MINISTRY OF EDUCATION AND TRAINING
CAN THO UNIVERSITY

School year: 2010-2012

LE THUY BINH PHUONG

# MITIGATION OF METHANE PRODUCTION FROM RUMINANTS: EFFECT OF NITRATE AND UREA ON METHANE PRODUCTION IN AN *IN VITRO* SYSTEM AND ON GROWTH PERFORMANCE AND METHANE EMISSIONS IN GROWING CATTLE

MASTER OF SCIENCE THESIS IN AGRICULTURAL SCIENCES
ANIMAL HUSBANDRY

Code Number: 60 - 62 - 40

Can Tho City, Viet Nam - 2012

**MINISTRY OF EDUCATION AND TRAINING**
**CAN THO UNIVERSITY**
**School year: 2010-2012**

**LE THUY BINH PHUONG**

# MITIGATION OF METHANE PRODUCTION FROM RUMINANTS: EFFECT OF NITRATE AND UREA ON METHANE PRODUCTION IN AN *IN VITRO* SYSTEM AND ON GROWTH PERFORMANCE AND METHANE EMISSIONS IN GROWING CATTLE

**MASTER OF SCIENCE THESIS IN AGRICULTURAL SCIENCES**
**ANIMAL HUSBANDRY**

**Code: 60 – 62 - 40**

**Scientific supervisors:**

**1-Associate professor Dr. Duong Nguyen Khang**

**2-Professor Dr. Thomas Reg Preston**

## AN APPROVAL OF THE SCIENTIFIC EVALUATION COMMITTEE

The thesis with the title: **"***Effect of sulphate and nitrate salts on methane production in an in vitro incubations and on growth performance and methane emissions in cattle fed molasses and cassava foliage"* implemented by Mrs. Le Thuy Binh Phuong was approved by the Scientific evaluatation Committee at the Can Tho University.

**Committee member** **Secretary**

**Opponent number 1** **Opponent number 2**

**Can Tho City, March, 2012**

**Chair person**

# CURRICULUM VITAE

## BIO-DATA

Full name: Le Thuy Binh Phuong Gender:Female
Date of birth: 19/08/1982 Place of birth: Ho Chi Minh city, Viet Nam
Father's birthplace: Nam Định Nationality: Viet Nam
Position *( before participating MSc program):* Lecturer and researcher
Workplace (*current or before participating MSc program):* Nong Lam university
Residence address *(house number, district, ward/district, city/province):* No. 6B, Vicasa hostel, 10 ward, An Binh precinct, Bien Hoa city, Dong Nai province.
Mailing address: 6B, Vicasa hostel, 10 ward, An Binh precinct, Bien Hoa city, Dong Nai province.
Tel: 0902689963 Fax:
E-mail: binhphuonglt@gmail.com, binhphuongty27@yahoomail.com

## II. EDUCATIONAL BACKGROUND

### 1. Undergraduate education

Type of training (*Permanent or impermanent)* : *Permanent*
Institution awarding degree: Fair
Major: Veterinary Medicine
Year earned: 2006
Country: Vietnam
Institution awarding degree: Faculty of Animal Science & Veterinary Medicine, Nong Lam University
Country: Viet Nam
Name of the thesis: Survey of MMA on sows and diarrhoea on piglets and sows in Tan Trung farm, Cu Chi district
Name of supervisor: Pathology and Parasitology Department Faculty of Animal Science & Veterinary Medicine, Nong Lam University, Vietnam

### 2. Master of Sciences

Type of training (*Permanent or un-permanent)*: *Permanent*
Institution awarding degree: Faculty of Animal Science, College of Agriculture and Applied Biology, Can Tho University, Can Tho city
Major: Animal production
Year earned: 2012
Country: Viet Nam
Institution awarding degree: Faculty of Animal Science, College of Agriculture and Applied Biology, Can Tho University, Can Tho city
Country: Viet Nam
Name of the thesis: Effect of sulphate and nitrate salts on methane production in in vitro incubations and on growth performance and methane emissions in cattle fed molasses and cassava foliage
Name of supervisor: Associate prof. Dr. Duong Nguyen Khang and prof. T R Preston

### 3. Foreign languages

1. English Level of proficiency (IELTS): Good (Overal score:5.0)
2. Mother' tongue name Level of proficiency: Excellent

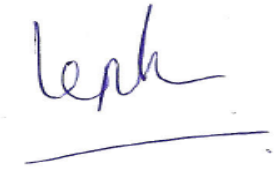
**4. BSc Degree awarding:**

- Major: Veterinary medicine

- Degree number: 779541 Issued date: 25 November 2006 Country: Vietnam

**Cantho City, 5st Feb, 2012**

**Signature**

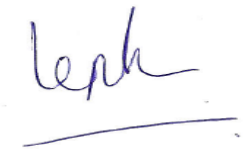


**Le Thuy Binh Phuong**

# COMMITMENT

I assure that this thesis is a scientific work which was implemented by myself. All the figures and results presented in the thesis are true and not published in any previous theses.

**Author**



**Le Thuy Binh Phuong**

# Acknowledgements

The research in this thesis was conducted at laboratory and farm of Research and Trainning of Ruminant Center, Binh Duong provice, with supported from Mekong Basin Animal Research Network (MEKARN) project and Norwegian Programme for Development, Research and Education (NUFU) project for funding this theses research and the scholarship for the MSc degree.

I would like to express my gratitude to:

Mekong Basin Animal Research Network (MEKARN) project and The Norwegian Programme for Development, Research and Education (NUFU) project, for the financial support of this study and research.

The Faculty of Animal science, Agriculture Forestry University and Agriculture and Apply Biology, Can Tho University, Vietnam.

Associate professor Dr. Duong Nguyen Khang, Head of Department of Animal Physiology and Biochemistry, Nong Lam University, my supervisor, for all your support, advice and explanations throughout the research, also for your reading and correcting my thesis papers.

Professor Dr. Thomas Reg Preston and Professor Dr. Ron Leng, my teacher and adviser, for all your valuable guidance and support during the study.

I would also like to extend sincere thanks to Professor Dr. Jan Erik Lindberg, International Coordinator MEKARN project; Professor Dr. Jan Berg, International Coordinator NUFU project; Dr Vanthong Phengvichith, National Agriculture and Forestry Researcher Institute (NAFRI), Associate professor Dr. Ngo Van Man, MEKARN regional coordinator and Mr. Nguyen Van Cong, MEKARN program officer and Mr. Nguyen Dzung, NUFU project assistant for their facilitation, help and support to the whole course. Associate professor Dr. Nguyen Van Thu, Head of the department of Animal Science, Faculty of Agriculture and Apply Biology, Can Tho University, Vietnam, Professors, Lecturers and assistant lectures in MEKARN courses, for giving me care and useful knowledge.

Warm thanks are extended to my parents, Mr. Le Van Thang and Mrs. Le Thi Huong for suppor and assistance and encouragement, my young brother Le Ba Thien for his supported and encouragement; to my husband, Mr. Than Van Dang and my daughter Than Ngoc Kim Nguyen for their love.

All of my friends in the MSc course 2010-2012 from the three countries: Laos, Vietnam and Cambodia, for sharing the culture, friendship and creating a warm atmosphere throughout the time of the course.

I would like to thank all the people who contributed to this study.

# CONTENTS

# Abstract

## Paper 1

The aim of this study was to evaluate the effect of replacing urea by calcium nitrate with or without supplementary sulphur (0, 0.4, 0.8% on DM basis) on methane production in an *in vitro* incubation medium inoculated with rumen fluid and using sugar cane stalk and cassava leaf meal as substrate. The design was a 3*2 factorial arrangement of the treatments with 4 replications.

Compared with urea, calcium nitrate reduced methane production. The effect was consistent over successive periods in the 48h incubation. Adding 0.4% sulphur, in the form of sodium sulphate, increased methane production, while 0.8% sulphur reduced methane production. When 0.8% sulphur was combined with nitrate the effects on methane reduction were additive. Methane production increased linearly with the length of the incubation on all treatments.



***Key words**: Calcium nitrate, climate change, fermentation, gas production, greenhouse gases, urea, substrate*



## Paper 2

This study aimed to evaluate the effect of three nitrate salts (calcium nitrate, potassium nitrate and sodium nitrate) compared with urea on methane production in an *in vitro* 24h incubation with molasses and cassava leaf meal as substrate.

Compared to urea treatment, all nitrate salts diminished total gas production, methane percentage in the gas and methane production per unit of substrate, with no differences among the nitrate salts.



***Key words:** Calcium nitrate, climate change, fermentation, gas production, greenhouse gases, potassium nitrate, sodium nitrate, urea*



## Paper 3

This *in vitro* incubation was arranged as a 2*3 factorial in a completely randomized block design with 6 treatments and 4 repetitions. The first factor was the source of fermentable N (potassium nitrate or urea); the second factor was the level of added sulphur (0, 0.4 and 0.8% of substrate DM, as sodium sulphate). The substrate was molasses and cassava leaf meal. Incubations were carried out on successive days for 6, 12, 18, 24 and 48h. At the end of each incubation, volume of gas and percentage methane was recorded, and the residual substrate filtered to determine the amount of substrate solubilized.

Gas production, percentage methane in the gas and methane production were reduced by replacing urea by nitrate at incubation times from 6 to 48h, and were increased with length of incubation. Added sulphur

increased methane emissions in the presence of nitrate over the early incubation periods indicating a greater fermentation rate in that period, but sulphur was additive in decreasing methane in longer incubations, indicating nitrate had been fully reduced and sulphur reduction commenced. The reduction in methane production after 48h of incubation, when sulphur and nitrate were combined, was greater than when nitrate was used alone.

***Key words:*** *Climate change, fermentation, gas production, greenhouse gases, potassium nitrate, urea*

## Paper4

The objective of this study was to determine the effect of potassium nitrate versus urea, and of supplementary sulphur, on growth performance of cattle fed molasses and cassava foliage. The experiment was designed as a 2*2*3 factorial with 4 replications. The factors were source of NPN (potassium nitrate: 6 % of diet DM basis or urea: 1.8 % of diet DM basis), level of added sulphur (0 or 0.8% S) and source of cassava foliage (fresh foliage or dried leaf meal).

DM intake was not affected by NPN source, but was depressed by adding 0.8% sulphur and was lower for the treatment with fresh cassava foliage compared with leaf meal. After correcting the data by covariance for differences in initial live weight, growth rate was depressed by adding 0.8% sulphur to the diet but was not affected by source of NPN or source of cassava foliage. The ratio of methane to carbon dioxide was reduced by feeding potassium nitrate rather than urea and by fresh cassava foliage compared with cassava leaf meal.

***Key words****: Feed conversion, foliage, leaf meal, potassium nitrate, urea*

## Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| AOAC | Association of Official Analytical Chemists |
| ATP | Adenosine tri-phosphate |
| $CH_4$ | Methane |
| CP | Crude protein |
| DM | Dry matter |
| FMD | Foot and mouth disease |
| GEI | Gross Energy Intake |
| HCN | Hydrogen cyanide |
| $HCO_3^-$ | Bicarbonate |
| LW | Live weight |
| Mekarn | Mekong basin animal research network |
| N | Nitrogen |
| $NH_3$ | Ammonia |
| NPN | Non-Protein Nitrogen |
| $NO_3$ | Nitrate |
| NRB | Nitrate-reducing bacteria |
| NRSOB | Nitrate-reducing sulphide-oxidizing bacteria |
| OM | Organic matter |
| pH | Power of/potential Hydrogen |
| Prob/P | Probability |
| RCD | Randomized complete design |
| SEM | Standard error of the mean |
| SRB | Sulphate-reducing bacteria |
| U | Urea |
| VFAs | Volatile fatty acids |

## Introduction

In recent years the sustainability of animal production has been stressed in view of the increasing human population and climate changes (Pollock 2008). According to FAO (2006) and Steinfeld et al (2006), the resultant trends in meat and milk consumption in developing and developed countries increased from 1980 to 2002 with estimates dramatically increasing in the future. Global livestock production has increased substantially since the 1960s and beef production has more than doubled (Thornton 2010).

Since feed costs are the major part of production costs in livestock production, an increase in the use of indigenous feed resources is an important way of helping farmers reduces their costs. Devendra and Leng (2011) suggested to shift priority for the development of ruminants (buffaloes, cattle, goats and sheep) in key agro-ecological zones towards making intensive use of the available biomass from the forage resources, crop residues, agro-industrial by-products and other non-conventional feed resources to improve efficiency of feed utilization and increase product output.

Fermentation of feeds in the rumen is the largest source of methane from enteric fermentation, and methane is one of the important gases contributing to the greenhouse effect. Moss et al (2000) indicated that agricultural emissions of methane in the EU-15 have recently been estimated at 10.2 million tonnes per year and two-thirds come from enteric fermentation. In particular, beef cattle and dairy cattle combined produced the highest level of methane emissions per head, accounting for 72% and 32% respectively (US. EPA, 2009). Leng (1993) indicated that the methane released from enteric fermentation reduced efficiency of conversion of feed to product (e.g. milk, beef, draught power).

Leng (2008) has suggested that supplementation strategies for ruminants to reduce methane production should be based on feeding of nitrate salts, which provide an alternative electron acceptor whilst still maintaining the efficiency of the fermentative digestion. Nitrate can also supply a high proportion of the total N in a feed, and poor quality forages are typically deficient in fermentable nitrogen.

The lack of conventional concentrate feeds and the need to expand meat production to provide protein for the population are aspects affecting the demand for feeds for livestock.

The use of molasses for cattle fattening has been developed in many countries because a high molasses feeding system has proved economically successful (Preston et al 1967b; Olbrich and Wayman 1972; Cuong et al 2010), since rates of animal performance and efficiency of feed utilization were almost doubled compared with the traditional method.

