Improvement of Nutritive Value and In vitro Ruminal Fermentation of Leucaena Silage by Molasses and Urea Supplementation

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ABSTRACT: Leucaena silage was supplemented with different levels of molasses and urea to study its nutritive value and in vitro rumen fermentation efficiency. The ensiling study was randomly assigned according to a 3×3 factorial arrangement in which the first factor was molasses (M) supplement at 0%, 1%, and 2% of crop dry matter (DM) and the second was urea (U) supplement as 0%, 0.5%, and 1% of the crop DM, respectively. After 28 days of ensiling, the silage samples were collected and analyzed for chemical composition. All the nine Leucaena silages were kept for study of rumen fermentation efficiency using in vitro gas production techniques. The present result shows that supplementation of U or M did not affect DM, organic matter, neutral detergent fiber, and acid detergent fiber content in the silage. However, increasing level of U supplementation increased crude protein content while M level did not show any effect. Moreover, the combination of U and M supplement decreased the content of mimosine concentration especially with M2U1 (molasses 2% and urea 1%) silage. The result of the in vitro study shows that gas production kinetics, cumulation gas at 96 h and in vitro true digestibility increased with the increasing level of U and M supplementation especially in the combination treatments. Supplementation of M and U resulted in increasing propionic acid and total volatile fatty acid whereas, acetic acid, butyric acid concentrations and methane production were not changed. In addition, increasing U level supplementation increased NH3-N concentration. Result from real-time polymerase chain reaction revealed a significant effect on total bacteria, whereas F. succinogenes and R. flavefaciens population while R. albus was not affected by the M and U supplementation. Based on this study, it could be concluded that M and urea U supplementation could improve the nutritive value of Leucaena silage and enhance in vitro rumen fermentation efficiency. This study also suggested that the combination use of M and U supplementation level was at 2% and 1%, respectively. (Key Words: Leucaena, Silage, Rumen Fermentation, In vitro Gas Production, Urea, Molasses)

INTRODUCTION

Tropical legume forages are a rich source of crude protein (CP) and minerals for animal nutrition, in addition to their contribution to a sustainable agro ecosystem (Bansi et al., 2014). The identification of alternative sources of dietary protein for ruminants is driven by the desire to reduce feeding cost and to ensure profitability and sustainability of livestock production systems. Ensiling is a method for preserving moist crops which ensures animal feed availability throughout the year. Silage processing is based on lactic acid fermentation under anaerobiosis and preserves the nutritive and sanitary qualities of the crops (Cazzato et al., 2011). Legume silages are better accepted by the animal than grass silages, with a tendency to higher animal performance. Leucaena (Leucaena leucocephala) is high in palatability, digestibility and digestible protein and is often recommended as ruminant feed (Barros Rodriguez et al., 2013). Ensiling may be an appropriate method for preservation and toxic reduction because Leucaena is harvested during the rainy season when drying is rather difficult. Sunagawa et al. (1989) reported that around 90% of mimosine is destroyed after 14 to 21 days of ensiling. Silage is widely used in farms and has a substantial role in animal production systems. High silage quality is a key factor in minimizing the cost of production and sustaining animal health. Increasing use of silage has resulted in
continuing efforts to minimize the quality losses. The main aim of ensiling is to preserve fodder under anaerobic condition, where anaerobic microbes build up organic acids, mainly lactic acid, by using fermentable carbohydrates, and aerobic stability describes the length of time that silage remains stable. As a result, the pH decreases, and the forage is preserved. A good additive increases the nutrient recovery, decreases heating of the silage and fungi development during the storage or feed out period and results in increased gas production and fermentation (Salem et al., 2013). Ensiling with additional carbon and nitrogen sources could improve the quality of silage. Therefore, the aim of this study was to investigate the effect of molasses and urea supplementation on *Leucaena* silage quality and in vitro gas production and ruminal fermentation profiles.

**MATERIALS AND METHODS**

**Dietary substrate, animals, experimental design and treatments**

*Leucaena* was harvested and immediately chopped in 2 to 3 cm lengths and ensiled to the respective supplementation treatments according to a 3×3 factorial arrangement in a completely randomized design (CRD). Factor A was molasses (M) supplementation at 0%, 1%, and 2%, and factor B was urea (U) supplementation at 0%, 0.5%, and 1.0% of the *Leucaena* dry matter (DM). A mixture of M and U was dissolved in water, sprayed onto the *Leucaena* which was then packed into plastic bags. The silage bags were kept in room temperature (about 25°C to 30°C). All treatments were done in triplicates at 1 kg each. After 28 days of ensiling, 200 g of *Leucaena* silage were sampled for analysis of DM, organic matter (OM) and CP (AOAC, 1990), and acid detergent fiber and neutral detergent fiber (NDF) (Van Soest et al., 1991). In addition, mimosine was analysed by the modified methods of Dalzell et al. (2012). Feed ingredients and chemical compositions of concentrate, rice straw and *Leucaena* leaf are shown in Table 1 and 2.

