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Supplementation of Urea Level and Malate in Concentrate Containing High Cassava Chip on Rumen Ecology and Milk Production in Lactating Cows

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Abstract: Four, lactation dairy cows were randomly assigned according to a 2 x 2 Factorial arrangement in a 4 x 4 Latin square design to study supplementation of urea level (U) at 2 and 4 % and malate (M) at 10 and 20 g/hd/d in concentrate. The treatments were as follows U2M10, U2M20, U4M10 and U4M20, respectively. The cows were offered the treatment concentrate at a ratio to milk yield at 1:2.5 and urea-treated rice straw was fed *ad libitum*. The results have revealed that rumen fermentation and blood metabolites were similar for all treatments. The populations of protozoa and fungal zoospores were significantly different as affected by urea level and malate. In addition, the viable bacteria were similar for amylolytic and proteolytic bacteria. Cellulolytic bacteria were significantly affected by level of malate especially *Selenomonas ruminantium* and *Megasphaera elsdenii* while *Butyrivibrio fibrisolvens* was significantly affected by level of urea supplementation. Yield of milk was greatest in cows fed cassava-based diets with M20U2, but were lowest when receiving M10U4 in diets. In addition, production of 3.5%FCM exhibited similar results for all treatments. In conclusion, the combined use of concentrate containing high level of cassava chip at 75% DM with urea at 4% in concentrate and malate at 20 g/hd/d with UTS as a roughage could improved rumen ecology and microbial protein synthesis efficiency in lactating dairy cows.

Key words: Malate, urea, rumen ecology, lactating cows

Introduction

Cassava (*Manihot esculenta*, Crantz) production in tropical areas has a potential use in ruminant livestock nutrition and feeding. Cassava root contains high levels of energy and has been used as a source of readily fermentable energy in ruminant rations (Wanapat *et al*., 2003; Kiyothong and Wanapat, 2004; Promkot and Wanapat, 2005). One strategy for using high degradable carbohydrates is to use in combination with readily available NPN sources such as urea. Urea is commonly used as N source when highly soluble carbohydrates are fed and maintained (Wohlt *et al.,* 1978). However, efficient utilization of protein and non-protein nitrogen (NPN) in ruminants depends upon knowledge of the basic principles underlying ruminal microbial N metabolism (Fernandez *et al.,* 1987). Moreover, ruminal pH has great impact on rumen fermentation efficiency (Wanapat, 2003).

Some strictly anaerobic bacteria use a reductive or reverse citric acid cycle known as the succinate-propionate pathway to synthesize succinate and (or) propionate (Gottschalk, 1986). Both malate and fumalate are key intermediates in the succinate propionate pathway, and *S. ruminantium* uses this pathway (Gottschalk, 1986). The fact dicarboxylic acids, especially malate and fumalate, stimulate lactate utilization is consistent with the presence of this pathway in this ruminal anaerobe (Callaway and Martin, 1996). Previous studies by Sanson and Stallcup (1984) reported that supplementation of malate in ruminant diets has been shown to increase nitrogen retention in sheep and steers, and to improve average daily gain and feed efficiency in bull calves. However, the use of malate in cassava based-diets has not yet been investigated. Therefore, the objective of this experiment was to investigate the supplementation of malate level and urea supplementation in concentrates containing high level of cassava chip with urea-treated rice straw as a basal roughage on ruminal ecology and milk production in lactating cows.

Materials and Methods

Animals, diets and experimental design: Four, Holstein-Friesian crossbred cows (75%) in the first lactation were used in experiment. Milk yield pre-experiment was 10±2 kg/day and the body weight were 390±10 kg. Cows were randomly assigned according to a 2 x 2 Factorial arrangement in a 4 x 4 Latin square design to study two levels urea with sodium dl-malate supplementation on ruminal ecology and milk production. The dietary treatments were as follows: T1 = supplementation of urea at 2 % with malate at 10 g/hd/d in concentrate (U2M10); T2 = supplementation of urea at 2 % with malate at 20 g/hd/d in concentrate (U2M20); T3 = supplementation of urea at 4 % with malate at 10 g/hd/d in concentrate (U4M10); T4 = supplementation of urea at 2 % with malate at 10 g/hd/d in concentrate (U4M20), respectively. The composition of dietary

treatments and urea-treated rice straw (UTS) used are shown in Table 1.

