Improvement of nutritive value of cassava pulp and in vitro fermentation and microbial population by urea and molasses supplementation

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ABSTRACT
This study aimed to investigate the effect of urea (U) and molasses (M) supplement on the nutritive value of cassava pulp and in vitro gas fermentation. The ensiling study was randomly assigned according to a 4 × 4 factorial arrangement in a completely randomized design. The first factor was U supplementation at U0, U2, U4, and U6% and the second was M supplementation at M0, M2, M4, and M6% of dry matter, respectively. After 14 days of ensiling, treated cassava pulp was sampled for chemical composition and subsequently used to study in vitro fermentation. The results revealed that increasing U supplement levels could increase crude protein and decrease fibre contents of treated cassava pulp (P < .05). Increasing U and M supplement levels increased gas production and in vitro true digestibility (P < .05). Total bacteria, Fibrobacter succinogenes and Ruminococcus flavefaciens were increased in cassava pulp treated with U and M supplemented groups (P < .05). Based on this experiment, it could be concluded that U4 and M4% supplement could improve the nutritive value of treated cassava pulp and increase gas production, in vitro digestibility, and the growth of dominant celluloytic bacterial population. However, further research should be conducted on the use of treated cassava pulp in ruminant feeding.

1. Introduction
Cassava root contains high levels of energy and has been used as a source of readily fermentable energy in ruminant rations (Wanapat 2003). Cassava starch production is a huge and growing industry in Thailand, with approximately 10 million tonnes of fresh cassava roots used annually (Chauynarong et al. 2009). Cassava pulp is a by-product which constitutes approximately 30% of the original weight of roots (Ghimire et al. 2015). Approximately 1.5–2.0 million tonnes of cassava pulp are produced annually from the entire cassava starch industry. It contains abundant starch (60%), while the other main carbohydrates are cellulose and hemicelluloses (20%) (Sriroth et al. 2000; Manish and Banerjee 2008; Kosugia et al. 2009). Cassava pulp offers an alternative to high-starch grains, and can be used as an energy source in ruminant diets, but is low in crude protein (CP) (Lounglawan et al. 2010). Ensiling is a feed preservation method based on natural lactic acid fermentation under anaerobic conditions where anaerobic microbes build up organic acids, mainly lactic acid, by using fermentable carbohydrates (Gollop et al. 2005; Ki et al. 2009). To improve animal feed quality, additives such as chemicals, enzymes, and bacterial agents can be employed. Wanapat, Kang, Khejornsart et al. (2013) reported that supplementation of urea (U) and molasses (M) could improve the quality of whole-crop rice silage in terms of nutritive value and rumen degradation. Addition of carbon and nitrogen sources could improve the quality of feed and have subsequent effects on rumen degradation characteristics and production in ruminants. Therefore, the objective of this study was to determine the effect of U and M treatment on the nutritional value of cassava pulp and in vitro rumen fermentation.

2. Materials and methods
2.1. Ensiling of cassava pulp
Cassava pulp was treated to the respective supplementation according to a 4 × 4 factorial arrangement in a completely randomized design (CRD). Factor A was U supplementation levels at 0%, 2%, 4%, and 6% and factor B was M supplementation levels at 0%, 2%, 4%, and 6% of cassava pulp dry matter (DM), respectively. Cassava pulp was mixed with U and M, and then packed into plastic containers kept at ambient temperature (about 25–30°C). All treatment combinations were done in triplicates at 300 g each. After 14 days, the treated cassava pulp was sampled for chemical compositions analysis. The samples were divided into two parts: the first part was for DM analysis (AOAC 1997) and the second part was dried in a forced-air oven at 60°C for 96 hour, ground through a 1-mm stainless steel screen (Cyclotec 1093 Sample mill, Tecator, Hoganas, Sweden), and analysed for organic matter (OM) and...
CP according to the AOAC (1997) method. The method of Van Soest et al. (1991) was used to determine neutral detergent fibre (NDF) and acid detergent fibre (ADF) on an ash-free basis using an Ankom fibre analyser incubator (Ankom Technology, Fairport, NY).