Leng (1990) suggested that for maximum production from a particular feed, it is necessary to ensure optimal rumen condition for microbial growth and adjust protein to energy ratios in the nutrients absorbed with a bypass protein to optimize efficiency of utilization of the absorbed nutrients. Most feed resources for ruminants are forages that can be described only as poor quality in which the limitations to production are a low protein supply from the microbial ecosystem and a virtual absence of dietary bypass protein (Leng 1993). Cassava is a crop grown widely in the tropics and sub-tropics and can be grown on soils with low organic matter, receiving low rainfall and high temperature. Particularly, cassava leaves contain high level of crude protein (25%) some of which can apparently by-pass the rumen since it is in the form of a tannin-protein complex (Wanapat 1995). The leaves residues after harvesting root can be available source of protein for feeding to many kinds of animals (Preston 2001).

## Hypotheses

The hypotheses to be tested were:

*Paper 1*

Supplementary sulphate will have a synergistic effect in reducing methane production in a basal diet of sugar cane in which the source of non-protein nitrogen (NPN) is nitrate rather than urea.

*Paper 2*

All nitrate salts (calcium nitrate, potassium nitrate and sodium nitrate) will reduce methane production compared with urea, but no difference among nitrate salts.

*Paper 3*

Supplementary sulphur in the synergistic interaction between sulphate and nitrate in lowering methane production in an *in vitro* fermentation of a substrate based on molasses and cassava leaf meal.

*Paper 4*

Feeding a molasses-based diet that is supplemented with potassium nitrate will support better growth and feed conversion than the same diet supplemented with urea.

# Literature review

## General metabolic pathways in the rumen

Nutritional components such as carbohydrates, proteins and lipids in feedstuffs are degraded by rumen microorganisms and are converted into microbial cells, wiith volatile fatty acids (VFAs) and gases as the by-product of the fermentation. Several types of carbohydrates (sugars, starch, pectins, hemi-cellulose and cellulose) are degraded by the microorganisms in the rumen. Also degradation of proteins occurs and a significant proportion of the amino acids are either incorporated in microbial protein or degraded further to VFA and ammonia. Lipids and free long-chain fatty acids are hardly fermented, but to some extent, long chain fatty acids may be incorporated in microbial matter (Harfoot and Hazlewood 1997). Within the diversified microorganism ecosystem of the rumen, substrates are fermented to VFA, $CO_2$, $H_2$, $NH_3$ and microbial matter is synthesized. The purpose of the fermentation is to generate energy for maintenance and energy for synthetic processes of microbial polymers which leads to the synthesis of more microbial cells which in turn increases available protein to the animal.

Most of the substrate energy is retained in the end of products of fermentation. According to Bergman (1990), current estimates are that VFA contribute approximately 70% to the caloric requirements of ruminants, such as sheep and cattle. The conserved energy is used mainly in the biosynthesis of cellular constituents (carbonhydrate, nitrogenous substancs, lipids and nucleic acids) that comprise microbial growth (Hobson and Stewart 1997). Figure 1 shows that the mass of microbial cells produced in the rumen is a function of the amount of ATP generated within the cells by catabolism of feed organic matter (pathway A), and the efficiency of ATP utilization for polymer synthesis (pathway B) (Nolan 1998).

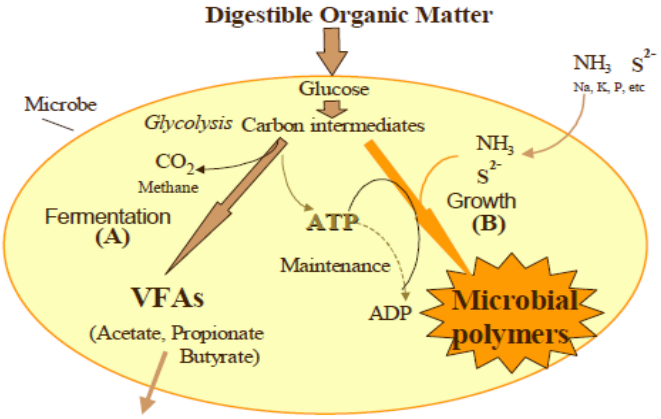



**Figure 1.** A diagram describing digestion of organic matter in the rumen (Nolan 1998)

## The process of methane production in the rumen

The methane is produced through the integrated activities of different microbial species, with the final step carried out by methanogenic bacteria (Moss et al 2000). Methane synthesis is regarded as one such relationship between hydrogen-producing microbes and hydrogen consuming methanogens. Since the hydrogen-producing microbes include fibrolytic fungi and bacteria, their co-association with methanogens allows efficient removal of hydrogen that reduces $CO_2$ to $CH_4$, which facilitates continuous fiber degradation. Therefore, when methane reduction is attempted, it is necessary to consider alternative hydrogen sinks for methanogenesis (Kobayashi 2010).

## Volatile fatty acids pattern, hydrogen balance and methane yield

Moss et al (2000) established that $CH_4$ production can be calculated from the stoichiometry of the main volatile fatty acids (VFA) formed during fermentation: acetate ($C_2$), propionate ($C_3$) and butyrate ($C_4$). VFA are then absorbed and utilized by the host animal. The molar percentage of VFA influences the production of $CH_4$. With acetate and butyrate production, $H_2$ is produced, whereas with propionate and valerate production, $H_2$ is utilized. It is generally assumed that the excess $H_2$ is almost completely converted into $CH_4$ by methanogens. Thus, acetate and butyrate production results in $CH_4$ production, while propionate formation serves as a competitive pathway for $H_2$ use in the rumen. With an increased molar proportion of propionate, the molar proportions of acetate and/or butyrate are reduced (Baker 1997). Reduction in $CH_4$ production can result from a decreased extent of fermentation in the rumen

or from a shift in the VFA pattern towards more propionate and less acetate (Boadi et al 2004).

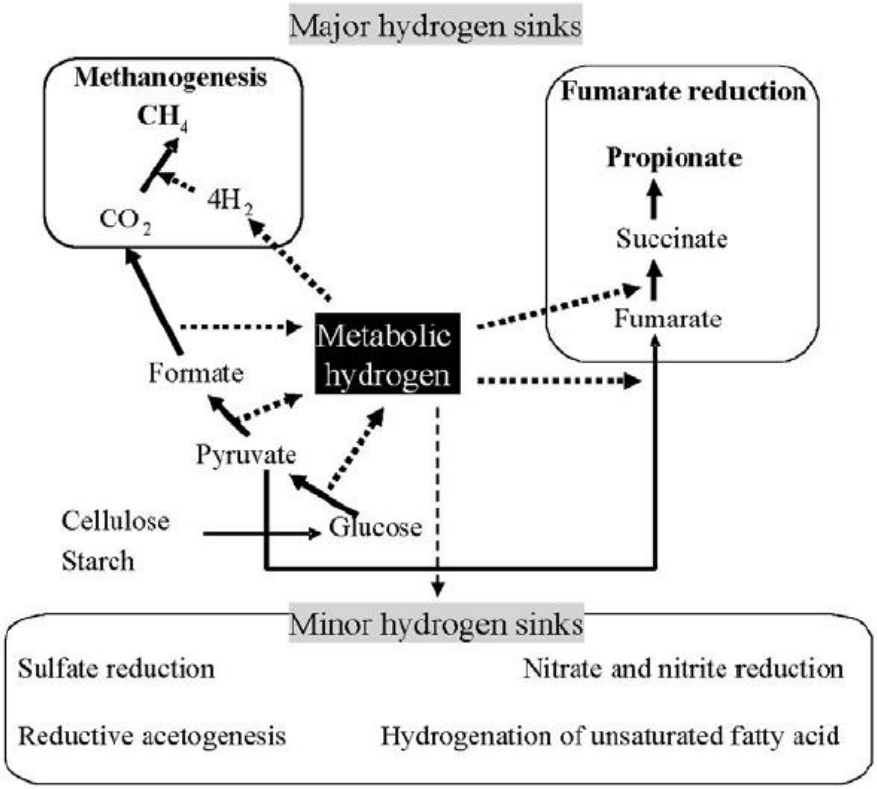



**Figure 2.** Hydrogen-consuming pathways recognized in the rumen (Source: Kobayashi 2010)

The most important effect on methane yield is dietary characteristics as well as the fermentation conditions in the rumen. According to Tamminga et al (2007), important dietary characteristics are daily feed intake and the resulting rumen fill, the proportion of concentrates in dietary dry matter, and the composition and the rate and extent of degradation of individual feed fractions (the types of carbohydrate and protein) in dietary dry matter. The factors to evaluate the fermentation condition include acidity (pH) of rumen fluid, the presence of unsaturated long chain fatty acids, the composition of the microbial population within the rumen, the dynamics of the passage of particles, fluid and the microbial population, the inflow of saliva and the absorption capacity of the rumen wall.

The type of carbohydrate fermented influences methane production most likely through impacts on ruminal pH and the microbial population. Fermentation of cell wall fiber yields higher acetic:propionic acid ratios and higher methane losses (Moe and Tyrrell 1979). Most *in vivo* studies concluded in general that fiber fermentation increases methanogenesis when

compared to fermentation of soluble carbohydrates (Moe and Tyrrell 1979). Torrent et al. (1994) assumed that non-cell wall carbohydrates should be separated according to their content of sugars and starch, since sugars seemed to be the most methanogenic of the two. Studies by Czerkawski and Breckenridge (1969) with pure carbohydrate sources confirms this assumption. Additionally, as a greater amount of any carbohydrate fraction is fermented per day, whether it is fiber or starch, methane production is decreased.

## Requirement of NPN source for cattle

Ammonia is produced in the rumen by metabolism of protein, peptides, amino acids, amides, urea, nitrates and some other non-protein nitrogen (NPN) sources. Dietary proteins are hydrolyzed in the rumen to varying degree depending upon their solubility (Allen and Kirpal 1969). NPN are used by bacteria in the rumen and broken down to ammonia ($NH_3$) during the normal fermentation process in the rumen. Microorganisms in the rumen combine the ammonia with products of carbonhydrate metabolism to form amino acids and hence protein for animal. Thus, the best option for assessing the availability of fermentable N is to measure the level of rumen ammonia in animals given the feed. There is an evidence that efficient utilization of $NH_3$ for microbial protein synthesis in the rumen occurs at relatively low concentration 5-8 mg $NH_3$-N/100 mL (Roffler et al 1975). Perdok and Leng (1990), however, suggested that the ammonia level should be at a minimum 100mg N/liter but that levels up to 200mg N/liter were even more efficiently used.

Urea is an important NPN source to form ruminal ammonia; it rapidly broken down into ammonia and carbondioxide by bacteria urease (Pearson and Smith 1943). In addition, urea enters the rumen endogenously via saliva and by diffusion through the rumen wall (Houpt 1959).

$$\text{Urea} \xrightarrow[\text{Urease}]{\text{Microbial}} NH_3 + CO_2$$

**Figure 3**. Metabolism of urea by bacteria

However, the efficiency of $NH_3$ utilization depends on the rumen environment and energy availability (Hungate 1966; Ali et al 2007). The supplementation of urea as NPN for intensive fattening of cattle on molasses-based diets was by Preston (1972). The report of Rush and Totusek (1976) suggested that live weight gain of livestock subsisting on low quality forage was improved with molasses. Yee Tong Wah et al (1981) studied the effect of different urea

levels in molasses on performance of cattle and showed that live weight gains were highest and feed conversion is best on the 2.5% urea level, which was therefore considered to be the optimum level. Kalmbacher et al (1995) measured the performance of mature cows fed creeping bluestem and supplements of either molasses-urea or molasses-cottonseed meal-urea, each containing 30% CP (DM basis). They concluded that the treatment did not affect cow weight loss on range or pregnancy rate but cows supplemented with molasses-urea tended to wean a heavier calf. A molasses-based supplement containing urea was of equal value to one containing a natural protein.

Lewis (1951) first demonstrated reduction of nitrate to ammonia, with nitrite as an intermediate, by rumen microorganisms. Accumulation of the toxic nitrite intermediate was believed due to the relatively rapid conversion of nitrate to nitrite and the slower conversion of nitrite to ammonia. Fewson and Nicholas (1961) have proposed the following scheme for nitrate reduction (Figure 3). The major end products, gaseous nitrogen or ammonia, vary with different microorganisms.

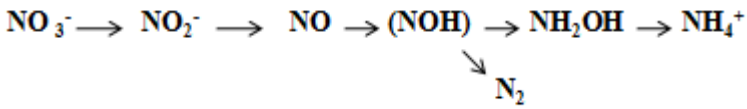



**Figure 4.** Scheme for nitrate reduction

Readily available carbohydrate, such as glucose, enhances the rate of nitrate reduction to ammonia (Barnett and Bowman 1957; Case 1957) and reduces toxicity of nitrate.

Allison and Reddy (1984) hypothesized that, with $NO_3$ as N source, there could be an increase in microbial growth efficiency which might reflect an increase in ATP yield through the functioning of issimilatory $NO_3$ reduction. Thauer et al (1977) reported that nitrate reduction to ammonium is more energetically favorable than $HCO_3^-$ reduction to $CH_4$. Thus, the ability of ruminal microorganisms to utilize nitrate as an electron acceptor suggested a potential approach to manipulation of fermentation end products. Satter and Esdale (1968) found acetate served as an electron acceptor and that, through the formation of butyrate, an electron sink was provided for the pair of hydrogen atoms released in lactate oxidation to pyruvate. Nitrate required eight electrons for reduction to ammonia, and could act as an electron sink in place of butyrate formation; acetate production would be increased also

butyrate production. Propionate or methane might also be decreased (Farra and Satter 1971). The potential of nitrate conversion to ammonia is to act as a hydrogen sink in the rumen (Leng 2008) but the major limitations to progress in using nitrate for animal have been the production of methaemoglobinaemia that results from nitrite generated in the rumen.