Silage samples were prepared and weighed (total substrate mixture 200 mg of DM) into 60 mL glass bottles for various times of incubation to study the rumen fermentation using in vitro gas techniques. All treatments were assigned according to a 3×3 factorial arrangement in a CRD with 3 bottles per treatment including triplicates of blank (medium only) in an incubation for 3 runs.

**Rumen inoculums**

Strict anaerobic techniques were used in all steps during the rumen fluid transferring and incubation periods. Rumen fluid samples were removed from swamp buffaloes (1 liter per animal) before morning feeding (7:00 h) under vacuum via the rumen fistula into a 2 liter plastic flask and transferred into 2 pre-warmed thermos flasks (1 liter) (Menke et al., 1979; Makkar et al., 1995). The fluid was then transported to the laboratory.

**Medium solution preparation**

In the present study, the medium was prepared for determination of gas production and fermented material during various incubation times. Therefore, the medium preparation was as described by Makkar et al. (1995). The mixture was kept stirring under CO2 at 39°C using a magnetic stirrer fitted with a hot plate. A portion (30 mL) of the rumen-fluid medium was transferred into each bottle and incubated in a water bath at 39°C.

**Substrate incubation**

The method used for in vitro fermentation was based on the technique described by Menke et al. (1979). The sets of sample incubations for the determination of fermentation end-products and gas production were prepared each time. The bottles with the mixture of substrate treatments were pre-warmed in a water bath at 39°C for 1 h before filling with 30 mL of rumen inoculums mixture. The bottles were then sealed with rubber stoppers and aluminum caps and incubated in a water bath set at 39°C.

**Sample collection and analysis**

*Gas production kinetics:* During the incubation, the gas production was recorded at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h. Cumulative gas production data was fitted to the

| Table 1. Feed ingredients and chemical composition of concentrate, rice straw, and *Leucaena* leaf |
|---------------------------------------------------------------|
| **Concentrate** | **Rice straw** | **Fresh Leucaena** |
| Ingredients (g/kg DM) | Ingredients (g/kg DM) | Ingredients (g/kg DM) |
| Cassava chip | 750 | 924 | 324 |
| Rice bran | 60 | 916 | 936 |
| Palm kernel meal | 50 | 121 | 21 |
| Coconut meal | 80 | 206 | 316 |
| Urea | 15 | 134 | 173 |
| Molasses | 15 | - | - |
| Tallow | 10 | 206 | 316 |
| Salt | 10 | - | - |
| Sulfur | 10 | - | - |
| Mineral premix | 10 | - | - |
| Chemical composition | | | |
| Dry matter (g/kg DM) | 924 | 911 | 324 |
| Organic matter | 916 | 895 | 936 |
| Crude protein | 121 | 23 | 21 |
| Neutral detergent fiber | 206 | 760 | 316 |
| Acid detergent fiber | 134 | 594 | 173 |
| Condensed tannins | - | - | 36 |
| Mimosine | - | - | 84 |
model of Ørskov and McDonald (1979) as follows:

\[ y = a + b (1 - e^{-ct}) \]

Where \( a \) = the gas production from the immediately soluble fraction, \( b \) = the gas production from the insoluble fraction, \( c \) = the gas production rate constant for the insoluble fraction (b), \( t \) = incubation time, \((a+b)\) = the potential extent of gas production. \( y \) = gas produced at time “t”.

**Determination of fermentation parameters:** The rumen inoculum mixtures were sampled at 0, 4, 8, and 12 h of fermenting post inoculation. Ruminal inoculum fluids were collected at 0, 4, 8, and 12 h post inoculation. Rumen fluid samples were then filtered through four layers of cheesecloth. Samples were divided into 2 portions. The first portion, around twenty milliliters of rumen inoculum, was put into plastic bottles for ammonia nitrogen (NH₃-N) and volatile fatty acid (VFA) analysis. The sample was centrifuged at 16,000×g for 15 min, and the supernatant was stored at –20°C before NH₃-N analysis by using the micro Kjeldahl methods (AOAC, 1990) and VFA analysis by high performance liquid chromatography (HPLC; Instruments by controller water model 1525, Waters Corporation, Milford MA, USA) water model 2707 auto sampler; water model 2489 UV detector and BREEZE software; column novapak C18; column size 3.9 mm×300 mm; mobile phase 10 mM H₂PO₄ [pH 2.5]) (Samuel et al., 1997). The second portion was fixed with 10% formalin solution in sterilized 0.9% saline solution. The total direct count was made by the methods of Galyen (1989) based on the use of a haemocytometer (Boeco, Hamburg, Germany). The last portion was stored at –20°C for DNA extraction (Yu and Morrison, 2004).