2.2. In vitro gas techniques

2.2.1. Experimental design and dietary treatments
The experimental design was a 4 × 4 factorial arrangement in a CRD, with three replications per treatment including triplicates of blank (medium only) in three incubation runs. The dietary treatments were 16 kinds of treated cassava pulp. Substrates of treated cassava pulp were milled to pass through a 1-mm screen (Cyclotec 1093 Sample mill, Tecator, Hoganas, Sweden).

2.2.2. Animals and preparation of rumen inoculums
Two rumen-fistulated dairy steers with body weight (BW) of 230 ± 15 kg were used as rumen fluid donors. The animals were fed on concentrated diet (16% CP) at 0.5% BW and rice straw was used as the main roughage before collecting the rumen fluid. Animals were placed on a routine for at least 20 days and kept in individual pens with free choice of clean fresh water and mineral blocks. On day 21, about 2000 ml of rumen liquor was obtained from both animals before the morning feeding. The rumen fluid was filtered through four layers of cheesecloth into pre-warmed thermo flasks and then transported to the laboratory.

2.2.3. In vitro fermentation of substrates
Samples of 0.2 g of treated cassava pulp were weighed into 60 ml serum bottles, respectively. The method used for in vitro fermentation was based on the technique described by Menke et al. (1979) and strict anaerobic techniques were used in all steps during the rumen fluid transferring and incubation periods (Menke et al. 1979; Makkar et al. 1995). The bottles were categorized into separate sets of sample incubations for the determination of gas production kinetics, in vitro digestibility, and fermentation end products with three replications per treatment including triplicates of blank (medium only). The bottles with the mixture of substrate treatments sealed with rubber stoppers and aluminium caps were pre-warmed in a water bath at 39°C for 1 h before filling with 30 ml of rumen inoculum mixture (Menke and Steingass 1988).

2.2.4. Medium solution preparation
The medium preparation per run (4500 ml) was as described by Makkar et al. (1995). The reduced medium (3000 ml) consists of 1425 ml distilled water, 720 ml rumen buffer solution (35.0 g NaHCO3 and 4 g NH4HCO3 made up to 1 l with distilled water), 720 ml macro-mineral solution (6.2 g KH2PO4, 5.7 g Na2HPO4, 2.22 g NaCl, and 0.6 g MgSO4.7 H2O made up to 1 l with distilled water), 0.36 ml micro-mineral solution (10.0 g MnCl2, 4H2O, 13.2 g CaCl2.2H2O, 1 g CoCl2.6H2O, and 8.0 g FeCl3.6H2O made up to 100 ml with distilled water), 3.66 ml resazurin (0.1 g made up to 100 ml with distilled water), and 148.5 ml freshly prepared reduction solution (142.5 ml distilled water, 1008 mg Na2S.9H2O, and 6 ml 1 M NaOH). Then, 1500 ml of rumen fluid from dairy steers (2:1; reduced medium: rumen fluid) was mixed with the reduced medium as described by Makkar et al. (1995). The mixture was stirred continuously under CO2 at 39°C using a magnetic stirrer fitted with a hot plate under continuous flushing with CO2. A portion (30 ml) of the rumen fluid medium was transferred into each bottle and incubated in a water bath at 39°C as described by Blummel and Ørskov (1993).

2.2.5. Sample collection and analysis
Gas production kinetics: During the incubation, the gas production kinetics was recorded at 0, 1.5, 3, 6, 8, 12, 18, 24, 48, 72, and 96 h following extraction using glass syringes. Cumulative gas production data were calculated based on the gas production at each time using the Fit Curve program fitted to the model of Orskov and McDonald (1979) as follows: \( y = a + b (1 - e^{-ct}) \), where \( a \) is the gas production from the immediately soluble fraction, \( b \) is the gas production from the insoluble fraction, \( c \) is the gas production rate constant for the insoluble fraction \( b \), \( t \) is the incubation time, \( (a + b) \) is the potential extent of gas production, and \( y \) is gas produced at time \( t \).