Leng (2008) indicated that nitrate could be used as a fermentable N source by ruminants provided the rumen ecosystem was allowed to adapt over a sufficiently long period and provided certain nutritional conditions were met. In particular the availability of sulphur appears to be a crucial issue. It was proved by the result of Sophea and Preston (2010) that goats showed no signs of ill-health, even at the highest levels of potassium nitrate (6% of DM basis). There are several experiments *in vitro* and *in vivo* that showed the potential role of nitrate for ruminants. The result of Guo et al (2009) on using nitrate in *in vitro* system showed that nitrate addition diminished methane production and increased microbial nitrogen synthesis compared with urea. Sangkhom et al (2011) concluded that lime can replace NaOH as a means of increasing the fermentability of rice straw and that methane production is decreased with potassium nitrate as NPN source.

In *in vivo* studies where nitrate has replaced urea as a fermentable N source in ruminant diets it has been well demonstrated that energy metabolism is not perturbed and the animals have consumed the same quantity of dry matter and have grown at the same rate (Nguyen Ngoc Anh et al 2010). In high yielding dairy cows fed a maize silage based diet, it was found by calorimetry that replacing 1.5% urea with 2.2% nitrate reduced methane production by 16% and that the effects, following adaptation to nitrate (over 4 weeks), were persistent over a 4 month period (Van Zijderveld et al 2010b).

## Requirement of sulphur by cattle

Rumen microorganisms and the host ruminant animal require many macro and micro minerals for normal growth and development. Among these minerals, sulphur is a necessary component of the amino acids cystine and methionine that are building blocks of proteins (Kung et al 1998), some vitamins and several important hormones (NRC 1984). A critical sulphur level in the rumen is 1 µg/ml according to Bray and Till (1975). Lower levels than this are likely to deplete the size of the microbial pool, eventually decreasing digestibility in addition to lowering the protein:energy (P: E) ratio in the nutrients absorbed (Leng 1990). In ruminants, many inorganic forms of sulphur (e.g. potassium sulphate and sodium sulphate)

and some common feeds and minerals that have moderate to high levels of sulphur (e.g. corn gluten meal, molasses) and can be used because sulphate is reduced in the rumen to hydrogen sulfite and hydrogen sulfide by a group of bacteria referred to as the sulphur reducing bacteria (Kung 2008). Some reports showed that sulphate-reducing bacteria (SRB) exist in the rumen and adaptive mechanism increased SRB activity. Cummings et al (1995) did not detect a change in numbers of ruminal sulphate-reducing bacteria as percentages of sulphur in the diet increased. However, after being exposed to high levels of sulphur, ruminal organisms did have a greater capacity to produce sulfide after 10 to 12 days. Oliveira et al (1996) reported that high dietary sulphur resulted in a faster rate of sulphate reduction by ruminal bacteria after several weeks on that diet. Van Zijderveld et al (2010a) showed that enhanced levels of sulphate in diet increased the number of SRB.

Hegarty et al (1994) reported improved dry matter digestion, increased total volatile fatty acids concentration, and more bacteria in the rumen of sheep fed a high versus a low sulphur diet (< 0.25%, dry matter basis). Moderately high percentages of sulphur (8.5g S/kg DM) in the diet have generally had no effects on ruminal volatile fatty acids and ammonia-nitrogen concentrations on sheep (Van Zijderveld et al 2010a). Especially, no clinical signs of polioencephalomalacia (PEM) were observed during this experiment. The effect of extremely high percentages of sulphur (1.3% sulphur in the diet) inhibited microbial protein synthesis in the rumen (Kahlon et al 1975). The explanation for inhibited synthesis is the imbalance of sulphur and nitrogen in diet. The NRC (1984) recommendations on dietary S allowances are based on a perceived optimal N:S ratio for ruminal microbial growth.

The inhibition of methanogenesis owing to the presence of sulphate has been reported for marine and freshwater sediments (Cappenberg et al 1974; Oremland and Taylor 1978) and also for anaerobic digesters (Kroiss and Wabnegg 1983). The inhibition of methanogenesis is interpreted in relation to the levels of sulfide produced by the microbial reduction of sulphate. Speece and Parkin (1983) found that methane production from an unacclimated batch digester was inhibited by a sulfide level as low as 50 mg of $S_2^-$ per liter (1.6 mM). However, with a submerged anaerobic filter, they noticed that sulfide levels up to 400 mg of $S_2^-$ per liter had no significant effect on methane production. At 800 mg of $S_2^-$ per liter methane production was reduced by about 30%. The sulfide produced by the microbial reduction of sulphate is distributed between $H_2S$, $HS^-$, and $S_2^-$ in solution and $H_2S$ in biogas (Isac et al 1986).

## Interaction of nitrate and sulphur in rumen metabolism

Nitrate may provide a competitive advantage for nitrate-reducing bacteria over sulphate-reducing bacteria during the competition for available electron donors. Londry and Suflita (1999) reported that nitrate amendment controlled the formation of sulphide in oily waste stream. Leng and Preston (2010) suggested that numerous rumen organisms are able to utilize energetically more favourable electron acceptors such as nitrate and sulphate, generating ammonia and hydrogen sulphide respectively. Nitrate-reducing bacteria (NRB) can be categorized according to whether they use organic (hNRB) or inorganic (NR-SOB) electron donors and whether nitrate reduction proceeds via denitrification or dissimilatory nitrate reduction. The NRB appear to effectively compete with SRB for degradable organic electron donors and thus potentially inhibit SRB metabolism (Leng 2008). Thus, in the electron donor competition between NRB and SRB in the anaerobic rumen, NRB always outcompete SRB. Qi et al (1994) reported that ruminal fluid ammonia N tended to decreased quadratically with sulphate supplementation while the bacterial N/S ratio tended to decreased linearly.

Studies with anaerobic ecosystems showed that in the presence of fermentable organic matter and a source of sulphur and nitrate, nitrate reducing organisms developed that can both reduce nitrate to ammonia and oxidize sulphide to sulphate (NRSOB) without release of nitrite. At least one prominent rumen organism has this capacity (*Wollinella succinogenese*) and may be termed an NRSOB (Leng and Preston 2010).

## The utilization of feed sources to improve ruminant production rates under tropical conditions

### Effect of dietary molasses on performance of ruminants

Molasses is a valuable by-product from the Sugar Industry. The molasses referred to in this article is blackstrap molasses that is the molasses from the production of raw sugar from sugar cane. The feeding value of molasses is based on the fact that it contains approximately 50% sugars in the form of sucrose and invert sugar but only 4% crude protein (Cleasby 1963). An important feature of sugar cane molasses is that it contains 3-7g S/kg DM (Suttle 2010).

Sugar is more rapidly fermented in the rumen than starch (Chamberlain et al 1993). Thus, molasses is considered a source of readily soluble carbonhydrate and a major source of dietary energy for ruminants. However, abnormal problem encountered with *ad libitum*

molasses feeding system was a high incidence of molasses toxicity in the feedlot program. Molasses toxicity appears to be precipitated by a low intake of forage in the high-molasses feeding system developed by Preston et al (1967a). Thus, to improve the efficiency of using molasses in the feeding system, the molasses-urea was fed with high protein forages (cassava and sweet potato leaves) (Ffoulkes and Preston 1978). With *ad libitum* feeding of molasses/urea mixtures, there is rarely any risk of urea toxicity since the sugars in molasses and ammonia from urea are quickly used in microbial cell growth (Preston 1986). Therefore carbonhydrate available for ruminal fermentation is the key factor for improving the efficiency of ruminal ammonia and the overall dietary nitrogen utilization in ruminants. Sirirat Buphan et al (2011) showed that dietary soluble protein (up to 60% of the crude protein) can be fed to crossbred Thai steers receiving sugar at a level of 12% of the DM had more positive effect on the intake, ruminal fermentation and nutrient digestibility compared with higher levels of soluble protein. Cuong et al (2010) concluded that molasses-based diets (48% on DM basis) with rice straw or pangola hay as a fiber source can support growth rates of finishing cattle better than with the traditional finishing diets.

**Utilization of cassava products by ruminants**

Cassava (*Manihot esculenta*, Crantz) is a crop of major importance in the tropics. It is grown mainly by smallholder farmers within existing farming systems primarily for the starchy root which is used for human food or as an energy source for non-ruminant or ruminant livestock feed (Khang et al 2005; Khampa et al 2006; Wanapat and Khampa 2007). Cassava can be grown to produce cassava foliage as a by-pass protein feed source (Wanapat 2003; Kiyothong and Wanapat 2004; Khang et al 2005). Wanapat (1995) reported that cassava leaves contain high level of crude protein (25%) some of which can apparently by-pass the rumen since it is in the form of a tannin-protein complex. Tannins, therefore, potentially alter the use and value of tree foliages and may at times be responsible for the poor utilization of such forages by ruminants. Tannins from *Leucaena leucocephala* afford a good level of protection of the protein (Wheeler et al 1995), but tannins from *Lotus pedunculatus* appear to overprotect protein from ryegrass fed to sheep (Waghorn and Shelton 1995) with subsequent increased fecal loss of protein. In general, animal responses to the utilization of cassava foliage as a protein roughage supplement is higher and better responses were obtained when cassava foliage was used as a supplement to molasses-urea based diets (Fernandez et al 1977, Ffoulkes and Preston 1978).

Moore (1976) first demonstrated the feed value of cassava foliage for ruminants in a trial in which steers weighing 250 kg were fed *Pennisetum purpureum* with varying levels of cassava foliage in the diet. Feed intake and growth rate were improved in diets containing cassava foliage supplements compared without cassava foliage in diet. Khang (1999) reported that cassava leaf meal at the rate of 18% of dry matter fed in the diet improved the intake, ruminal $NH_3$-N, ruminal microflora population and degradability of feeds.

Cassava foliage especially fresh cassava foliage contains cyanogenic glucosides, linamarin and lotaustralin. These are hydrolyzed by the endogenous enzyme linamarase to cyanohydrins after plant tissue damage. Further hydrolysis to HCN is responsible for chronic toxicity (Promkot et al 2011). Varieties of cassava are traditionally designated as "bitter" or "sweet" considered to reflect different levels of cyanogenic glucosides, the precursors of the highly toxic HCN (Chhay Ty et al 2007). Detoxification of cassava leaves may be partially accomplished by heating or boiling to inactivate linamarase and to drive off free hydrogen cyanide. The report of Chhay Ty et al (2007) showed that wilting cassava leaves for up to 48 h reduced HCN content to minimal values, the rate of decrease being more pronounced in the bitter than in the sweet variety. Sangkhom et al (2011a) showed that N solubility was decreased by ensiling and with severity of drying and methane produced per unit of fermented DM in an *in vitro* incubation system was inversely related to N solubility. They concluded that the higher level of cyanide present in fresh cassava leaves could have been responsible for the reduced methane production compated with over-dried leaves. Tham et al (2008) concluded that DM intake, live weight gain and feed conversion were improved with significant linear trends according to the level of supplementation with cassava leaf meal crude protein. Another factor that also contributes to reduce the toxicity of HCN in fresh cassava foliage is the presence of sulphur. Blakley and Coop (1949) found that the metabolism of HCN in rumen fluid was enhanced by the presence of sulphur donors which facilitated the detoxification of HCN by rumen microbes. Recently, Promkot et al (2011) suggested that the utilization of cassava foliage for rumen microorganisms in terms of fermentation and HCN detoxification could be improved by sulphur supplementation of 0.5% of DM.

## Conclusion

- There are interactions between nitrate and sulphate as electron acceptors for hydrogen generated by rumen fermentation of carbohydrates. It is not clearly defined under what

dietary conditions there will be a need to provide extra sulphate as well as nitrate in order to ensure maxium reduction in enteric methane.

## References


**Ali C S, Khaliq T, Javaid A, Sarwar M, Khan M A, Shazad M A and Zakir S 2007** *In vitro* utilization of NPN sources by increasing levels of corn starch in straw based diets. Pakistan Vet. J.,27(2): 95-101.

**Allison M J and Reddy C A 1984** Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate. In: Proceedings of the Third International Symposium on Microbial Ecology (Ed. M. J. Klug and C. A. Reddy). American Society for Microbiology. Washington, DC. pp. 248-256.

**Baker S K 1997** Gut microbiology and its consequences for the ruminant. Proc. Nutr. Soc. Aust. 21: 6–13.

**Blakley R L and Coop I E 1949** The metabolism and toxicity of cyanides and cyanogenetic glucosides in sheep. II.Detoxication of hydrocyanic acid. New Zealand J. Sci. Thechnol. 31A(3):1-16.

**Barnett A J G and Bowman I B R 1957** *In vitro* studies on the reduction of nitrate by rumen liquor J. Sei. Food Agr.,8 : 243.

**Bergman E N 1990** Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev April 1, 1990 70:567-590.

**Case A A 1957** Some aspects of nitrate intoxication in livestock. J. Amer. Vet. Med. Ass., 130: 323.

**Chamberlain D G, Robertson S, and Choung J J 1993**. Sugars versus starch as supplements to grass silage: Effects of ruminal fermentation and the supply of microbial protein to the small intestine, estimated from the urinary excretion of purine derivatives, in sheep. J. Sci. Food Agric. 63:189–194.