**Extraction of genomic DNA and real-time polymerase chain reaction:** Community DNA was extracted from 0.5 g of rumen content (fluid and digesta) by the RBB+C method (Yu and Morrison, 2004). In brief, the RBB+C method employs two rounds of bead beating in the presence of NaCl and sodium dodecyl sulphate, followed by sequential ammonium acetate and isopropanal precipitations. The precipitated nucleic acids were then treated with RNase A and proteinase K, and the DNA was purified using columns from QIAGEN DNA Mini Kit (QIAGEN, Valencia, CA, USA), according to manufacturer's recommendations. The targeted bacteria were total bacteria, the three predominant cellulolytic bacteria (\( F.\) succinogenes, \( R.\) flavifaciens, and \( R.\) albus) and protozoa.Primers for \( F.\) succinogenes, Fs219f (5′-GGTATGGGATGAGCTTGC-3′) and Fs654r (5′-GCCTGCCCCTGAACTATC-3′) were selected to allow amplification of all 10 \( F.\) succinogenes strains deposited in Gene Bank. For \( R.\) albus primers, Ra1281f (5′-GCTGTGCCCTGAACTATC-3′) and Fs654r (5′-GCCTGCCCCTGAACTATC-3′) were selected to allow amplification of all 10 \( F.\) succinogenes strains deposited in Gene Bank. For \( R.\) flavifaciens primers, Rf154f (5′-TCTGGAAACGGATGGA-3′) and Rf425r (5′-CTTGAAGACGGAGTGTAA-3′) were also selected to allow species-specific amplification of all seven \( R.\) flavifaciens strains deposited in Gene Bank.

| Treatment¹ | DM | Ash | OM | CP | NDF | ADF | Mimosine |
|------------|----|-----|----|----|-----|-----|----------|
| T1 (Control) | 332 | 76 | 924 | 215 | 387 | 224 | 27.1 |
| T2 (M1%) | 324 | 83 | 917 | 214 | 371 | 221 | 26.5 |
| T3 (M2%) | 322 | 93 | 907 | 218 | 372 | 210 | 25.6 |
| T4 (U0.5%) | 335 | 78 | 922 | 223 | 374 | 220 | 26.7 |
| T5 (U1.0%) | 337 | 79 | 921 | 235 | 376 | 218 | 27.0 |
| T6 (M1.0% U0.5%) | 321 | 84 | 916 | 222 | 374 | 217 | 26.6 |
| T7 (M1.0% U1.0%) | 328 | 94 | 906 | 237 | 367 | 212 | 16.4 |
| T8 (M2.0% U0.5%) | 319 | 96 | 904 | 232 | 368 | 209 | 15.5 |
| T9 (M2.0% U1.0%) | 325 | 97 | 903 | 248 | 363 | 207 | 15.3 |
| SEM | 0.87 | 5.53 | 1.02 | 3.14 | 2.98 | 1.05 | 8.72 |

Contrast

| | Con vs Supp | Con vs M | Con vs U | Con vs MU | M1.0% vs M2.0% | U0.5% vs U1.0% |
|-----|-------------|---------|---------|---------|----------------|----------------|
| | ns | ns | ns | * | ns | ns |
| | ns | ns | ns | ns | ns | ns |
| | ns | ns | ns | ns | * | ns |
| | * | ns | ns | ns | ns | ns |
| | | ns | ns | ns | ns | ns |
| | | ns | ns | ns | ns | ns |
| | | ns | ns | ns | ns | ns |

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; SEM, standard error of the mean; ns, non significant. ¹ M1% and M2% were molasses supplementation at 1% and 2% of Leucaena DM, respectively and U0.5% and U1.0% were urea supplementation at 0.5% and 1.0% of Leucaena DM, respectively.
Primers for total bacteria and protozoa were F (5’-GC-clamp-CTTACGGGAGCGACGAG3’), R (5’GWATTAC CGCGGCKGCTG3’) and F (5’-GCTTTCGWTGGA GTGTTC-3’), R (5’-ACTTGCCCTCYAATCGTWCT-3’). These primers were chosen from previously published sequences that demonstrated species specific amplification (Koike and Kobayashi, 2001). The conditions of the real-time polymerase chain reaction (PCR) for F. succinogenes were as follows: 30 s at 94°C for denaturing, 30 s at 60°C for annealing and 30 s at 72°C for extension (48 cycles), except for 9 min denaturation in the first cycle and 10 min extension in the last cycle. Amplification of 16s rDNA for R. albus and R. flavefaciens was carried out similarly except an annealing temperature of 55°C.