Cows were housed in individual pens and individually fed concentrate at a ration to milk yield of 1:2.5, twice daily at 0600 a.m. and 1600 p.m. after milking. All cows were fed *ad libitum* of UTS with water and a mineral-salt block. Feed intake of concentrate and roughage were measured separately and refusals recorded. The experiment was run in four periods, each experimental period lasted for 21 days, the first 14 days for treatment adaptation and for feed intake measurements whist the last 7 days were for sample collections of rumen fluid and faeces. Body weights were measured daily during the sampling period prior to feeding. Milk yield was recorded during the 21 day-period and samples were collected during the last 7 day of each period.

UTS was prepared by using 5% (W/W) urea mixed with 100 kg of water in 100 kg of rice straw (RS) batches (50:50, water to straw) and poured over a stack of straw and then covered with a plastic sheet for a minimum of 10 days before feeding to animals (Wanapat, 1990).

Data collection and sampling procedures: UTS and concentrate were sampled daily during the collection period and were composted by period prior to analyses. Cows were milked twice daily, and milk weights were recorded at each milking of each period. Milk samples were composite daily, according to yield, for both the a.m. and p.m. milking, preserved with 2-bromo-2 nitropropane-1, 3-dial, and stored at 4°C until analysis for fat, protein, lactose, totals solids and solids-not-fat content by infrared methods using Milko-Scan 33 (Foss Electric, Hillerod, Demark). Milk urea nitrogen (MUN) was determined using Sigma kits #640 (Sigma Diagnostics, St. Louis, MO) (Valadares *et al.*, 1999). Moreover, milk samples were homogenized using a T1500 homogeniser (Ystral, Dottingen, Germany). Five hundred µl aliquots of milk were diluted with 4.5 ml of HPLC grade acetonitrile and vortex-mixed for 15 s. A 2 ml aliquot of this mixture was passed through a 13 mm disposable syring filter containing a 0.45 µm PTFE menbrane (HPLC Technology, Cheshire, UK) into a HPLC vial (Shingfield and Offer, 1998).

Rumen fluid samples were collected at 0 and 4 h post-feeding. Approximately 200 ml of rumen fluid was taken from the middle part of the rumen by a stomach tube connected with a vacuum pump at each time at the end of each period. Rumen fluid was immediately measured for pH and temperature using (HANNA instruments HI 8424 microcomputer) after withdrawal. Rumen fluid samples were then filtered through four layers of cheesecloth. Samples were divided into two portions. One portion was used for NH_3-N analyses where 5 ml of H_2SO_4 solution (1M) was added to 50 ml of rumen fluid. The mixture was centrifuged at 16,000 g for 15 minute and the supernatant stored at -20°C prior to NH_3-N analysis using the micro Kjeldahl methods (AOAC, 1985) and volatile fatty acids (VFAs) analyses using a HPLC according to Zinn and Owen (1986). Another portion was fixed with10% formalin solution in normal saline (Galyean, 1989).

The total count of bacteria, protozoa and fungal zoospores were made using the methods of Galyean (1989) based on the use of a haematocytometer (Boeco) and culture groups of bacteria using the roll-tube method described by Hungate (1969), for identifying of bacteria groups (cellulolytic, proteolytic, amylolytic and total viable count bacteria). In addition, specific bacteria namely *Butyrivibrio fibrisolvens* was grown anaerobically at 39°C in basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_47H_2O$, 64 mg of $CaCl_22H_2O$, 4,000 mg of Na_2CO_3 600 mg of cysteine hydrochloride, 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, MD.), 2.5 g of yeast extract, and branched-chain volatile fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals. *Megasphaera elsdenii* was grown in a basal medium that was prepared anaerobiaclly under O_2-free CO_2 (per liter) K_2HPO_4, 292 mg; KH_2PO_4, 292 mg; $(NH_4)_2SO_4$, 480 mg; NaCl, 480 mg; $MgSO_47H_2O$, 100 mg; $CaCl_22H_2O$, 64 mg; Na_2CO_3, 4000 mg; cystein hydrochloride, 600 mg; vitamins and micromineral mixture (Cotta and Russell, 1982).