**Chhay Ty, Preston T R and Borin K 2007:** Effect of variety and wilting on HCN content of cassava leaves and on intake, digestibility and N retention by growing pigs.*Livestock Research for Rural Development. Volume 19, Article #135.* RetrievedJanuary 27, 2012, from http://www.lrrd.org/lrrd19/9/chha19135.htm

**Cummings B A, Gould D H, Caldwell D R, and Hamar D W 1995a** Ruminal microbial alterations associated with sulfide generation in steers with dietary sulphate-induced polioencephalomalacia. Am. J. Vet. Res. 56:1390-1395.

**Cuong V C, Tuyen D V, McCrabb G J and Verstegen M W A 2010** Performance of Laisind cattle in Vietnam as affected by a sugar cane molasses diet. J. Sci. Dev. 2010, 8 (Eng.Iss.1): 76 - 84

**Czerkawski J W 1969.** Methane production in ruminants and its significance. World Review of Ruminants and Dietetics 11:240-282.

**Czerkawski J W and Breckenridge G1969** Fermentation of various soluble carbohydrates by rumen micro-organisms with particular reference to methane production. Br. J. Nutr. 23: 925–937.

**Devandra C and Leng R A 2011.** Feed Resources for Animals in Asia: Issues, Strategies for Use, Intensification and Integration for Increased Productivity. Asian-Aust. J. Anim. Sci.Vol. 24, No. 3 : 303 – 321

**FAO 2006** *World agriculture: towards 2030/2050.* Interim report, Global Perspective Studies Unit. Rome, Italy: Food and Agriculture Organization of the United Nations.

**Farra P A and Satter L D 1971** Manipulation of the Ruminal Fermentation. III. Effect of Nitrate on Ruminal Volatile Fatty Acid Production and Milk Composition. Journal of daily science Vol.54, No.7

**Fernandez A, MacLeod N A, and Preston T R 1977**. Cassava forage as a combined source of protein and roughage for cattle fed on molasses/urea. Tropical Animal Production 2:195-199.

**Fewson C A and Nicholas D J D 1961**. Utilization of nitrate by microorganisms. Nature, 190: 2.

**Ffoulke S D and Preston T R 1978**. Cassava and sweet potato forage as combined sources of protein and roughage in molasses based diets: effect of supplementation with soybean meal. Tropical Animal Production 3:186-192.

**Guo W S, Schaefer D M, Guo X X, Ren L P, Meng Q X 2009** Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis in vitro. *Asian-Australian Journal of Animal Science* **22**, 542-549

**Harfoot C G and Hazlewood G P 1997** Lipid metabolism in the rumen. Pages 382-426 In: The Rumen Microbial Ecosystem, Second Edition, Eds: P.N. Hobson and C.S. Stewart. Blackie Academic and rofessional, London.

**Hegarty R S, Nolan J V and Leng R A 1994**. The effects of protozoa and of supplementation with nitrogen and sulfur on digestion and microbial metabolism in the rumen of sheep. Aust. J. Agric. Res. 54:1215-1227.

**Hindrichsen I K, Wettstein H R, Machmüller A, Soliva C R, Bach Knudsen K E, Madsen J and Kreuzer M 2004** Effect of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84: 265–276

**Houpt T R 1959**. Utilization of blood urea in ruminants. Am. J. Physiol. 197:115.

**Hobson N P and Stewart C S 1997**. Rumem microbial ecosystem. Second edition. http://books.google.com.vn/books?id=--OAI30q6NgC&pg=PA524&lpg=PA524&dq=The+ATP+from+VFA,+methane,+rumen+fermentation&source=bl&ots=o9_ZAAZEiz&sig=I72uiVYhX2pRYZtljv8Ti-lEfH0&hl=en&sa

**Hungate R E 1966**. The rumen and its microbes. Academic Press, London, UK

**Iv Sophea and Preston T R 2010** Effect of different levels of supplementary potassium nitrate replacing urea on growth rates and methane production in goats fed rice straw, mimosa foliage and water spinach.International Conference on Livestock, Climate Change and Resource Depletion, (Editor: Reg Preston), Champasack University, Pakse, LAO PDR, 9-11 November 2010 .htm://www.mekarn.org/workshops/pakse/html/soph.htm

**Johnson K A and Johson D E1995.** Methane emissions from cattle. J. Anim. Sci. 73:2483-2492

**Kalmbacher R S, Brown W F and Pate F M 1995** Effect of molasses-based liquid supplements on digestibility of creeping bluestem and performance of mature cows on winter range. J Anim Sci. 1995 Mar;73(3):853-60

**Khampa S, Wanapat M, Wachirapakorn C, Nontaso N and Wattiaux M 2006** Effects of Urea Level and Sodium DL-malate in Concentrate Containing High Cassava Chip on Ruminal Fermentation Efficiency, Microbial Protein Synthesis in Lactating Dairy Cows Raised under Tropical Condition. Asian-Aust. J. Anim. Sci. 19:837-844.

**Kahlon T S, Meiske J C and Goodrich R D 1975** Sulphur metabolism in ruminants.I. In vitro availability of various chemical forms of sulphur. J. Anim. Sci. 41:1147-1153.

**Khang D N 1999** Effects of cassava leaf meal on rumen environment and as a replacement for cotton seed meal in napier grass for dairy cows. MSc thesis in the programme "Tropical Livestock Systems". SLU, Dept of Animal nutrition and management, Uppsala, Sweden.

**Khang D N, Wiktorsson H and Preston T R 2005** Yield and chemical composition of cassava foliage and tuber yield as influenced by harvesting height and cutting interval. AsianAust. J. Anim. Sci. 18:1029-1035

**Kiyothong K and Wanapat M 2004** Supplementation of cassava hay and stylo 184 hay to replace concentrate for lactating dairy cows. Asian-Aust. J. Anim. Sci. 17(5):670-677.

**Kiyothong K and Wanapat M 2004**. Growth, hay yield and chemical composition of cassava and stylo 184 grown under intercropping. Asian-Aust. J. Anim. Sci. 17:799-807.

**Kroiss H and Wabnegg F P 1983** Sulfide toxicity with anaerobic wastewater treatment, p. 72-85. In W. J. van der Brink (ed.), Proceedings of the European Symposium on Anaerobic Wastewater Treatment (AWWT). AWWT Symposium Secretariat, TNO Corporate Communication Department, The Hague, Netherlands.

**Kung Jr L, Bracht J P, Hession A O, and Travares J Y 1998** High sulphate induced poilioencephalomalacia (PEM) in cattle – burping can be dangerous if you are a ruminant. Proc. Chr.Hansen's Technical Symposium. Pacific Northwest Nutriotion Conference. Vancouver, BC, Canada. http://ag.udel.edu/anfs/faculty/kung/H2S.htm. Accessed July 23, 2007.

**Kung Jr L 2008**. Metabolism of sulphur in the rumen.Proc. Sulphur in Ruminant Diets Symposium.Midwestern Section Animal Science Meeting. Des Moines, Ia. http://ars.sdstate.edu/extbeef. Accessed March 30, 2008.

**Leng R A and Preston T R 1976** Sugarcane for cattle production: present constraints, perspectives and research priorities. Tropical Animal Production 1: 1 22

**Leng R A 1990** Factors affecting the utilization of 'poor quality' forages by ruminants particularly under tropical conditions. *Nutrition Research Reviews* **3,** 277-303.

**Leng R A 1993** Quantitative ruminant nutrition - A green science. Australian Journal of Agricultural Research 44: 363-80.

**Leng R A 2008** The potential of feeding nitrate to reduce enteric methane production in ruminants. Report to Department of Climate Change, Commonwealth Government, Canberra. 82 pp.

**Leng R A and Preston T R 2010** Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. *Livestock Research for Rural Development. Volume 22, Article #221* . Retrieved , from http://www.lrrd.org/lrrd22/12/leng22221.htm

**Lewis D 1951**. The metabolism of nitrate and nitrite in the sheep. I. The reduction of nitrate in the rumen of the sheep. Biochem.*J., 48: 175*

**Mathison G W 1997** Effect of canola oil on methane production in steers. Can. J. Anim. Sci. **77**: 545–546 (Abstr.).

**McCaughey W P, Wittenberg K and Corrigan D 1997** Methane production by steers on pasture. Can. J. Anim. Sci. **77**:519–524.

**Mercer J R and Annison E F 1976** Utilization of nitrogen in ruminants. In: Protein Metabolism and Nutrition. D. J. A. Cole, K. N. Boorman, P. J.Buttery, D. Lewis, R. J. Neale and H. Swan (eds). Butterworths, London, UK.

**Moe P W and Tyrrell H F 1979** Methane production in dairy cows. J. Dairy Sci. 62: 1583–1586

**Moss A R, Jouany J P and Newbold J 2000** Methane production by ruminants: its contribution to global warming. Ann. Zootech. **49**: 231–253.

**Moore C P 1976**. The utilization of cassava forage in ruminant feeding. International Seminar on Tropical Livestock Products, 8-12 March, Acapulco, Mexico. 21p.

**Nolan J V 1998** Stoichiometry of rumen fermentation and gas production. Meeting the Kyoto target. Implications for the Australian Livestock Industries. Workshop proceedings
Canberra, 4–5 November 1998

**NRC 1984** Nutrient Requirements of Beef Cattle (6th Rev. Ed.). National Academy of Press, Washington, DC.

**Oliveira d L A, Jean-Balin C, Corso V D, Benard V, Durix A, and Komisarczuk-Bony S 1996** Effect of high sulphur diet on rumen microbial activity and rumen thiamine status in sheep receiving a semi-synthetic, thiamine-free diet. Reprod. Nutr. Dev. 36:31-42

**Olbrich S E and Wayman O 1972**. Effect of feeding raw sugar on growth performance and rumen fluid parameters of fattening beef cattle. J. Anim. Sci. 34: 820-825.

**Oremland R S and Taylor B F 1978**. Sulfate reduction and methanogenesis in marine sediments. Geochim. Cosmochim. Acta 42:209-214.

**Pearson R M and Smith J A B 1943**. The utilization of urea in the bivine rumen. 2. The conversasion of urea to ammonia. Biochem. J.37:148.

**Perdok H B and Leng R A 1990**. Effect of supplementation with protein meal on the growth of cattle given a basal diet of untreated or ammoniated rice straw. Asian-Australasian J. of Agric. Sci. 3**:**269-279.

**Pollock C J 2008.** Impacts on livestock agriculture of competition for resources. Pp:16-18. In: (Eds) P Rowlinson, M Steele and A Nefzaoui, Livestock and Global Climate Change, Proceedings of International Conference May 2008, Hammamet, Tunisia Cambridge University Press, British Society of Animal Science, ISBN 978-0-906562-62-8.

**Preston T R, Elias A, Willis W B and Sutherland T M 1967a** Intensive beef production from molasses and urea. Nature 216: 721-722

**Preston T R, Willis M B and Elias A 1967**. Intensive beef production from sugar cane. 1. Different levels of urea in molasses given ad libitum to fattening bulls as a supplement to a grain diet. Cuban Journal of Agricultural Science 1, 33-37.

**Preston T R 1972**. Molasses as an energy source for cattle World Review of Nutrition and Dietetics 17:250-311

**Preston T R 1986.** Molasses as animal feed: an overview. http://www.fao.org/docrep/003/s8850e/S8850E19.htm

**reston T R 2001** Potential of cassava in integrated faming systems. In: Proceedings of International Workshop on Current Research and Development on Use of Cassava as Animal feed. (Editors: T R Preston, B Ogle and M Wanapat), organized by Khon Kaen University and SIDA-SAREC, Sweden. July 23-24, 2001 http://www.mekarn.org/procKK/pres.htm

**Promkot C, Wanapat M , Wachirapakorn C and Navanukraw C 2011.** Influence of Sulphur on Fresh Cassava Foliage and Cassava Hay Incubated in Rumen Fluid of Beef Cattle. Asian-Aust. J. Anim. Sci. Vol. 20, No. 9 : 1424 – 1432.

**Qi K, Lu C D, Owens F N, Lupton C J 1994** Effects o f sulfate supplementation on performance, acid -base balance, and nutrient metabolism in Angora kids. Small Ruminant Research 15 (1994) 19-29

**Roffler R E, Schwab C G, Satter L D 1975**. Relationship Between Ruminal Ammonia and Nonprotein Nitrogen Utilization by Ruminants. III. Influence of Intraruminal Urea Infusion on Ruminal Ammonia Concentration. Journal of Dairy Science Volume 59, Issue 1, January 1976, Pages 80-84

**Rush I G, and Totusek R 1976**. Supplemental value of feed grade buiret and urea-molasses for cows an dry winter grass. J. Animal Sci. 42: 497-505.

**Satter L D and Esdale W J 1968**. *In vitro* lactate metabolism by ruminul ingests. Appl. Microbiol., 16: 680.

**Sangkhom Inthapanya, Preston T R and Duong Nguyen Khang T R 2011** Mitigating methane production from ruminants; Effect of nitrogen solubility in cassava leaves as source of protein in an *in vitro* incubation using cassava root as source of carbohydrate. Workshop on Reducing Greenhouse Gas Emissions from Livestock and Soils (Editors: Reg Preston and Sisomphone Southavong). National Institute of Animal Science, Hanoi, 14-15 November 2011. http://www.mekarn.org/workshops/GHG-CC/sang2.htm

**Sangkhom I, Khang D N, Leng R A and Preston T R 2011:** Effect of potassium nitrate as modifier of the fermentation in an in vitro incubation using as substrate NaOH and/or lime treated straw supplemented with fresh cassava leaves. Livestock Research for Rural Development. Volume 23, Article #204. http://www.lrrd.org/lrrd23/10/sang23204.htm

**Sirirat Buphan, Virote Pattarajinda, Mark A Froetshel, Monchai Duangjinda and Yanin Opatpattanakit 2011**. Effect of soluble protein and sugar concentration on ruminal fermentation and nutrient digestibility in crossbred steer. Journal of Animal and Veterinary Advances 10(13): 1724-1730.