To establish a quantitative assay, amplified target 16s rDNA of each species by using specific primers and PCR conditions as described previously, the purified DNA were quantified by spectrophotometry with multiple dilutions. The target DNA was quantified by using serial 10-fold dilutions from 10¹ to 10⁸ DNA copies of the previously quantified DNA standards. Real-time PCR amplification and detection were performed in a Chromo 4TM system (Bio-Rad, Hercules, CA, USA). In brief, Biostools QuantiMix Easy SYG Kit was used for PCR amplification and samples were assayed in duplicate in a 20 µL reaction mixture contained 4 to 6 mM MgCl₂, 10 µL of Mastermix (including: Taq DNA polymerase, reaction buffer, dNTP mixture, MgCl₂ and SybrGreen), 2 µL of DNA template and 0.8 µL of each primer (10 µM/µL).

Digestibility: At 12 and 24 h post inoculation, the in vitro true digestibility (IVTD) of a set of samples was determined. In brief, the content of the bottle was transferred quantitatively to a spout-less beaker by repeated washing with 100 mL neutral detergent solution. The content was refluxed for 1 h and filtered through pre weighed Gooch crucibles. The DM of the residue was weighed and IVTD of feed was calculated based on the following equation:

\[
\text{IVTD} = \frac{(\text{DM of feed taken for incubation} - \text{NDF residue}) \times 100}{\text{DM of feed taken for incubation}}
\]

Statistical analysis

Data used for the statistical analyses consisted of 3 levels of molasses supplementation, 3 levels of urea supplementation, 3 replications, and runs making a total of 27 observations. All obtained data were subjected to the general linear models procedures of the Statistical Analysis System Institute (SAS, 1998) according to a 3×3 factorial arrangement in CRD. The statistical model including molasses level, urea level and interaction effects were: \( Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ij} \), where \( Y_{ij} \) is an observation, \( \mu \) is the overall mean, \( A \) is molasses level effect (\( i = 1, 2, 3 \)), \( B \) is urea level effect (\( j = 1, 2, 3 \)), \( AB \) is interaction effect of molasses level and urea level, and \( \varepsilon \) the residual effect. Multiple comparisons among treatment means were performed by Duncan’s New Multiple Range Test (DMRT) and orthogonal contrast (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Feed ingredients and chemical compositions

The feed ingredients and chemical composition of concentrate, rice straw and fresh Leucaena leaf are shown in Table 1. Rice straw was used as roughage source. However, the CP of rice straw was low (23 g/kg DM) and high in NDF (760 g/kg DM). Leucaena silage (Table 2) contained CP 215 to 248 g/kg of DM and mimosine 15.3 to 27.1 g/kg of DM. Increasing U supplementation increased the CP content of the silage and this was similar to the finding of Wanapat et al. (2013) who investigated whole crop rice silage. Energy is usually the limiting factor for growth of anaerobic microbes and provision of U and M might have increased the microbial mass that lead to increased CP (Staples et al., 1981). The provision of carbon skeleton and energy for microbial growth might have synchronized with ammonia released from urea hydrolysis, consequently increasing the CP content of forages ensiled (Salem et al., 2013). Furthermore, fermentation decreased 85% to 90% of mimosine. The result was in agreement with that reported by Sunagawa et al. (1989) who found mimosine reduction over 90% in Leucaena silage either with or without additives. The reduction of mimosine by ensiling being higher than by sun drying (14.5% to 51.1% of the original samples) was reported by Wee and Wang (1987). These results indicated that Leucaena silage is an interesting alternative for feed preservation.

Gas production kinetics and in vitro digestibility

Cumulative gas production for each of the substrate treatments presented as gas production and values for kinetics of gas production models for substrates studied are given in Table 3. The values for the estimated parameters obtained from the kinetics of gas production models for supplements studied revealed that the intercept value (a) for the different treatments representing gas production from soluble fractions and gas production rate constants for the insoluble fraction (c) ranged from -4.72 to -1.82 and 0.04 to 0.12, respectively. Gas production from the insoluble fraction (b), potential extent of gas production (a+b) and cumulative gas production at 96 h were significantly different with U supplementation (p<0.01). The treatments with U supplementation showed the higher gas production. Effect of M and U supplementation on digestibility from in vitro incubation are shown in Table 3. There was no interaction effect between M and U on DM digestibility at

\[
\text{IVTD} = \frac{(\text{DM of feed taken for incubation} - \text{NDF residue}) \times 100}{\text{DM of feed taken for incubation}}
\]

\( Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ij} \)
hours 12 and 24 of incubation. According to Cone and van Gelder (1999), comparison of gas production data of samples differing widely in CP content can lead to misinterpretations. Generally, low gas production would indicate low degradability, but feedstuffs high in CP normally produce less gas during fermentation, even if their extent of degradation is high, because protein fermentation produces ammonia, which influences the carbonate buffer equilibrium by neutralizing H⁺ ions from VFA without release of carbon dioxide. In the present study Leucaena silage contained high CP but produced more gas. The addition of M and U to Leucaena silage increased digestibility after 12 and 24 h of incubation, suggesting that during the ensiling process molasses might have removed some chemical linkages of hemicelluloses and thus enhanced their solubility in detergent solutions and also possibly due to the ability of rumen microorganism to degrade the plant secondary metabolites like alkaloids and saponins (Hart et al., 2008) and utilize them as an energy source.

