect feed intake and growth performance (Animal performance section)

Animal performance

Animals received inoculated silage showed greater voluntary feed intake and average daily gain. It is *interesting* to note that voluntary feed intake of the animals receiving inoculated silage was higher than that of the control group by only5.21% whereas average daily gain showed huge improvement of 42.29% (Table 5). These findings are consistent with previous studies. Winters at al. (2001) [22] reported that Charolais beef steers received inoculated Italian ryegrass silage *ad libitum* without concentrate supplementation showed significantly greater dry matter fed intake and live weight gain. Muck and Kung (1997) [23] reviewed several studies showing that *L. plantarum* inoculated silage led to an increase dry matter intake and live weight gain in both growing beef steers and finishing beef steers. *L. plantarum* inoculated silage has a higher lactic acid content, which can be converted to propionate in the rumen and utilized as an energy source for growth in beef cattle [24] . Moreover, feeding ovine with LAB inoculated grass silage increase total bacterial production in the rumen [6, 25] and improved nitrogen retention [26, 27]. In addition, The LAB used for silage inoculants may survive in the rumen which, can be beneficial for the fermentation process in the rumen [28] by inhibiting the growth of undesirable bacteria [29] resulting in improving efficiencies of rumen fermentation. Chen et al. (1992) [30] also reported that

high dry matter intake increased microbial synthesis in the rumen and also *bacterial* N *flow* to *the small intestine.*

Conclusion

This study showed that Napier Pakchong 1 grass silage fermented with *L. plantarum* BCC65951 has a better fermentative quality in all parameters measured namely lower pH value, higher lactic acid and lower acetic acid concentration when compare with control fermentation. In addition, the ensiling Napier grass silage with *L. plantarum* BCC6595 improved voluntary feed intake and growth performance of Brahma cattle. *L. plantarum* BCC65951 is suggested as a starter culture organism for making good quality silage in the tropical zone from forage with low dry matter content and/or low epiphytic lactic acid bacteria.

Abbreviations

DM: Dry matter; CP: Crude protein; EE: Ether extract; CF: Crude fiber; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin; MBY: Microbial biomass yield; VFAs: Volatile fatty acids; GC: Gas chromatography; HPLC-UV: High-Performance Liquid Chromatography-Ultraviolet

Declarations

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Authors' contributions

SS, SY and VP conceived and designed the experiments. SK and KT, performed animal experiments, analyzed the data and wrote the manuscript. KK and IP assisted with data analysis and paper writing. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval

The Institutional Animal Care and Use Committee, National Center for Genetic Engineering and Biotechnology, Thailand, has approved this research project in accordance with the Ethical Principles for the Use of Animals for Scientific Purposes issued by the National Research Council of Thailand. The approval for the Care and Use of Animals for Scientific Purposes Code BT-Animal 15/2559 was grant on September 2016.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Napier Pakchong 1 at 45 days of mutualit