**Speece R E and ParkinG F 1983**. The response of methane bacteria to toxicity, p. 23-35. In R. L. Wentworth, D. A. Stafford, B. I. Wheatley, W. E. Edelmann, G. Lettinga, Y. Minoda, P. Mulas del Pozo, E. J. Nyns, F. G. Pohland, J. F. Rees, L. van den Berg, W. Verstraete, and R. F. Ward (ed.), Proceedings of the 3rd International Symposium on Anaerobic Digestion. Evans and Faulkner, Inc., Watertown, Mass.

**Steinfeld H, Gerber P, Wassenaar T, Castel V,Rosales M, de Haan C 2006** Livestock's long shadow: environmental issues and options. Rome, Italy: FAO.

**Suttle F 2010**. Mineral nutrition of livestock ,4th edition. http://books.google.com/books?id=RwlJJbjATXEC&pg=PA207&dq=molasses+contain+sulphur&hl=vi&ei=3dNETоSvG6XomAWg9OnkBg&sa=X&oi=book_result&ct=result&resnum=3&ved=0CDQQ6

**Thauer R K, Jungermann K and Decker K 1977**. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100-180.

**Tamminga S, Bannink A, Dijkstra J, Zom R 2007.** Feeding strategies to reduce methane loss in cattle. Report 34. Animal science group**.** http://www.asg.wur.nl/NR/rdonlyres/F81D8745-6596-4296-A292-8553950E2B98/42707/34.pdf

**Tham H T, Man N V and Preston T R 2008**. Performance of young cattle fed rice straw sprayed with mixture of urea and molasses supplemented with different levels of cassava leaf meal. Livestock Research for Rural Development. Volume 20, supplement. http://www.lrrd.org/lrrd20/supplement/tham1.htm

**Thornton Philip K 2010**. Livestock production: recent trends, future prospects. Phil. Trans. R. Soc. B 2010 365, 2853-2867; doi: 10.1098/rstb.2010.0134

**Tillman A D and Kirpal S Sidhu 1969**. Production and Nitrogen Utilization by Ruminants A Review Nitrogen Metabolism in Ruminants: Rate of Ruminal Ammonia. J. Anim. Sci. 1969, 28:689-697.

**U.S. EPA 2009** US Greenhouse Gas Inventory Report. United States Enviromental Protection Agency. April 2009. Accessed June 23, 2009. http://epa.gov/climatechange/emissions/usinventoryreport.html.

**Van Zijderveld S M, Gerrits W.J.J. Apajalahti J.A, Newbold J.R,Dijkstra, J, Leng R.A. and Perdok H.B. 2010a.** Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J Dairy Sci. 2010 Dec;93(12):5856-66

**Van Zijderveld S M, Dijkstra J, Gerrits W J J, Newbold J R, Perdok H B 2010b** Dietary nitrate persistently reduces enteric methane production in lactating dairy cows In Greenhouse gases and animal agriculture conference October 3-8, 2010 Banff, Canada ( Quoted by Hulshof et al 2010)

**Waghorn G C and Shelton I D 1995** Effect of condensed tannins in *Lotus pedunculatus* on the nutritive value of rye grass *Lolium perenne* fed to sheep. Jounal of Agricultural Science. 125: 291-297.

**Wanapat M 2003**. Manipulation of Cassava Cultivation and Utilization to Improve Protein to Energy Biomass for Livestock Feeding in the Tropics. Asian-Aust. J. Anim. Sci. 16:463-472.

**Wanapat M 1995** The use of local feed resources for livestock production in Thailand. Proceedings of the International Conference on Increasing Animal Production with Local Resources (Editor: Guo Tingshuang). China Forestry Publishing House, Ministry of Agriculture, China.

**Wanapat M and Khampa S 2007**. Effect of levels of supplementation of concentrate containing high levels of cassava chip on rumen ecology, microbial n supply and digestibility of nutrients in beef cattle. Asian-Aust. J. Anim. Sci. 20:75-81.

**Wheeler R A, Norton B W and Shelton H M 1995**. Condensed tannins in Leucaena species and hybrids and implications for nutritive value. In: Leucaena - Opportunities and limitations, Proceedings of a workshop held in Bogor, Indonesia, 24-29th January, 1994, pp.112-118 (H.M. Shelton, C.M. Piggin and J.L. Brewbaker, editors) Canberra: ICIAR.

**Wythes J R, Wainwright D H and Blight G W 1978.** Nutrient composition of Queensland molasses. *Australian Journal of Agriculture and Animal Husbandry*. 18:629-634.

**Yasuo Kobayashi** 2010. Abatement of Methane Production from Ruminants: Trends in the Manipulation of Rumen Fermentation. Asian-Aust. J. Anim. Sci. Vol. 23, No. 3 : 410 – 416

**Yee Tong Wah R L, Rulman B and Preston T R 1981.** Effect of urea level on performance of cattle on a molasses/urea and restricted forage feeding system. Trop Anim Prod 1981 6:1

**Zaid Isa, Stephane grusenmeyer, Willy Verstraete 1986**. Sulfate Reduction Relative to Methane Production in High-Rate Anaerobic Digestion: Technical Aspects. Applied and environmental microbiology, Mar. 1986, p. 572-579. Vol. 51, No. 3

# Mitigating methane production from ruminants; effect of supplementary sulphate and nitrate on methane production in an *in vitro* incubation using sugar cane stalk and cassava leaf meal as substrate

**Le Thuy Binh Phuong, T R Preston* and R A Leng****

*Nong Lam University, Viet Nam*

*binhphuongty27@yahoo.com*

** Finca Ecologica, TOSOLY, UTA (Colombia)*

*AA#48, Socorro, Santander, Colombia*

*** University of New England, Armidale NSW, Australia*

## Abstract

The aim of this study was to evaluate the effect of replacing urea by calcium nitrate with or without supplementary sulphur (0, 0.4, 0.8% on DM basis) on methane production in an *in vitro* incubation medium inoculated with rumen fluid and using sugar cane stalk and cassava leaf meal as substrate. The design was a 3*2 factorial arrangement of the treatments with 4 replications.

Compared with urea, calcium nitrate reduced methane production. The effect was consistent over successive periods in the 48h incubation. Adding 0.4% sulphur, in the form of sodium sulphate, increased methane production, while 0.8% sulphur reduced methane production. When 0.8% sulphur was combined with nitrate the effects on methane reduction were additive. Methane production increased linearly with the length of the incubation on all treatments

***Key words**: Calcium nitrate, climate change, fermentation, gas production, greenhouse gases, urea, substrate*

## Introduction

Emissions of greenhouse gases (GHG) from anthropogenic sources (man made) are considered to be the main cause of global warming (IPCC 2007). Livestock production is a major contributor to GHG levels, accounting for about 18% of the anthropogenic GHG emissions (Steinfield et al 2006). Ruminants produce globally about 80 million tonnes of

methane gas annually which represents some 28% of anthropogenic methane emissions (Leng 2008).

Methane is produced in the fore-stomach of ruminants as a result of microbial fermentation of feed components. In the process of digestion of organic matter reduced cofactors are generated and a requirement for continuous fermentation is that theses cofactors are re-oxidised. The oxidation of theses reduced cofactors is coupled to high affinity electron acceptors mainly sulphur and nitrate. However if these are in low concentrations or absent, which is the case under normal feeding conditions, carbon dioxide is reduced to methane.

Recently, Leng (2008) has emphasised that nitrate as a feed component replacing urea has a dual role as an electronic sink for hydrogen produced by fermentation, and the ammonia produced is the preferred source of fermentable nitrogen in diets having a low content of crude protein. The dietary conditions which favour utilization of nitrate to lower the production of methane are a source of easily fermentable carbohydrate, a low content of soluble protein, an adequate level of sulphur and a source of bypass protein (Leng 2008). It is thus logical to test whether nitrate can be used to lower enteric methane production and at the same time maintain or promote microbial growth in the rumen. Sugar cane contains 50% of sugars on DM basis and almost no protein. A diet of sugar cane with 3% urea (in DM) and with rice polishing as a bypass protein source has been shown to support growth rates of 800 g/day in fattening cattle (Preston et al 1976). Leng and Preston (2010) have emphasized that the reduction of both nitrate and nitrite could be stimulated by a source of ruminal sulphide produced locally. As most organisms that reduce sulphate are also capable of reducing nitrate or nitrite, the feeding of nitrate and sulphate is likely to have important interactions. Recently, Van Zijderveld et al (2010) demonstrated an added effect on lowering of methane production by feeding a combination of nitrates and sulphate to sheep.

**Hypothesis**

Supplementary sulphate will have a synergistic effect in reducing methane production in a basal diet of sugar cane in which the source of non-protein nitrogen (NPN) is nitrate rather than urea.

**Objectives**

To study the effect of sulphate and nitrate on methane production in an *in vitro* system inoculated with rumen fluid using sugar cane as the basal substrate and cassava leaf meal as the source of protein.

## Materials and methods

### Location and duration

The experiment was conducted in the laboratory of An Giang University from September to November, 2010.

### Experimental design

Three levels of sulphur and two sources of NPN were compared in an *in vitro* fermentation system. The design was a 3*2 factorial arrangement with 4 replications. The levels of sulphur (as sodium sulphate) were 0, 0.4 and 0.8% (in DM); the NPN sources were urea (2% in DM) and calcium nitrate (3.8% in DM).

The individual treatments were:

- CaN: Calcium nitrate
- U: Urea
- CaN-0.4S: CaN + 0.4% sulphur as sodium sulphate
- U-0.4S: Urea + 0.4% sulphur as sodium sulphate
- CaN-0.8S: CaN + 0.8% sulphur as sodium sulphate
- U-0.8S: Urea + 0.8% sulphur as sodium sulphate

### Material preparation and implementation of the method

Fresh sugar cane stalk was purchased in the market. The outer rind was removed with a knife and the central part (the sugar-rich pith) chopped into small pieces (about 2mm section). Cassava leaves were sun-dried and ground through a 4 mm sieve. These two components (cassava leaf meal and sugar cane stalk) were mixed with the sources of N (urea or nitrate) and sulphate (according to the proportions shown in Table 1). Representative samples of the mixtures (12g DM) were put in the incubation bottle to which were added 960ml of buffer solution (Table 2) and 240ml of rumen fluid obtained from a buffalo, prior to filling each bottle with carbon dioxide. The rumen fluid was taken at 10-11pm in the slaughter-house from a buffalo immediately after the animal was killed. A representative sample of the rumen contents (including feed residues) was put in a vacuum flask and stored until 8-9am the

following morning when the contents were filtered through one layer of cloth before being added to the incubation bottle. The gas space inside the bottle was then flushed with carbon dioxide prior to the incubation at 38°C for 48h. A simple *in vitro* system was used (Photo 1) based on the procedure reported by Sangkhom et al (2011).

**Table 1**.Ingredients in the substrate (g DM)

| | **CaN0.4S** | **U0.4S** | **CaN0.8S** | **U0.8S** | **CaN** | **U** |
|---|---|---|---|---|---|---|
| Sugar cane stalk | 8.13 | 8.55 | 7.92 | 8.33 | 8.34 | 8.76 |
| Cassava leaf meal | 3 | 3 | 3 | 3 | 3 | 3 |
| Urea | | 0.24 | | 0.24 | | 0.24 |
| $Ca(NO_3)_2.4H_2O$ | 0.66 | | 0.66 | | 0.66 | |
| $Na_2SO_4$ | 0.21 | 0.21 | 0.42 | 0.42 | | |

**Table 2.** Ingredients of the buffer solution (adapted from Tilly and Terry 1964)

| Ingredients | **$CaCl_2$** | **$NaHPO_4.12H_2O$** | **NaCl** | **KCl** | **$MgSO_4.7H_2O$** | **$NaHCO_3$** | **Cysteine** |
|---|---|---|---|---|---|---|---|
| (g/liter) | 0.04 | 9.30 | 0.47 | 0.57 | 0.12 | 9.80 | 0.25 |

**Photo 1**. The *in vitro* incubation system

## Measurements

The gas volume was measured at 8, 24, and 48h by water displacement from the receiving bottle suspended in water which was calibrated at intervals of 50ml. On each occasion, after measuring the volume, the gas was ejected from the receiving bottle though a tube attached to a Crowcom meter (Crowcom Instruments Ltd, UK) fitted with an infra-red sensor to measure methane (Photo 2). Undigested substrate at the end of the incubation after 48h was filtered by cloth and was then dried at $105^0$C for 24h to determine residual DM. The DM and crude protein contents of the substrates were determined according to AOAC (1990) methods.

**Photo 2.** $CH_4$ measurement with Crowcom meter

**Statistic analysis**

The data were analyzed by the General Linear Model (GLM) option in the ANOVA program of the Minitab Software (version13.2) (Minitab 2000). Sources of variation in the model were: levels of sulphur, NPN source, interaction sulphur*NPN source and error

## Results and Discussion

At each stage of the fermentation and overall, gas production, per cent of methane in the gas, was less when nitrate rather than when urea, was the source of NPN (Table 3). Increasing the level of sulphur reduced gas production but the effect on methane production was variable. Methane production increased with sulphur level in samples taken after 8 and 24h. Only the last samples (25-48h) and overall values showed reduction in methane with level of sulphur. There was an interaction between NPN source and level of sulphur, such that when nitrate was the supplementary source of N, the relative reduction in methane with added 0.8% sulphur was greater than when 0.8% sulphur was given with urea as the fermentable N source, indicating suppression of sulphur-reducing bacteria by nitrate.

The substrate DM fermented in 48h incubation was less with nitrate than with urea; and was reduced with added sulphur.