### Rumen fermentation

In the in vitro gas production technique, NH₃-N concentration was highest in T9 (M2.0% U1.0%). While NH₃-N was found to be the lowest in control. The concentration of NH₃-N was increased dramatically based on the time incubation. This result could be due to the effects of tannins contained in Leucaena silage which protect CP from degradation by the formation of tannin-protein complexes in the rumen, thereby increasing metabolizable protein supply to the duodenum (Waghorn, 2008). NH₃-N concentration was found higher in the treatments with high level of U supplementation. There was no effect of M supplementation on NH₃-N. Availability of NH₃ is an important determinant of microbial protein production as the majority of rumen bacteria use NH₃ as a nitrogen source. It is essential to know what concentration of NH₃ will support maximal microbial growth in order to make judgments regarding utilization of non-protein N. The NH₃–N concentration of all treatments ranged from 16.8 to 22.8 mg/dL (Table 4). However, Satter and Slyter (1974) suggested NH₃ concentrations from 3 to 5 mg/dL as optimal to produce ruminal microorganism growth, which was relatively less than those observed in this work. It appears that, once NH₃ starts to accumulate, the growth of bacteria utilizing NH₃ is not enhanced by increasing NH₃ concentration (Satter and Slyter, 1974).

Interaction between M and U supplementation affected the proportion of propionic acid (Table 4). Total VFA concentrations in M2.0% U1.0% was higher than other treatments (p<0.05). In addition, supplementation of M2.0% U0.5% and M2.0% U1.0% resulted in a higher
(p<0.05) proportion of propionic acid (34.4 and 34.1 mole/100 mole, respectively) and a lower (p<0.01) proportion of acetic acid which was highest in the control. While the proportion of butyric acid was not affected by M and U supplementation. Calculation of ruminal methane (CH₄) production using VFA proportions according to Moss et al. (2000) showed that methane production was not influenced by interaction of U and M supplementation. In contrast, Anantasook and Wanapat (2012) reported that a high proportion of propionic acid was caused by a decreased methane production due to tannins contained in rain tree pot meal. Effects of tannins on increased propionic acid and reduced acetic to propionic ratio have been found to vary with diets and applications.

**Rumen microbes**

In the present study, effects of M and U supplementation on microbes from *in vitro* incubation are shown in Table 5 and 6. The results revealed a significant effect on bacterial populations by M and U supplementation, while protozoa and fungi zoospores were not effect by supplementation. As compared with the control group, a supplementation resulted in a larger bacteria population (p<0.05). This effect may be due to the cause of *Leucaena* silage supplemented with U an M which contains high level of nitrogen and carbon source. The additional protein provided by the *Leucaena* would have increased availability of ammonia for rumen micro flora, stimulating microbial growth and increasing rate of breakdown of the forage (Barros-Rodriguez et al., 2013).

The effect of M and U supplementation on microbes from *in vitro* incubation with swamp buffalo rumen fluid is shown in Table 6. The real-time PCR for quantification of ruminal microbes with specific targets (total bacterial, *R. albus*, *F. succinogenes*, *R. flavefaciens*, and protozoa) are reported in Table 6. The total bacteria and three dominant cellulolytic bacteria were found affected by M and U supplementation. Predominant cellulolytic bacteria in *in vitro* incubation were affected by the M and U supplementation, except for *R. albus*. Supplementation of M and U decreased the population of protozoa (p<0.05). Koike and Kobayashi (2001) reported that *F. succinogenes* was the most dominant bacteria among the three species of cellulolytic bacteria. Changes of the population size or the proportion of cellulolytic bacterial numbers in the rumen may be due to some effect of tannins in *Leucaena*. Goel et al. (2008) reported that the *F. succinogenes* population was increased when supplementation with *S. sesban* leaves and Fenugreek seeds, while the *R. flavefaciens* population increased with *Cardus* leaves and fenugreek supplementation. Moreover, McSweeney et al. (2001) reported that the protein-tannin complexes reduce the