A blood sample (about 10 ml) was drawn from the jugular vein at the same time as rumen fluid sampling, separated by centrifugation at 5000 *g* for 10 minutes and stored at -20°C until analysis of blood urea nitrogen (BUN) according to the method of Crocker (1967).

Statistical analysis: All data obtained from the experiment were subjected to ANOVA for a 4x4 Latin square design with 2 x 2 Factorial arrangement of treatments using the General Linear Models (GLM) procedures of the Statistical Analysis System Institute (SAS, 1998). Treatment means were compared by Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980).

Results and Discussion

Chemical composition of feeds: The chemical composition of roughage and concentrate diets fed in dairy cows are presented in Table 1. Concentrate diets contained similar concentrations of DM, OM, CP, NDF, ADF, EE and non-structural carbohydrate (NSC). Diets containing high levels of cassava chip based diets had a slightly higher NSC and lower NDF due to increased level of cassava chip in the diets. Furthermore, the chemical composition of UTS is presented in Table 1. Similar values for UTS has been similar to those reported by Wanapat (2000).

Table 1: Chemical composition of concentrates and UTS used in the experiment (% DM basis)

Item	Dietary treatments		
	Concentrate I	Concentrate II	UTS
Ingredient (%DM)			
Cassava chip	70	75	
Palm meal	1.5	4	
Soybean meal	17	3.5	
Molasses	1	5	
Coconut oil	4	4	
Urea	2	4	
Sulfur	1	1	
Salt	1	1	
Limestone	1	1	
Mineral mix	1.5	1.5	
Chemical compositions (%)			
DM	88.7	89.1	55.8
OM	91.1	91.2	88.9
CP	16.1	16.0	8.0
NDF	13.1	12.1	73.2
ADF	8.1	7.2	52.3
NSC[1]	58.1	59.0	6.7
Ash	8.9	8.8	11.1
EE	4.0	3.9	1.2
Ca	1.7	1.8	-
P	0.7	0.6	-
TDN	80.1	80.2	55.1
ME, Mcal/kg (DM)[2]	2.9	2.9	1.9
NE_L, Mcal/kg (DM)[3]	1.8	1.8	1.2
Feed cost, US$/kg	0.21	0.11	0.09

[1]Estimated: NSC = 100 – ((NDF – NDF protein) + Protein + EE +Ash). [2]Estimated: Metabolizable energy (ME, Mcal/kg, DM)=TDNx0.04409x0.82. [3]Estimated: Net energy for lactation (NE_L, Mcal/kg)= (0.0245xTDN)-0.12.

Effect on feed intake: The effects of urea level with malate on feed-intake of lactating dairy cows are presented in Table 2. Feed intakes were not significantly affected by urea level and malate supplementation (2.8-2.9 %BW). This data indicated that urea level with malate supplementation had no effect on feed-intake in lactating dairy cows. These result was in agreement with earlier work by (Sommart *et al.,* 2000) which reported that inclusion of cassava chip in diets resulted in satisfactory animal performance and had no negative effects on animal health in finishing beef cattle and lactating dairy cows.