In vitro digestibility: At 48-hour post-incubation, the contents of the incubation substrate were washed with 100 ml of neutral detergent solution and then filtered through pre-weighed Gooch crucibles (40 mm porosity), and residual DM was weighed and estimated for in vitro true digestibility based on the following equation according to Van Soest and Robertson (1985): True digestibility (TD) = \( (\text{DM of feed taken for incubation-NDF residue}) \times 100)/\text{DM of feed taken for incubation} \).

Extraction of genomic deoxyribonucleic acid (DNA): Community DNA was extracted from 0.5 g of rumen content (fluid and digesta) by the repeated bead beating plus column (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 sulfate, 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), according to the manufacturer’s recommendations.

Primers and real-time polymerase chain reaction (real-time PCR): The targeted microorganisms were total bacteria and three cellulolytic bacteria (Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens). The primers used for the real-time PCR were as follows: primers for F. succinogenes, Fs219f (5′-GGT ATG GGA TGA GCT TGC-3′) and Fs654r (5′-GCC TGC CCC TGA ACT ATC-3′), were selected to allow the amplification (446-bp product) of all 10 F. succinogenes strains deposited in GenBank. For R. albus, primers were Ra1281f (5′-CCC TAA AAG CAG CAG TCT TAG TTC G-3′) and Ra1439r (5′-CCT CTC TGC GGT TAG AAC A-3′) (175-bp product). R. flavefaciens primers, Rf154f (5′-TCT GGA AAC GGA TGG TA-3′) and Rf425r (5′-CTT TTA AGA CAG GAG TTT ACA A-3′), were also selected to allow species-species amplification (295 bp) of all seven R. flavefaciens strains deposited in GenBank. All these primer sets were previously published by Koike and Kobayashi (2001). Primers for total bacteria were F (5′-GC-clamp-CTT AAC GGA GGC AGC AG-3′) and R (5′-GWA TTA CCG CGG CKG CTG-3′) according to Lane (1991).
Regular PCR conditions 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 rRNA for the other two species was carried out similarly, except for an annealing temperature of 55°C was used. Regular PCR conditions 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. The targeted populations were total bacteria and cellulolytic bacteria (i.e. *R. albus, F. succinogenes*, and *R. flavefaciens*). Four sample-derived standards (for total bacteria, *R. albus, F. succinogenes*, and *R. flavefaciens*) were prepared from a pooled set of all treatments of community DNA. The regular PCR was used to generate sample-derived DNA standards for each real-time PCR assay. Then the PCR product was purified using a QiA quick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using a spectrophotometer. For each sample-derived standard, copy number concentration was calculated based on the length of the PCR product and the mass concentration (Yu et al. 2005). The target DNA was quantified by using serial 10-fold dilutions from 10¹ to 10⁹ DNA copies of the previously quantified DNA standards (Yu et al. 2005). Real-time PCR amplification and detection were performed in a Chromo 4™ system (Bio-Rad, USA). All samples were assayed in duplicate in a 20 μl reaction mixture containing 4–6 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).

### 2.3. Statistical analysis

All obtained data were subjected to the General Linear Models (GLM) procedures of SAS (1996) according to a 4 × 4 factorial arrangement in CRD. Differences among treatment means were contrasted by Tukey’s Multiple Comparison Test (Crichton 1999). Comparison between plant oil source supplementation was tested by orthogonal contrast.