**Table 4. Effect of *Leucaena* silage on ammonia nitrogen, volatile fatty acid and methane production from *in vitro* incubation with swamp buffalo rumen fluid**

| Treatment¹ | NH₃-N (mg/dL) | TVFA (mM/L) | C₂ | C₃ | C₄ | C₂:C₃ | CH₄² |
|------------|---------------|-------------|----|----|----|-------|------|
| T1 (Control) | 16.8 | 86.4 | 68.7 | 22.6 | 8.7 | 1.7 | 20.2 |
| T2 (M1%) | 17.1 | 93.0 | 61.3 | 30.1 | 8.6 | 1.8 | 20.8 |
| T3 (M2%) | 17.6 | 92.9 | 62.2 | 30.4 | 7.4 | 1.8 | 20.6 |
| T4 (U0.5%) | 18.7 | 87.7 | 60.3 | 32.3 | 7.4 | 1.6 | 18.6 |
| T5 (U1.0%) | 20.7 | 92.7 | 58.8 | 32.6 | 8.6 | 1.7 | 19.6 |
| T6 (M1.0% U0.5%) | 18.6 | 94.0 | 59.6 | 32.2 | 8.2 | 1.8 | 21.3 |
| T7 (M1.0% U1.0%) | 20.9 | 99.0 | 57.8 | 32.7 | 9.5 | 1.8 | 20.8 |
| T8 (M2.0% U0.5%) | 19.4 | 95.3 | 57.4 | 34.1 | 8.5 | 1.8 | 21.2 |
| T9 (M2.0% U1.0%) | 22.8 | 100.4 | 56.6 | 34.5 | 8.9 | 1.8 | 21.1 |
| SEM | 0.30 | 4.01 | 0.84 | 1.07 | 0.50 | 0.09 | 0.77 |

NH₃-N, ammonia nitrogen; TVFA, total volatile fatty acid; C₂, acetic acid; C₃, propionic acid; C₄, butyric acid; C₂:C₃, acetic acid:propionic acid ratio; SEM, standard error of the mean; ns, non significant; DM, dry matter.

¹ M1% and M2% were molasses supplementation at 1% and 2% of *Leucaena* DM, respectively and U0.5% and U1.0% were urea supplementation at 0.5% and 1.0% of *Leucaena* DM, respectively.

² Methane production (mM/L) calculated by Moss et al. (2000) = 0.45 (C₂)-0.275 (C₃)+0.4 (C₄).

*p<0.05; **p<0.01; ***p<0.001.
availability of fermentable N for microbial activity in the rumen. Kumar and Singh (1984) reported that tannins in tree leaves inhibited proteolysis of casein and subsequent ammonia production in vitro. Therefore, U addition would provide fermentable N for stimulating microbial fermentation in the rumen. Wanapat and Cherdthong (2009)

### Table 5. Effect of Leucaena silage on microorganisms from in vitro incubation with swamp buffalo rumen fluid

| Treatment | Protozoa (×10^5 cell/mL) | Fungi (×10^6 cell/mL) | Bacteria (×10^8 cell/mL) |
|-----------|--------------------------|-----------------------|--------------------------|
|           | 4 h  | 12 h  | Mean | 4 h | 12 h | Mean | 4 h | 12 h | Mean |
| T1 (Control) | 1.4  | 1.8  | 1.6  | 2.3 | 2.8  | 2.6  | 15.6 | 14.2 | 14.9 |
| T2 (M1%)    | 1.6  | 1.4  | 1.5  | 3.4 | 3.2  | 3.3  | 14.2 | 18.8 | 16.5 |
| T3 (M2%)    | 2.3  | 2.2  | 2.2  | 5.1 | 4.7  | 4.9  | 17.5 | 20.6 | 19.1 |
| T4 (U0.5%)  | 1.7  | 2.2  | 2.0  | 5.3 | 5.6  | 5.5  | 20.4 | 22.4 | 21.4 |
| T5 (U1.0%)  | 3.2  | 1.8  | 2.5  | 6.2 | 4.3  | 5.3  | 26.8 | 21.5 | 24.2 |
| T6 (M1.0% U0.5%) | 2.5  | 1.5  | 2.0  | 6  | 5.1  | 5.6  | 24.6 | 26.3 | 25.5 |
| T7 (M1.0% U1.0%) | 2.2  | 1.9  | 2.1  | 5.4 | 6.2  | 5.8  | 30.2 | 28.7 | 29.5 |
| T8 (M2.0% U0.5%) | 3.4  | 1.7  | 2.6  | 5.2 | 6.1  | 5.7  | 33.5 | 30.2 | 31.9 |
| T9 (M2.0% U1.0%) | 3.2  | 2.1  | 2.7  | 5.6 | 6.3  | 6.0  | 35.2 | 40.3 | 37.8 |
| SEM        | 0.43 | 0.22 | 0.51 | 0.34 | 0.60 | 1.12 | 0.31 | 1.08 | 2.01 |

**Contrast**

- Con vs Supp: ns ns ns ns ns ns
- Con vs M: ns ns ns ns ns ns ns ns ns
- Con vs U: ns ns ns ns ns ns ns ns ns
- M1.0% vs M2.0%: ns ns ns ns ns ns ns ns ns
- U0.5% vs U1.0%: ns ns ns ns ns ns ns ns ns

SEM, standard error of the mean; ns, non significant; DM, dry matter.