Effect on rumen fermentation: Rumen ecology parameters were measured for temperature, pH, CH_4, lactic acid, NH_3-N, VFA (Table 3). In addition, BUN and MUN were determined to investigate their relationships with rumen NH_3-N and protein utilization. Rumen pH at 0 and 4 h post-feeding were unchanged by dietary treatments and the values were quite stable at 6.5-6.6, but all treatment means were within the normal range which has been reported as optimal for microbial digestion of fiber and also digestion of protein (6.0-7.0) (Hoover, 1986). The concentrations of lactic acid in rumen were significant ($p < 0.05$) when dairy cows received different level of malate supplement with high cassava-based diets. However, the values of lactic acid in rumen for dairy cows received high cassava-based diets were non-exceeding levels (range between at 21.7-26.7 mM). As reported, the concentrations of lactic acid in rumen exceeding 40 m*M* were indicative of severe acidosis in cattle (Owens *et al.,* 1998).

The concentrations of methane production in rumen were not affected ($p > 0.05$) by dairy cows receiving different urea level and malate supplementation with concentrates containing high cassava-based diets. In previous studies by Asanuma *et al.* (1999) reported that the use of malate and fumalate as feed additives could reduce methanogenesis and increase propionate production in the rumen.

Ruminal NH_3-N, BUN and MUN concentrations were not altered by urea level and malate supplement in diets containing high cassava-based diets. As NH_3-N is regarded as the most important nitrogen source for microbial protein synthesis in the rumen. In addition, the result obtained was closer to optimal ruminal NH_3-N (15-30 mg/dl, Wanapat and Pimpa, 1999; Chanjula *et al.,* 2003, 2004) for increasing microbial protein synthesis, feed digestibility and voluntary feed intake in ruminant fed on low-quality roughages.

The influence of urea level with malate supplementation on total VFA concentrate, production of total VFA, acetic acid proportion, propionic acid proportion, butyric acid proportion and acetic to propionic ratio are shown in Table 3. Mean total VFAs and propionate concentrations in the rumen were increased with increasing urea level and malate in the diet. However, the concentration of propionic acid was slightly higher in U2M20 than U4M10, U4M20 and U2M10, respectively. However, it was found that total VFA concentration in all diets ranged from 70 to 130 m*M*. Although the acetate to propionate ratio was decreased by the level of malate, but the supplementation of urea level with malate increased the daily output of propionate without decreasing the production of acetate, and it was in agreement with the results reported by other authors (Callaway and Martin, 1996; Khampa *et al.,* 2006).

Rumen microorganisms populations: Table 4 presents rumen microorganism populations. Total viable bacteria counts cellulolytic, *Selenomonas ruminantium*, *Megasphaera elsdenii* and *Butyrivibrio fibrisovens* bacteria were significantly different, whilst populations of amylolytic and proteolytic bacteria were similar by urea level and malate supplementation in diets containing high cassava-based diets. Nevertheless, fungal zoospores, protozoa and total bacteria direct counts were significantly different and populations of bacteria had higher numbers in lactating cows receiving at 20 than 10 g/hd/d of malate. In contrast, the present number of protozoa in the rumen was decreased by urea level

Table 2: Influence of urea level and malate on feed-intake in lactating cows

Item	Treatments[1]					Contrast[2]		
	U2M10	U2M20	U4M10	U4M20	S.E.M.	U	M	UxM
DM intake (%BW)								
UTS	1.5	1.6	1.5	1.5	0.08	NS	NS	NS
Concentrate	1.3	1.3	1.3	1.4	0.03	NS	NS	NS
Total	2.8	2.9	2.8	2.9	0.03	NS	NS	NS

[a, b, c]Values on the same row with different superscripts differ (p< 0.05). [1]U2M10 = Urea at 2 % with malate at 10 g/hd/d; U2M20 = Urea at 2 % with malate at 20 g/hd/d; U4M10 = Urea at 4 % with malate at 10 g/hd/d; U4M20 = Urea at 2 % with malate at 20 g/hd/d. [2]Probability of main effects of level urea in concentrates (2 vs 4 %), levels of malate (10 vs 20 g/hd/d), or the UxM interaction. * = P < 0.05, ** = P < 0.01, NS = P > 0.05.