Methane production per unit substrate fermented was lowered by some 30% when nitrate was the NPN source but there was no difference due to added sulphur. On all levels of added sulphur, the nitrate consistently reduced methane production (Figure 1), with the greatest reduction being observed when 0.8% sulphur was added to the substrate.

**Table 3**. Mean value for gas production, methane percentage in the gas, substrate fermented and methane production per substrate fermented

| | **NPN source** | | | | **% added sulphur** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CaN | Urea | SEM | P | 0S | 0.4S | 0.8S | SEM | P | **P(S*NPN)** |
| **Gas production, ml** | | | | | | | | | | |
| 0-8h | 770 | 817 | 23.6 | 0.18 | 875 | 700 | 806 | 28.9 | 0.001 | 0.02 |
| 9h-24h | 552 | 817 | 34.3 | 0.001 | 925 | 575 | 553 | 34.3 | 0.001 | 0.63 |
| 25-48h | 321 | 363 | 28.5 | 0.31 | 450 | 344 | 231 | 34.9 | 0.001 | 0.22 |
| Total | 1644 | 1996 | 54.2 | 0.001 | 2250 | 1619 | 1590 | 66.4 | 0.001 | 0.09 |
| **Methane, %** | | | | | | | | | | |
| 0-8h | 14.2 | 17.7 | 0.43 | 0.001 | 14.4 | 17.3 | 16.1 | 0.52 | 0.001 | 0.49 |
| 9h-24h | 19.8 | 28.6 | 0.55 | 0.001 | 19.6 | 26.8 | 26.1 | 0.68 | 0.001 | 0.03 |
| 25-48h | 25.6 | 39.6 | 1.92 | 0.001 | 37.4 | 33.9 | 26.5 | 2.36 | 0.01 | 0.001 |
| Total methane, ml | 313 | 513 | 11.7 | 0.001 | 474 | 397 | 367 | 14.3 | 0.001 | 0.012 |
| **Methane, ml/g substrate** | | | | | | | | | | |
| 0-8h | 8.96 | 12.0 | 0.36 | 0.001 | 10.4 | 10.1 | 10.9 | 0.44 | 0.47 | 0.09 |
| 9h-24h | 8.80 | 18.8 | 0.56 | 0.001 | 15.2 | 13.3 | 12.9 | 0.69 | 0.063 | 0.12 |
| 25-48h | 8.28 | 11.9 | 0.68 | 0.001 | 13.9 | 9.57 | 6.83 | 0.83 | 0.001 | 0.11 |
| 0-48h | 26.0 | 42.7 | 0.97 | 0.001 | 39.5 | 33.0 | 30.6 | 1.20 | 0.001 | 0.072 |
| **DM solubilized after 48h, %** | 44.0 | 49.4 | 1.43 | 0.02 | 55.8 | 41.4 | 42.90 | 1.75 | 0.001 | 0.245 |
| **Methane, ml/g DM solubilized** | 60.8 | 89.7 | 3.71 | 0.001 | 72.6 | 81 | 72.2 | 4.53 | 0.33 | 0.151 |

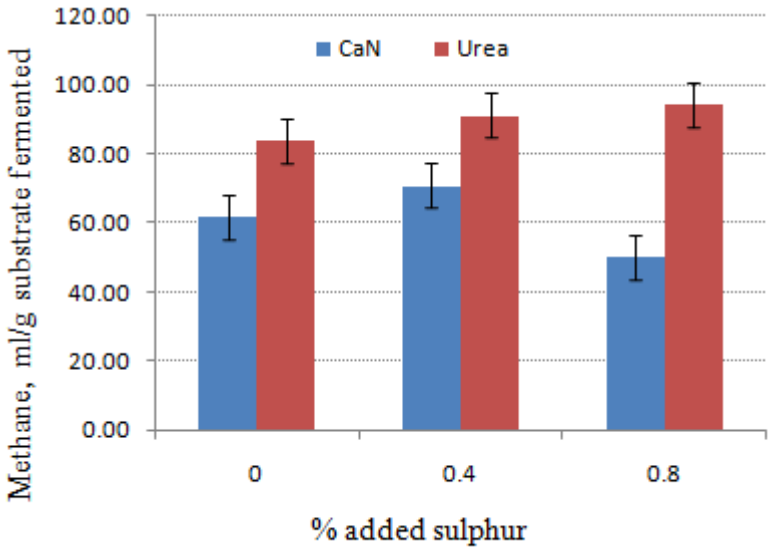



**Figure 1.** Effect on methane production of replacing urea by calcium nitrate as NPN source in an *in vitro* fermentation of sugar cane stalk and cassava leaf meal with different levels of added sulphur.

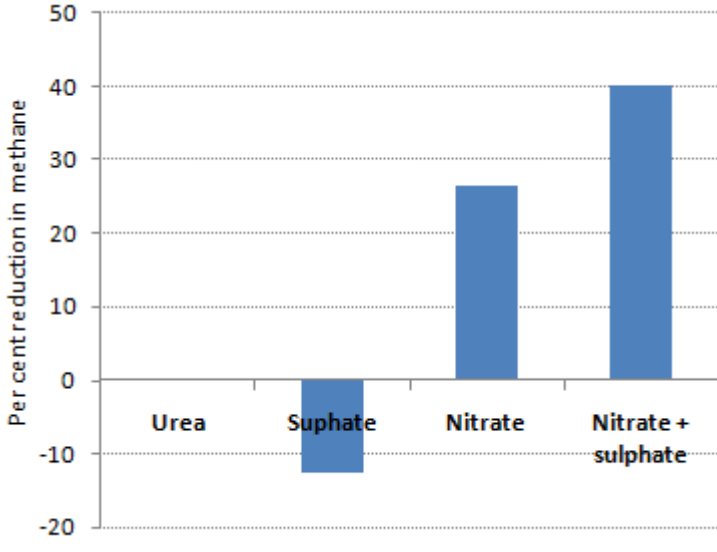



**Figure 2**. Reduction in methane due to addition of 0.8% sulphur, 3.8 calcium nitrate or 3.8% nitrate plus 0.8% sulphur.

The interaction between nitrate and sulphur is apparent in the incubations when methane production rate was compared on the treatments with zero and 0.8% sulphur with urea or nitrate as NPN source. Sulphur alone appeared to increase methane production whereas in combination with nitrate the lowering in methane production was greater than on nitrate alone (Figure 2). The effects with 0.8% sulphur are similar to those reported by Van Zijderveld et al (2010) and Silivong et al (2011) when 0.8% sulphur and nitrate had additive effects in lowering methane production. A low level of sulphur appears to stimulate fermentation rate as it actually increased methane output indicating that the incubation medium on the 0% sulphur treatment was actually deficient in S. At higher sulphate additions undoubtedly the extra S was reduced through the production of hydrogen sulphide. As the electron acceptors in the medium are used up there will be a swing with time from nitrate conversion to ammonia to sulphate reduction to hydrogen sulphide and when sulphate is exhausted it appears that methane will be generated from reduction of carbon dioxide. These are governed by, and in accord with, the Gibbs free energy change of the reactions.

The curvilinear increase in methane production with duration of incubation (Figures 3 and 4), indicative of the transition to a secondary fermentation of the VFA to methane, supports the findings of Sangkhom et al (2011), who used cassava leaves or mimosa foliage as supplements to cassava root meal, and Outhen et al (2011) who employed fresh or dried cassava leaves with sugar cane stalk, as substrate in a similar *in vitro* system.

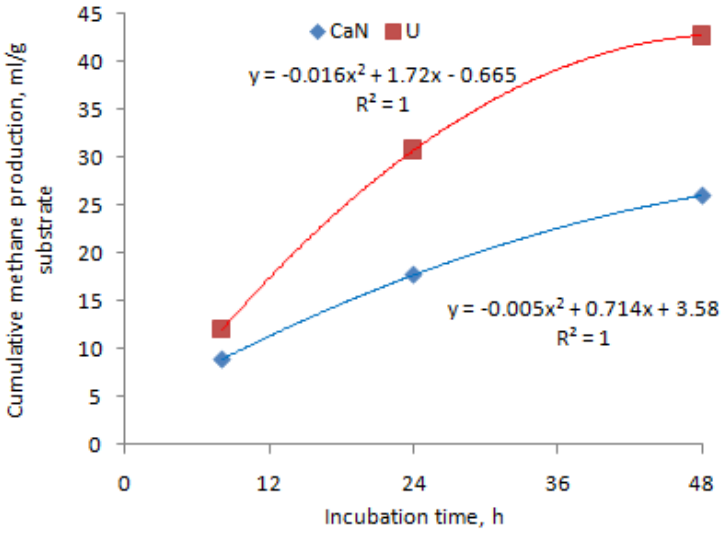



**Figure 3**. Effect of nitrate vs urea on methane production at intervals during the incubation..

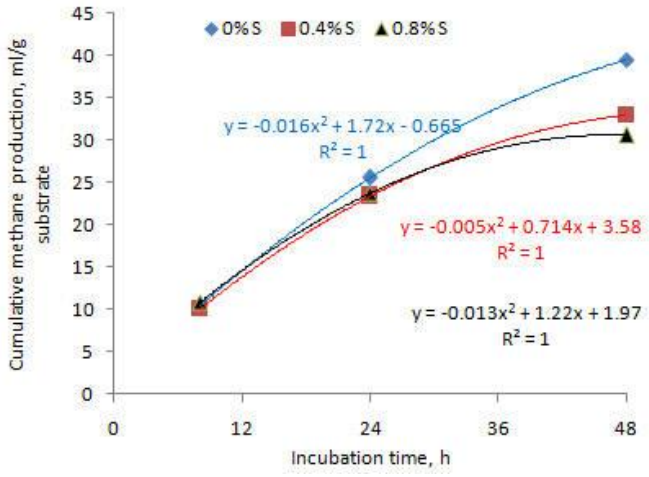



**Figure 4.** Effect of level of added sulphur on methane production at intervals during the incubation

The lowering of methane production when nitrate replaced urea is in accord with its high affinity for electron capture but following the depletion of nitrate would see the reversion to methane generation by reduction of hydrogen to methane Moreover, the transition from a primary fermentation to a secondary fermentation where VFA are degraded is more likely to produce relatively higher increments of methane per unit of organic acid degraded. However, with higher sulphur level in treatment 0.8% S, the effect of sulphur in lowering methane production may be more prolonged, while nitrate is depleted Nitrate is known to inhibit sulphur reducing bacteria (see Hubert and Voordouw 2007). As nitrate is depleted by dissimulatory conversion to ammonia, sulphur reduction will return and hydrogen sulphide production may dilute the methane percentage in the gas (see Bracht and Kung 1997).

## Conclusions

- Compared with urea, calcium nitrate reduced methane percentage in the gas produced in an *in vitro* system with sugar cane stalk and cassava leaf meal as substrate. The effect was consistent over successive periods in the 48h incubation.
- Adding 0.4% sulphur, in the form of sodium sulphate, increased methane production indicating S deficiency in the medium; however, at the 0.8% level there appeared to be an additive effect on methane reduction when combined with nitrate.

- Both nitrate compared with urea and increasing level of sulphur decreased rate of gas production and the rate of fermentation of the substrate.
- The methane production increased linearly with the length of the incubation.

## Acknowledgements

This research was carried out in the laboratory of the Faculty of Agriculture, An Giang University, An Giang province, Vietnam. We wish to thank SIDA-SAREC for funding this research as part of the MSc course at Cantho University through the regional MEKARN project. Special thanks to Ms. Le Thi Thuy Hang and Mr. Ho Xuan Nghiep who helped us in the laboratory. We thank the administration at An Giang university laboratory for support in providing the facilities to carry out the research.

## References

**AOAC 1990** Official methods of analysis. Association of official Analysis (15th edition). Washington, D.C, USA.

**Hubert C and Voordouw G 2007** Oil field souring by nitrate reducing *Sulphospirillium* spp that out-compete sulphate reducing bacteria for organic electron donors Applied and Environmental Microbiology. 73, 2644-2652

**IPCC 2007** Climate Change 2007: Synthesis Report. http://www.ipcc.ch/publications_and_data/publications_ipcc_fourth_assessment_report_synthesis_report.htm

**Leng R A 2008** The potential of feeding nitrate to reduce enteric methane production in ruminants. A Report to The Departmernt of Climate Change Commonwealth Government of Australia. ACT Canberra Australia For paper and PPT presentation see http://www.penambulbooks.com

**Leng R A and Preston T R 2010** Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. Livestock Research for Rural Development. Volume 22, Article #221. http://www.lrrd.org/lrrd22/12/leng22221.htm

**Minitab 2000** Minitab Release 13.31 for windows, Windows* 95/98/2000/xp. Minitab Inc., State College Pennsylvania, USA.