1 M1% and M2% were molasses supplementation at 1% and 2% of Leucaena DM, respectively and U0.5% and U1.0% were urea supplementation at 0.5% and 1.0% of Leucaena DM, respectively.

* p<0.05; ** p<0.01; *** p<0.001.

### Table 6. Effect of Leucaena silage on cellulolytic bacteria and protozoa populations in in vitro incubation in swamp buffalo fluid as determined by real-time PCR

| Treatment | Total bacteria (×10^9 cell/mL) | F. succinogenes (×10^7 cell/mL) | F. flavefaciens (×10^6 cell/mL) | R. albus (×10^6 cell/mL) | Protozoa (×10^4 cell/mL) |
|-----------|---------------------------------|---------------------------------|---------------------------------|--------------------------|-------------------------|
| T1 (Control) | 2.44                           | 3.42                            | 1.21                            | 2.14                     | 3.32                    |
| T2 (M1%)    | 3.15                           | 3.17                            | 1.32                            | 2.21                     | 2.16                    |
| T3 (M2%)    | 4.24                           | 3.56                            | 1.47                            | 1.97                     | 2.31                    |
| T4 (U0.5%)  | 5.87                           | 3.61                            | 1.84                            | 2.35                     | 2.55                    |
| T5 (U1.0%)  | 6.32                           | 3.80                            | 1.68                            | 2.66                     | 2.49                    |
| T6 (M1.0% U0.5%) | 6.01                           | 3.72                            | 2.03                            | 3.34                     | 2.03                    |
| T7 (M1.0% U1.0%) | 6.55                           | 3.88                            | 2.15                            | 3.41                     | 2.18                    |
| T8 (M2.0% U0.5%) | 6.83                           | 4.01                            | 2.65                            | 3.32                     | 1.97                    |
| T9 (M2.0% U1.0%) | 7.35                           | 4.22                            | 2.43                            | 3.17                     | 1.89                    |
| SEM       | 0.07                           | 0.42                            | 0.06                            | 0.14                     | 0.12                    |

**Contrast**

- Con vs Supp: * * * * ns
- Con vs M: * ns ns ns ns
- Con vs U: * ns ns ns ns
- Con vs MU: ** * * * ns
- M1.0% vs M2.0%: * ns ns ns ns
- U0.5% vs U1.0%: * ns ns ns ns

PCR, polymerase chain reaction; SEM, standard error of the mean; ns, non significant; DM, dry matter.

1 M1% and M2% were molasses supplementation at 1% and 2% of Leucaena DM, respectively and U0.5% and U1.0% were urea supplementation at 0.5% and 1.0% of Leucaena DM, respectively.

* p<0.05; ** p<0.01; *** p<0.001.
reported that increasing protein supplementation for ruminants lead to a higher population of ruminal microbes. The reason could be due to a nitrogen source which could support microbial production in the rumen. The population of \textit{F. succinogenes} was higher than those of \textit{R. albus}. The finding of this study was similar to that of Wanapat and Cherdthong (2009), who studied rumen cellulolytic bacteria population using real-time PCR. They found that the population of \textit{F. Succinogenes} was more abundant than \textit{R. albus} (3.0×10^8 vs 2.93×10^6 copies/mL of rumen fluid).

CONCLUSION

Based on this study, it could be concluded that supplementation of molasses and urea could efficiently improve \textit{Leucaena} silage quality in terms of the chemical composition being high in protein and low in NDF contents. The present results suggest that supplementation of urea and molasses to \textit{Leucaena} silage enhanced \textit{in vitro} rumen fermentation efficiency, especially by the addition of urea at 1% and molasses at 2% of crop DM. However, further study using \textit{Leucaena} silage supplemented with urea and molasses in feeding trials emphasizing lactating dairy cows and fattening beef cattle should be investigated.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The authors would like to express their most sincere gratitude and appreciation to the Thailand Research Fund (TRF) via “The Royal Golden Jubilee Ph.D. Program”, Tropical Feed Resources Research and Development Center (TROFREC), and the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, for their kind financial support and provision of research facilities.

REFERENCES

Anantasook, N. and M. Wanapat. 2012. Influence of rain tree pod meal supplementation on rice straw based diets using \textit{in vitro} gas fermentation technique. Asian Australas. J. Anim. Sci. 25:325-334.

AOAC. 1990. Official Methods of Analyses, 15th edn. Association of Official Analytical Chemists, Arlington, VA, USA.

Bansi, H., E. Wina, P. R. Matataputy, and V. Tufarelli. 2014. Evaluation of Zappoteca tetragona forage as alternative protein source in ruminants’ feeding. Ital. J. Anim. Sci. 13:147-150.