Table 3: Influence of urea level and malate on rumen fermentation blood metabolites and VFA characteristics in lactating cows

Item	Treatments					Contrast		
	U2M10	U2M20	U4M10	U4M20	S.E.M.	U	M	UxM
Ruminal pH	6.5	6.5	6.6	6.6	0.13	NS	NS	NS
CH_4 (mmol/L)	10.6	11.4	13.0	11.2	3.77	NS	NS	NS
Lactic acid (mg/ml)	24.7[a]	21.7[b]	26.7[a]	22.1[b]	0.67	NS	**	NS
NH_3-N (mg/dl)	15.8	14.7	16.2	16.1	1.96	NS	NS	NS
BUN (mg/dl)	11.5	11.0	16.7	14.7	2.66	NS	NS	NS
Total VFA (mmol/L)	114.1	116.1	121.4	123.2	6.88	NS	NS	NS
Molar proportion of VFA (mol/100mol)								
Acetate (C2)	64.3	62.7	64.2	63.5	1.59	NS	NS	NS
Propionate (C3)	26.9	27.8	27.7	27.6	1.40	NS	NS	NS
Butyrate (C4)	8.7	9.4	8.0	8.8	0.57	NS	NS	NS
C2:C3 ratio	2.4	2.3	2.3	2.4	0.19	NS	NS	NS
C2+C4:C3 ratio	2.7	2.6	2.6	2.7	0.20	NS	NS	NS

[a, b]Values on the same row with different superscripts differ (p < 0.05).

Table 4: Influence of urea level and malate on rumen microorganisms in lactating cows

Item	Treatments					Contrast		
	U2M10	U2M20	U4M10	U4M20	S.E.M.	U	M	UxM
Total direct counts								
Bacteria (x10^{11} cell/ml)	6.3[a]	9.3[b]	5.6[a]	8.3[ab]	0.91	NS	**	NS
Protozoa								
Holotric (x10^4 cell/ml)	2.1[a]	1.0[b]	2.0[a]	1.5[ab]	0.26	NS	**	NS
Entodiniomorph (x10^5 cell/ml)	7.8[a]	3.4[b]	11.3[c]	5.0[b]	0.71	*	**	NS
Fungal zoospores (x10^4 cell/ml)	3.7[a]	7.0[b]	2.5[a]	5.6[b]	0.51	*	**	NS
Roll tube techniques								
Total viable bacteria								
(x10^7 CFU/ml)	31.2[a]	54.1[b]	27.6[c]	52.7[b]	0.73	*	**	NS
Amylolytic (x10^6 CFU/ml)	12.6	15.9	12.6	9.2	4.00	NS	NS	NS
Proteolytic (x10^6 CFU/ml)	6.8	8.6	9.9	11.5	3.99	NS	NS	NS
Cellulolytic (x10^7 CFU/ml)	10.6[a]	27.3[b]	28.6[b]	5.6[a]	7.15	NS	*	NS
Selenomonas ruminantium								
(x10^4 CFU/ml)	8.7[a]	13.7[b]	7.1[a]	14.1[b]	1.41	NS	**	NS
Megasphaera elsdenii								
(x10^4 CFU/ml)	10.4[a]	17.3[b]	10.3[a]	17.0[b]	1.69	NS	**	NS
Butyrivibrio fibrisolvens								
(x10^4 CFU/ml)	7.7[a]	7.0[a]	14.0[b]	15.6[b]	1.75	*	NS	NS

[a, b, c] Values on the same row with different superscripts differ (p < 0.05).

and malate supplementation in high cassava-based diets. In the experiment by Newbold *et al*. (1996) has shown that feeding 100 mg of malate per day in sheep caused an increase in the number of total bacteria and tended to increase the population of cellulolytic bacteria. In agreement with these observations, Lopez *et al*. (1999) reported that fumalate (another intermediate in the succinate to propionate pathway) increased the number of cellulolytic bacteria almost three-fold during fermentation in the RUSITEC system. As cassava chip can be readily degraded in the rumen and ruminal pH was decreased, malate could stimulate lactate utilization by *S. ruminantium* and could improve pH in the rumen. It is possible that supplementation of urea with malate