**Ngoc Huyen L T, Do H Q, Preston T R and Leng R A 2010:** Nitrate as fermentable nitrogen supplement to reduce rumen methane production. *Livestock Research for Rural Development. Volume 22, Article #146.* Retrieved August 25, 2010, from http://www.lrrd.org/lrrd22/8/huye22146.htm

**Nguyen Ngoc Anh, Khuc Thi Hue, Duong Nguyen Khang and Preston T R 2010** Effect of calcium nitrate as NPN source on growth performance and methane emissions of goats fed sugar cane supplemented with cassava foliage. MEKARN Conference on Live stock production, climate change and resource depletion (Editors: Reg Preston). Pakse, Laos, November 7-9 2010.http://www.mekarn.org/workshops/pakse/abstracts/anh_grrc.htm

**Nolan J V, Hegarty R S, Hegarty J, Godwin I R and Woodgate R 2010** Effects of dietary nitrate on rumen fermentation, methane production and digesta kinetics in sheep. Animal Production Science 50 (8) 801–806

**Phommasack Outhen, T R Preston T R and Leng R A 2011** Effect of supplementation with urea or calcium nitrate and cassava leaf meal or fresh cassava leaf in an *in vitro* fermentation using a basal substrate of sugar cane stalk. Livestock Research for Rural Development. Volume 23, Article #023. http://www.lrrd.org/lrrd23/2/outh23023.htm

**Preston T R, Carcaño C, Alvarez F J and Gutierrez D G 1976** Rice polishings as a supplement in a sugar cane diet effect of level of rice polishings and of processing the sugar cane by derinding or chopping. Tropical Animal Production Volume 1, (3). http://www.utafoundation.org/TAP/TAP13/1_3_1.pdf

**Sangkhom Inthapanya, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of calcium nitrate as modifier of the fermentation in an in vitro incubation using cassava root as the energy source and leaves of cassava or *Mimosa pigra* as source of protein. Livestock Research for Rural Development. Volume 23, Article #021. http://www.lrrd.org/lrrd23/2/sang23021.htm

**Silivong Phonevilay, Preston T R and Leng R A 2011** Effect of sulphur and calcium nitrate on methane production by goats fed a basal diet of molasses supplemented with Mimosa (*Mimosa pigra)* foliage. Livestock Research for Rural Development. Volume 23, Article #047. http://www.lrrd.org/lrrd23/2/sili23047.htm

**Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M and de Haan C 2006** Livestock's Long Shadow: Environmental Issues and Options. Rome: Food and Agriculture Organization of the United Nations; 2006.

**Tilley J M A and Terry R A 1963** A two stage technique for the in vitro digestion of forage crops. Journal of the British Grassland Society 18 : 104.

**Van Zijderveld S.M, Gerrits W.J J, Apajalahti J A, Newbold J R, Dijkstra J, Leng R A and Perdok H B 2010a** Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. Journal of Dairy Science. Volume 93, 5856-5866
http://download.journals.elsevierhealth.com/pdfs/journals/0022-0302/PIIS0022030210006387.pdf

# Mitigating methane emissions from ruminants; comparison of three nitrate salts as sources of NPN (and sinks for hydrogen) in an *in vitro* system using molasses and cassava leaf meal as substrates

**Le Thuy Binh Phuong, Duong Nguyen Khang, T R Preston* and R A Leng****

*Nong Lam University, Viet Nam*

*binhphuongty27@yahoo.com*

** Finca Ecologica, TOSOLY, UTA (Colombia)*

*AA#48, Socorro, Santander, Colombia*

*** University of New England, Armidale NSW, Australia*

## Abstract

This study aimed to evaluate the effect of three nitrate salts (calcium nitrate, potassium nitrate and sodium nitrate) compared with urea on methane production in an *in vitro* 24h incubation with molasses and cassava leaf meal as substrate.

Compared to urea treatment, all nitrate salts diminished total gas production, methane percentage in the gas and methane production per unit of substrate. but there were no differencesamong the three nitrate salts.

***Key words:*** *Calcium nitrate, climate change, fermentation, gas production, greenhouse gases, potassium nitrate, sodium nitrate, urea*

## Introduction

It has been established that calcium nitrate acts as an alternative sink for hydrogen in *in vitro* incubations using molasses, sugar cane or cassava root meal as energy substrates and cassava leaf meal as the source of protein (Inthapanya Sangkhom et al 2011; Outhen et al 2011; Binh Phuong et al 2011; Du Thuy Thanh et al 2011). Potassium nitrate was used successfully by Trinh Phuc Hao et al (2009) in feeding trials with goats while sodium nitrate

was studied by Ngoc Huyen et al (2010) with cattle. However, there have been no comparisons of the different nitrate salts in the same experiment.

Calcium nitrate is marketed as Calcinit and contains a small percentage of ammonium ions. Ammonia under warm temperature reacts with sugars in molasses to form a toxic imidazole (Perdok and Leng 1987). In tropical conditions this could be factor in total mixed feeds, so it was decided to confirm that any nitrate salt would have the same effect on methane emissions before comparisons in the whole animal.

The objective of this study was to compare nitrate salts of calcium, sodium and potassium in an *in vitro* incubation using molasses as energy substrate and cassava leaf meal as the protein source. Urea served as the control treatment.

## Materials and methods

### Location and duration

The *in vitro* experiments were conducted in the laboratory of Nong Lam University, Ho Chi Minh city, Viet Nam, in July, 2011.

### Experimental design

The four treatments in a completely randomized design (CRD) were iso-nitrogenous levels of urea and nitrates of calcium, potassium and sodium (Table 1). The substrates were molasses and cassava leaf meal.

**Table 1**. Individual treatments

| Treatment | NPN sources | % N in NPN | % NPN in diet |
|---|---|---|---|
| CaN | $Ca(NO_3)_2.4H_2O$ | 11.9 | 7.87 |
| NaN | $NaNO_3$ | 16.5 | 5.67 |
| KN | $KNO_3$ | 13.9 | 6.73 |
| Urea | $(NH_2)_2CO$ | 46.7 | 2.00 |

A simple *in vitro* system was used based on the procedure reported by Inthapanya Sangkhom et al (2011).

## Material preparation and implementation of the method

Molasses was purchased in the market. Cassava leaves was sun-dried and ground through a 1 mm sieve. The ingredients in the substrate (molasses, cassava leaf meal, source of NPN) were mixed according to the proportions shown in Table 2. Representative samples of the mixtures (12g DM) were put in the incubation bottle to which was added 960ml of buffer solution (Table 4) and 240ml of cattle rumen fluid (taken immediately from a steer that was slaughtered at the local abattoir). The bottles with substrate were then incubated in a water bath at 39 $^{\circ}$C for 24h.

**Table 2**. Ingredients in the different treatments (g)

| Treatment | Molasses | Cassava leaf meal | NPN sources | | | |
|---|---|---|---|---|---|---|
| | | | Calcium nitrate | Sodium nitrate | Potassium nitrate | Urea |
| CaN | 8.06 | 3 | 0.94 | - | - | - |
| NaN | 8.32 | 3 | - | 0.68 | - | - |
| KN | 8.19 | 3 | - | - | 0.81 | - |
| Urea | 8.76 | 3 | - | - | - | 0.24 |

**Table 3.** Ingredients of the buffer solution (adapted from Tilly and Terry 1963)

| Ingredients | **$CaCl_2$** | **$NaHPO_4.12H_2O$** | **NaCl** | **KCl** | **$MgSO_4.7H_2O$** | **$NaHCO_3$** | **Cysteine** |
|---|---|---|---|---|---|---|---|
| (g/liter) | 0.04 | 9.30 | 0.47 | 0.57 | 0.12 | 9.80 | 0.25 |

## Measurements

The gas volume was measured by water displacement from the receiving bottle suspended in water. The bottle was calibrated at intervals of 50ml. The methane percentage in the gas was measured with a Crowcom meter (Crowcom Instruments Ltd, UK), and expressed as volume of methane per unit substrate.

The DM and crude protein contents of the substrates were determined according to AOAC (1990) methods. The sulphate in molasses was analyzed by Smart3 colorimeter. N solubility was determined by shaking 3g sample with 100ml NaCl 1M for 3 hours, filtering through Whatman No.4 filter paper and determining nitrogen in the filtrate.

## Results and discussion

### Chemical composition of ingredients in the substrate

Crude protein in the cassava leaf meal was high but very low solubility indicating that cassava leaf meal has potential by-pass protein characteristic. Compared with cassava leaf meal, the crude protein in molasses is low, beside that it contains significant sulphate source. Thus, molasses is easily fermentable substrate for anaerobic incubation and is sulphate source providing for microorganism.

**Table 4**. Chemical composition of ingredients \in the substrate

| | DM,% | CP in DM, % | N solubility, % | Sulphur, g/kg DM |
|---|---|---|---|---|
| Molasses | 57.2 | 5.77 | - | 2.53 |
| Cassava leaf meal | 88.0 | 22.6 | 24.3 | - |

### Gas and methane production

Compared with urea, all the nitrate treatments supported lower gas production (Figure 1). Percentages of methane in the gas were similar for all the nitrate salts, the values being some 25% less than with urea (Table 5; Figure 2). Expressed as volume of methane per unit substrate, the reduction due to the nitrate salts was some 40% (Figure 3). This finding is similar to the report of Ngoc Huyen et al (2010), for sodium nitrate and ammonium nitrate as the sources of fermentable N compared with urea, and Lin et al (2011) who compared sodium nitrate and urea in an *in vitro* experiment.

**Table 5**. Mean values for total gas production, methane volume, methane percentage and methane production per unit substrate

| | CaN | KN | NaN | Urea | SEM | P (nitrate) | SEM | P (NPN sources) |
|---|---|---|---|---|---|---|---|---|
| Gas production, ml | 1593 | 1490 | 1535 | 1927 | 31.5 | 0.12 | 29.1 | <0.001 |
| Methane, ml | 237 | 248 | 235 | 394 | 7.09 | 0.43 | 6.78 | <0.001 |
| Methane, % | 14.9 | 16.6 | 15.3 | 20.4 | 0.30 | 0.007 | 0.27 | <0.001 |
| Methane, ml/g substrate | 19.8 | 20.6 | 19.6 | 32.8 | 0.59 | 0.43 | 0.57 | <0.001 |

The higher gas production on the urea treatment is partially due to the carbon dioxide liberated from hydrolysis of the urea. One mole of urea produces 1 mole of carbon dioxide which is 22.4 litres of gas thus hydrolysis of 0.24 g of urea accounted for 90ml of the total gas production and would have been produced over a short time after initiation of the incubation. This would account for about 25% of the difference in gas production (about 400 ml) between use of urea compared with the nitrate salts. These results are similar to the

findings of Guo et al (2009), where addition of sodium nitrate in a 24h incubation lead to lower methane production and also less $CO_2$ production, compared with urea as NPN source.

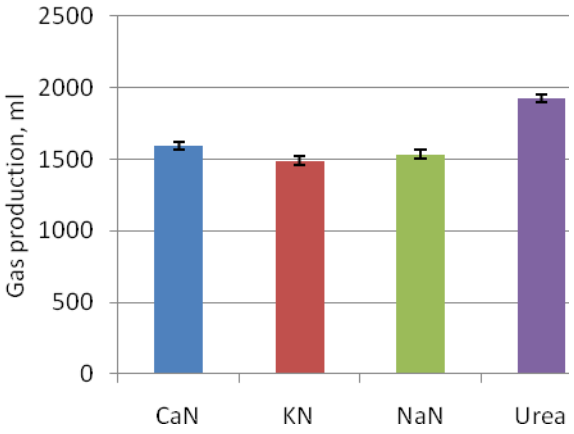



**Figure 1.** Effect of nitrate sources versus urea on gas production in an *in vitro* system with molasses and cassava leaf meal as substrate.

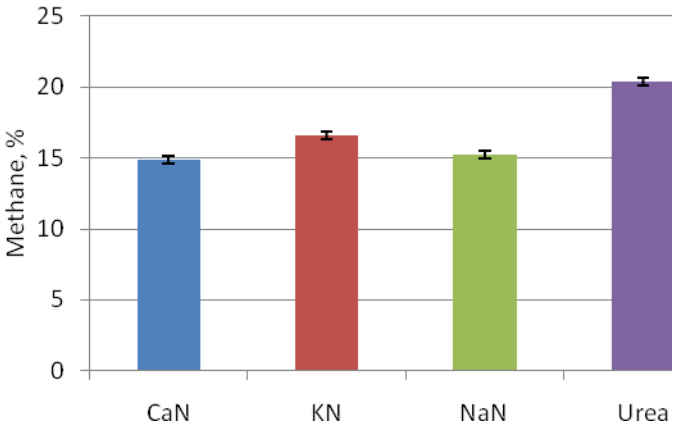



**Figure 2.** Effect of nitrate sources versus urea on methane percentag an *in vitro* system with molasses and cassava leaf meal as substrat

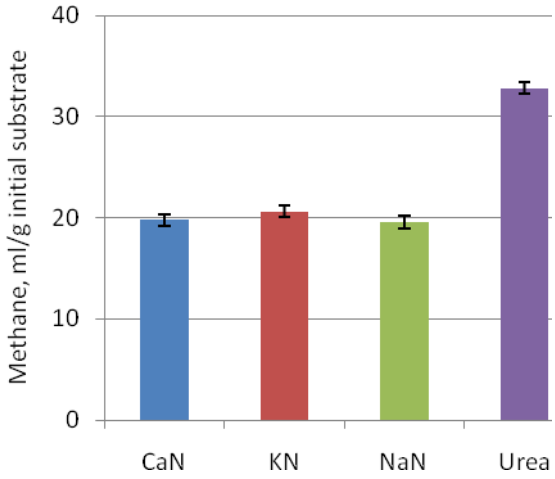



**Figure 3.** Effect of nitrate sources versus urea on methane production per unit substrate

## Conclusions

- Nitrate salts of calcium, potassium and sodium all reduced gas production, methane percentage in the gas and methane production per unit of a substrate composed of

molasses and cassava leaf meal. Thus in the rumen, it is expected that this will be also true unless there are other interactions with either the cations of the different salts and or ammonia (in the case of calcium salts).

## Acknowledgements

We wish to thank SIDA-SAREC for funding this research through the regional MEKARN project. We also acknowledge the administration at Nong Lam University for support in providing the facilities to carry out the research, which is part of the requirements for the degree of MScbe submitted by the Senior Author to Cantho University.