Barros-Rodriguez, M., J. Solorio-Sánchez, C. Sandoval-Castro, A. V. Klieve, E. B. Briceno-Poot, L. Ramirez-Aviles, and R. Rojas-Herrera. 2013. Effect of two intake levels of \textit{Leucaena leucocephala} on rumen function sheep. Trop. Grasslands-Frourajes Tropicales 1:55-57.

Cazzato, E., V. Laudatio, A. Corleto, and V. Tufarelli. 2011. Effects of harvest date, wilting and inoculation on yield and forage quality of ensiling safflower (\textit{Carthamus tinctorius} L.) biomass. J. Sci. Food Agric. 91:2298-2302.

Cone, J. W. and A. H. Van Gelder. 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76:251-264.

Cudjoe, N. and V. Mlambo. 2014. Buffer nitrogen solubility, \textit{in vitro} ruminal partitioning of nitrogen and \textit{in vitro} ruminal biological activity of tannins in leaves of four tree species. J. Anim. Physiol. Anim. Nutr. 98:722-730.

Dalzell, S. A., D. J. Burnett, J. E. Dowsett, V. E. Forbes, and H. M. Shelton. 2012. Prevalence of mimosine and DHP toxicity in cattle grazing \textit{Leucaena leucocephala} pastures in Queensland, Australia. Anim. Prod. Sci. 52:365-372.

Galyen, M. 1989. Laboratory procedures in animal nutrition research. New Mexico State University, Las Cruces, NM, USA.

Goel, G., H. P. S. Makkar, and K. Becker. 2008. Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fractions from different plant materials. J. Appl. Microbiol.105:770-777.

Hart, K. J., D. R. Yáñez-Ruiz, S. M. Duval, N. R. McEwan, and C. J. Newbold. 2008. Plant extracts to manipulate rumen fermentation. Anim. Feed Sci. Technol. 147:8-35.

Koike, S. and Y. Kobayashi. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: \textit{Fibrobacter succinogenes}, \textit{Ruminococcus albus}, and \textit{Ruminococcus flavefaciens}. FEMS Microbiol. Lett. 204:361-366.

Kumar, R., M. Singh. 1984. Tannins: their adverse role in ruminant nutrition. J. Agr. Food Chem. 32:447-453.

Makkar, H. P. S., M. Blummel, and K. Becker. 1995. Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in \textit{in vitro} techniques. Br. J. Nutr. 73:897-913.

McSweeney, C. S., B. Palmer, D. M. McNeil, and D. O. Krause. 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91:83-93.

Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor \textit{in vitro}. J. Agric. Sci. 93:217-222.

Moss, A. R., J. P. Jouany, and J. Newbold. 2000. Methane production by ruminants: its contribution to global warming. Anim. Res. 49:231-253.

Mathew, S., S. Sagarthavan, J. Thomas, and G. Mathen. 1997. An HPLC method for estimation of volatile fatty acids of rumen fluid. Indian J. Anim. Sci. 67:805-807.

Orskov, E. R. and I. McDonal. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92:499-503.

Salem, A. Z. M., C. S. Zhou, Z. L. Tan, M. Mellado, M. C. Salazar, M. M. Y. Elghandour, and N. E. Odongo. 2013. \textit{In vitro} ruminal gas production kinetics of four fodder trees ensiled with or without molasses and urea. J. Integr. Agric. 12:1234-1242.
SAS. 1998. User's Guide: Statistic, Version 6, 12th edn. SAS Inst. Inc., Cary, NC, USA.

Satter, L. D. and L. L. Slyter. 1974. Effect of ammonia concentration on ruminal microbial protein production in vitro. Br. J. Nutr. 32:199-208.

Staples, C. R., G. C. Fahey Jr, L. L. Berger, and R. B. Rindsig. 1981. Evaluation of dairy waste fiber as a roughage source for ruminants. J. Dairy Sci. 64:662-671.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. McGraw Hill Book Co., New York, NY, USA.

Sunagawa, K., F. Hongo, Y. Kawashima, and S. Tawata. 1989. The effect of mimosine reduced Leucaena feed on sheep. JPN. J. Zootech. Sci. 60:133-140.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—progress and challenges. Anim. Feed Sci. Technol. 147:116-139.

Wanapat, M. and A. Cherdthong. 2009. Use of real-time PCR technique in studying rumen cellulolytic bacteria population as affected by level of roughage in Swamp buffalo. Curr. Microbiol. 58:294-299.

Wanapat, M., S. Kang, P. Khejornsart, and R. Pilajun. 2013. Improvement of whole crop rice silage nutritive value and rumen degradability by molasses and urea supplementation. Trop. Anim. Health Prod. 45:1777-1781.

Wee, K. L. and S. S. Wang. 1987. Effect of postharvest treatment on the degradation of mimosine in Leucaena leucocephala leaves. J. Sci. Food. Agric. 39:195-201.

Yu, Z. and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques 36:808-812.