Table 5: Effects of urea and malate supplementation on milk yield and composition in lactating dairy cows

Item	Treatments					Contrast		
	U2M10	U2M20	U4M10	U4M20	S.E.M.	U	M	UxM
Production								
Milk yield (kg/d)	11.6	12.0	10.8	11.6	1.35	NS	NS	NS
3.5%FCM (kg/d)	12.5	13.6	11.2	12.9	1.37	NS	NS	NS
Milk composition (%)								
Milk fat	3.8	4.0	3.6	3.9	0.37	NS	NS	NS
Milk protein	3.1	3.0	3.0	3.1	0.09	NS	NS	NS
Lactose	4.7	4.6	4.7	4.3	0.23	NS	NS	NS
Solids not fat	9.2	8.6	8.8	8.4	0.28	NS	NS	NS
Total solids	13.2	12.5	13.3	12.0	0.73	NS	NS	NS
Milk urea nitrogen (mg/dl)	17.0	16.4	14.7	16.0	1.19	NS	NS	NS
Milk allantoic (µmol/L)	87.5^{ac}	103.4^{b}	82.9^{a}	96.6^{bc}	3.51	NS	**	NS

[a, b, c] Values on the same row with different superscripts differ ($p < 0.05$).

may play an important role in increasing bacterial populations. Moreover, Martin *et al.* (1999) reported that increasing dietary concentrations of malate might help to reduce problems associated with ruminal acidosis by stimulating lactate utilization by *S. ruminantium*.

The populations of *Megasphaera elsdenii* were significantly ($p < 0.05$) affected by different level of malate supplementation with high cassava-based diets (Table 4). In contrast, the populations of *Butyrivibrio fibrisolvens* were affected when receiving different urea level supplementation in concentrate containing high cassava-based diets. In the experiment by Kim *et al.* (2002) which reported that only a few ruminal bacteria can utilize lactate (e.g. *M. elsdenii* and *S. ruminantium*), in which *M. elsdenii* was the most important predominant ruminal bacteria that produces *trans*-10, *cis*-12 conjugated linoleic acid (CLA) from linoleic acid. The use of sodium dl-malate may enhance in these regards.

Milk production and composition: The influences of urea level and malate supplementation in lactating dairy cows receiving high cassava-based diets are shown in Table 5. All cows were able to maintain levels of milk yield during the 80 days of experiment. Yield of milk was greatest in cows fed cassava-based diets with U2M20, but were lowest ($p > 0.05$) when receiving U4M10 in diets. In addition, production of 3.5%FCM exhibited similar results for all treatments ($p > 0.05$). The supplementation of urea level and malate in high cassava-based diets fed in lactating dairy cows and UTS as roughage sources did not affect on milk compositions (Table 5). Milk allantoic concentrations were found to range between 82.9-103.4 µmol/L depending upon diets and production level, which were lower than those values (159-237 µmol/L) reported by Giesecke *et al.* (1994). Allantoic concentrations in bovine milk were similar to those in plasma but were much lower than urinary concentrations, which typically ranged between 0.7 and 29.4 m*M* (Shingfield and Offer, 1998).

Conclusions and Recommendations: Based on this experiment, it could be concluded that supplementation of urea level with malate in concentrate containing high level of cassava chip maintained could improved ruminal fermentation efficiency, increasing propionate production and decreased acetate to propionate ratio. Moreover, high level of cassava chip in diet resulted increase populations of bacteria, but decreased protozoal populations in rumen. These results suggest that the combined use of concentrates containing high level of cassava chip at 75 %DM with urea at 4 %DM in concentrate and malate at 20 g/hd/d could highest improved rumen ecology and milk production in lactating cows. However, further studies should be conducted, particularly in concentrate containing high level of cassava on milk compositions especially on conjugated linoleic acid (CLA) in lactating cows fed straw based-diets.

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