#### Table 1. Chemical compositions of treated cassava pulp affected by urea and molasses treatment.

| Treatments | Urea (%) | Molasses (%) | DM | OM | Ash | CP | NDF | ADF |
|------------|---------|--------------|----|----|-----|----|-----|-----|
| 1          | 0       | 0            | 83.8 | 95.7 | 4.3 | 1.8 | 45.2 | 15.1 |
| 2          | 2       | 2            | 38.6 | 93.8 | 6.2 | 2.0 | 40.3 | 12.3 |
| 3          | 4       | 4            | 29.4 | 93.6 | 6.3 | 2.0 | 39.2 | 12.0 |
| 4          | 6       | 6            | 45.6 | 93.4 | 6.5 | 2.0 | 39.5 | 11.8 |
| 5          | 2       | 0            | 34.0 | 93.4 | 6.6 | 5.7 | 36.6 | 11.0 |
| 6          | 2       | 2            | 34.2 | 93.4 | 6.6 | 5.8 | 36.2 | 10.8 |
| 7          | 4       | 4            | 38.0 | 93.5 | 6.5 | 5.8 | 36.0 | 10.3 |
| 8          | 6       | 6            | 40.1 | 93.3 | 6.7 | 5.8 | 36.3 | 10.5 |
| 9          | 4       | 0            | 33.3 | 93.5 | 6.5 | 9.3 | 35.3 | 9.5  |
| 10         | 2       | 2            | 37.0 | 93.3 | 6.7 | 9.4 | 35.1 | 9.1  |
| 11         | 4       | 4            | 34.5 | 93.3 | 6.7 | 9.3 | 34.9 | 9.3  |
| 12         | 6       | 6            | 43.2 | 93.2 | 6.8 | 9.4 | 34.6 | 9.6  |
| 13         | 0       | 0            | 44.1 | 93.3 | 6.6 | 12.2 | 33.6 | 8.6  |
| 14         | 2       | 2            | 40.7 | 93.6 | 6.4 | 12.3 | 33.8 | 8.3  |
| 15         | 4       | 4            | 41.7 | 93.5 | 6.5 | 12.3 | 33.1 | 8.5  |
| 16         | 6       | 6            | 37.6 | 93.2 | 6.8 | 12.4 | 33.4 | 8.0  |
| SEM        | 1.77    | 0.63         | 0.63 | 0.63 | 0.72 | 1.87 | 1.20 |

**Comparison**

| Level of urea | ** | NS | NS | ** | ** | ** | ** |
| Level of molasses | ** | NS | NS | NS | NS | NS | NS |
| Interaction | ** | NS | NS | NS | NS | NS | NS |

Note: DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; SEM, standard error of mean; NS, non-significant. **P < .01
microorganisms growth resulting from the presence of more available N in the form of ammonia from the hydrolysis of U (Boucher et al. 2007; Khattab et al. 2013; Kang et al. 2015). In addition, Wanapat, Kang, Khejornsart et al. (2013) reported that the supplementation of U and M could increase rumen degradability of whole-crop rice silage.

The application of real-time PCR to quantify predominant cellulolytic bacteria (16S rRNA) targets revealed that total bacteria, \textit{F. succinogenes}, and \textit{R. flavefaciens} were significantly increased (Table 3 and Figure 2; \(P < .05\)) with the increasing level of M in treated cassava pulp. Similarly, Salais et al. (1977) reported that both the nature of fibre and protein content of the forage are important contributing factors in determining the responses to M/U diets. It is well known that cellulolytic bacteria utilize NH\(_3\) for their growth (Bryant 1973). They were unable to grow on other nitrogen sources in the absence of NH\(_3\) (Russell et al. 2009). Cassava root contains high levels of readily fermentable energy and could be used in combination with readily available

![Figure 1](https://example.com/gas-production.png)

**Figure 1.** Effect of fermented cassava pulp on cumulative gas production at different times of incubation in dairy steers rumen fluid.
NPN sources such as U in ruminant rations and this could improve the growth of rumen bacteria (Wanapat and Kang 2013; Wanapat, Kang, Polyorach 2013). All groups of bacteria were enhanced by the supplementation group, especially in the treatment with high M and U ratio \((P < .05)\).

### 4. Conclusion and recommendations

Based on this study, it could be concluded that cassava pulp nutritive value could be enhanced by U and M supplementation. Moreover, the treatment of U and M on cassava pulp could improve gas production kinetics, in vitro digestibility, and the growth of dominant cellulolytic bacterial population. It is suggested that the level of U and M treatment be at U4 and M4\% of cassava pulp DM. However, further research should be conducted on the use of treated cassava pulp in ruminant feeding.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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