## References

**AOAC 1990** Official methods of analysis. Association of official Analysis (15th edition). Washington, D.C, USA.

**Binh Phuong L T, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of supplementary sulphate and nitrate on methane production in an in vitro incubation using sugar cane stalk and cassava leaf meal as substrate. Livestock Research for Rural Development. Volume 23, Article #22. http://www.lrrd.org/lrrd23/2/phuo23022.htm

**Du Thuy Thanh, Preston T R and Leng R A 2011** Effect on methane production of supplementing a basal substrate of molasses and cassava leaf meal with mangosteen peel (Garcinia mangostana) and urea or nitrate in an in vitro incubation. Livestock Research for Rural Development. Volume 23, Article #98. http://www.lrrd.org/lrrd23/4/than23098.htm

**Guo W S, Schaefer D M, Guo L P, Ren L P and Meng Q X 2009** Use of Nitrate-nitrogen as a Sole Dietary Nitrogen Source to Inhibit Ruminal Methanogenesis and to Improve Microbial Nitrogen Synthesis *In vitro.* Asian-Australasian Journal of Animal Science Volume 24, No. 4, 542 – 549.

**Inthapanya S, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of calcium nitrate as modifier of the fermentation in an in vitro incubation using cassava root as the energy source and leaves of cassava or Mimosa pigra as source of protein. Livestock Research for Rural Development. Volume 23, Article #21. http://www.lrrd.org/lrrd23/2/sang23021.htm

**Lin M, Schaefer D M, Guo W S, Ren L P and Meng Q X 2011** Comparisons of *In vitro* Nitrate Reduction, Methanogenesis, and Fermentation Acid Profile among Rumen Bacterial, Protozoal and Fungal Fractions. Asian-Australasian Journal of Animal Science Volume 24, No. 4 : 471 - 478

**Ngoc Huyen LT, Ho Quang Do, Preston T R and Leng R A 2010** Nitrate as fermentable nitrogen supplement to reduce rumen methane production. Livestock Research for Rural Development. Volume 22, Article #146.http://www.lrrd.org/lrrd22/8/huye22146.htm

**Outhen P, Preston T R and Leng R A 2011** Effect of supplementation with urea or calcium nitrate and cassava leaf meal or fresh cassava leaf in an in vitro incubation using a basal substrate of sugar cane stalk. Livestock Research for Rural Development. Volume 23, Article #23. http://www.lrrd.org/lrrd23/2/outh23023.htm

**Perdok H B and Leng R A 1987** Hyper-excitability in cattle fed ammoniated roughages. Animal Feed Science and Technology, 17: 121-143.

**Trinh Phuc Hao, Ho Quang Do, Preston T R and Leng R A 2009** Nitrate as a fermentable nitrogen supplement for goats fed forage based diets low in true protein. Livestock Research for Rural Development. Volume 21, Article #10. http://www.lrrd.org/lrrd21/1/trin21010.htm

**Tilley J M A and Terry R A 1963** A two stage technique for the in vitro digestion of forage crops. Journal of the British Grassland Society 18 : 104.

# Mitigating methane production from ruminants; effect of supplementary sulphate and nitrate on methane production in an *in vitro* incubation using molasses and cassava leaf meal as substrate

**Le Thuy Binh Phuong, Duong Nguyen Khang, T R Preston* and R A Leng****

*Nong Lam University, Viet Nam*

*binhphuongty27@yahoo.com*

** Finca Ecologica TOSOLY, AA#48, Socorro, Santander, Colombia*

*** University of New England, Armidale NSW, Australia*

## Abstract

This *in vitro* incubation was arranged as a 2*3 factorial in a completely randomized block design with 6 treatments and 4 repetitions. The first factor was the source of fermentable N (potassium nitrate or urea); the second factor was the level of added sulphur (0, 0.4 and 0.8% of substrate DM, as sodium sulphate. The substrate was molasses and cassava leaf meal. Incubations were carried out on successive days for 6, 12, 18, 24 and 48h. At the end of each incubation, volume of gas and percentage methane were recorded, and the residual substrate filtered to determine the amount of substrate solubilized.

Gas production, percentage methane in the gas and methane production were reduced by replacing urea by nitrate at incubation times from 6 to 48h, and were increased with length of incubation. Added sulphur increased methane emissions in the presence of nitrate over the early incubation periods indicating a greater fermentation rate in that period, but sulphur was additive in decreasing methane in longer incubations, indicating nitrate had been fully reduced and sulphur reduction commenced The reduction in methane production after 48h of incubation, when sulphur and nitrate were combined, was greater than when nitrate was used alone

***Key words:*** *climate change, fermentation, gas production, greenhouse gases, potassium nitrate, urea*

## Introduction

Livestock are a significant source of global methane ($CH_4$) emissions, producing some 80 million tonnes annually which represents about 28% of anthropogenic methane emissions

(Steinfeld et al 2006). Enteric methane from ruminant livestock is also an energy cost reducing available feed energy. Blaxter and Clapperton (1965) indicated that 8-12% of the gross feed energy was lost in the form of methane resulting from the ruminant digestion process.

In the absence of hydrogen sinks other then carbon dioxide in the rumen, methane production is indispensable as it maintains molecular hydrogen levels low at the sites in the biofilm (MacAllister et al 1994) close to hydrolytic breakdown of polymers, such as cellulose and other structural carbohydrates, and fermentative degradation of the solubilized organic matter to VFA. This process is a critical requirement for continuous fermentation because of the low partial pressure of hydrogen that is required to re-oxidise reduced cofactors produced in the pathways of metabolism (Cheng et al 1980).

Leng (2008) suggested it was more energetically favourable to use nitrate and sulphate as potential high affinity electron acceptors, competing with carbon dioxide for hydrogen and thus maintaining the availability of oxidized cofactors generated in fermentative degradation of carbohydrates, while at the same time lowering methane production. The products of nitrate and sulphate reduction are then ammonia and sulphide, respectively.

There are several potential benefits from using nitrate as an alternative electron acceptor in the diets of ruminants. These include potentially higher microbial growth efficiency as ATP is generated in the reduction of nitrate to ammonia and the nitrate can substitute fermentable N (e.g. urea) in a low protein diet. The conditions which appear to favor the use of nitrate as a fermentable N source, that also mitigates methane production, are a source of readily fermentable carbohydrate, an adequate level of sulphur, and a protein supplement low in content of soluble protein and high in "bypass" protein (Leng 2008). A source of supplementary sulphur may have an important interaction as sulphide availability in the rumen may be a critical issue in conversion of nitrate to ammonia without releasing nitrite into the medium (Leng and Preston 2010).

Molasses contains approximately 50% of sugars (Cleasby 1963) and may have 3-7g sulphur/kg dry matter (Suttle 2010), depending on the process used for clarification of the sugar-rich juice before centrifugation of the sugar. It is therefore convenient to use molasses as the source of sulphur and fermentable carbohydrate.

Cassava forage has been shown to be a good source of bypass protein in diets based on molasses-urea (Ffoulkes and Preston 1978). Keo Sath et al (2008) and Tham et al (2008) reported that cassava leaf meal improved growth performance and feed conversion in cattle diets based on urea-supplemented rice straw. However, the presence of cyanogenic glucosides in cassava leaves may increase the requirement for sulphur to detoxify the cyanide produced in the rumen. Blakeley and Coop (1949) concluded that approximately 1.2g of sulphur was required to detoxify 1.0g of HCN. Wheeler et al (1975) showed that the supply of sulphur licks to ruminants effectively protected them against chronic cyanide toxicity.

The research reported here examines the role of supplementary sulphur in the synergistic interaction between sulphate and nitrate in lowering methane production in an *in vitro* fermentation of a substrate based on molasses and cassava leaf meal.

## Materials and methods

### Location and duration

The experiment was conducted in the laboratory of Nong Lam University, Ho Chi Minh city, Viet Nam, from July to August, 2011.

### Experimental design

The experiment was arranged as a 2*3 factorial in completely randomized block design (CRBD) with 6 treatments and 4 repetitions (Table 1). The first factor was the source of fermentable N (potassium nitrate [6% of substrate DM] or urea [1.8% of substrate DM); the second factor was the level of added sulphur (0, 0.4 and 0.8% of diet DM, as sodium sulphate). The substrates were molasses and cassava leaf meal.

**Table 1**. Individual treatments (as % of substrate DM)

| Treatment | NPN levels | Sulphur levels |
|---|---|---|
| KN0S | 6.0 | 0 |
| U0S | 1.8 | 0 |
| KN0.4S | 6.0 | 0.4% |
| U0.4S | 1.8 | 0.4% |
| KN0.8S | 6.0 | 0.8% |
| U0.8S | 1.8 | 0.8% |

The experiment was conducted using 24 incubation flasks every day (equivalent to 6 treatments and 4 repetitions) for 5 days corresponding to the lengths of each incubation, which were for 6, 12, 18, 24 and 48h. The *in vitro* procedure was that described by Sangkhom Inthapanya et al (2011).

**Table 2**. Plan of the incubations

| Day | Number of flasks | Length of incubation, h |
|---|---|---|
| 1 | 24 | 6 |
| 2 | 24 | 12 |
| 3 | 24 | 18 |
| 4 | 24 | 24 |
| 5 | 24 | 48 |

**Material preparation and implementation of the method**

Molasses was purchased in the market. Cassava leaves (from cassava plants managed for foliage production) were sun-dried and ground through a 1 mm sieve. The ingredients in the substrate (molasses, cassava leaf meal, source of NPN) were mixed according to the proportions shown in Table 3. Representative samples of the mixtures (12g DM) were put in the incubation bottle to which was added 960ml of buffer solution (Tilly and Terry 1963) and 240ml of cattle rumen fluid. The rumen fluid was taken immediately from a steer that was slaughtered at the local abattoir, and held in a thermos flask for about 1hr until placed in the incubation bottles. The bottles with substrate were then incubated in a water bath at 39 $^{\circ}$C for the different lengths of incubation (6, 12, 18, 24 and 48h).

**Table 3** . Ingredients in the treatments (g DM)

| | KN | U | KN-0.4S | U-0.4S | KN-0.8S | U-0.8S |
|---|---|---|---|---|---|---|
| Molasses | 8.28 | 8.784 | 8.06 | 8.57 | 7.85 | 8.35 |
| Cassava leaf meal | 3 | 3 | 3 | 3 | 3 | 3 |
| Urea | 0 | 0.22 | 0 | 0.22 | 0 | 0.22 |
| KNO3 | 0.72 | 0 | 0.72 | 0 | 0.72 | 0 |
| $Na_2SO_4$ | 0 | 0 | 0.21 | 0.21 | 0.42 | 0.42 |

**Measurements**

The gas volume was measured by water displacement from the receiving bottle suspended in water which was calibrated at intervals of 50ml. Methane percentage was measured by

Crowcom meter (Crowcom Instruments Ltd, UK). At the end of each incubation period, residual substrate was filtered through 2 layers of cloth and absorbent cotton, followed by drying of the residue at 100°C for 48h to determine the DM residue.

The DM and N in the molasses and cassava leaf meal, and in the residue after incubation, were determined according to AOAC (1990) methods. The sulphate in molasses was measured with a " Smart 3" colorimeter. N solubility was determined by shaking 3g sample with 100ml 1M NaCl for 3 hours, filtering through Whatman No.4 filter paper and determining nitrogen in the filtrate.

### Statistic analysis

The data were analyzed by the General Linear Model (GLM) option in the ANOVA program of the Minitab Softwar (Minitab 2000). Sources of variation in the model were: levels of sulphur, NPN source, interaction sulphur*NPN source and error

## Results and Discussion

### Chemical composition of ingredients in the substrate

Crude protein in the cassava leaf meal was high but of very low solubility indicating that cassava leaf meal has potential bypass protein characteristics (Table 4). At the levels used, molasses contributed an additional 0.18% sulphur to the substrates.

**Table 4**. Chemical composition of ingredients \in the substrate

| | DM,% | CP in DM, % | N solubility, % | Sulphur, g/kg DM |
|---|---|---|---|---|
| Molasses | 57.2 | 5.77 | - | 2.53 |
| Cassava leaf meal | 88.0 | 22.6 | 24.3 | - |

In all incubations, gas production, percentage methane in the gas and methane production, were: (i) reduced by replacing urea by nitrate at incubation times from 6 to 48h; and (ii) were increased with length of incubation (Table 5). There were no effects of added sulphur (Figures 1 and 2) with incubation times of 6 and 12h but for incubations of 18, 24 and 48h, methane was reduced by both levels of added sulphur where urea provided the fermentable N source In the presence of nitrate sulphur appeared to increase methane production over the short incubation period possibly indicating an increased fermentation of the substrate but in the longer incubation periods reversed this effect indicating that sulphur reduction increased

with time, possibly through depletion of the energetically more favourable nitrate reduction. The lowering in methane production after 48h of incubation, when sulphur and nitrate were combined, was greater than when nitrate was used alone (Figure 3). These results are similar to our earlier report (Binh Phuong et al 2011) when added sulphur had no effect on methane production over 8h of fermentation, but decreased methane when the incubation was for 48h, with more pronounced effects when nitrate was the NPN source. After 48 hr incubation all the soluble sugar from the added molasses was obviously utilized and the overall effect of nitrate vs urea, and their interactions can be calculated according to the amount of total dry matter included in the flasks that was solubilized (this was not done for the earlier incubations since soluble sugars could lead to error). These results clearly indicate the lowered methane production per g of digestible carbohydrate (Figure 3) owing to nitrate and sulphate additions, relative to urea.

**Table 5**. Mean value for gas production, methane production, substrate fermented and methane production per unit substrate fermented for lengths of incubation of 6, 12, 18, 24 and 48h

| | K-nitrate | Urea | SE | P (NPN) | 0S | 0.4S | 0.8S | SEM | P ( sulphur) | P (NPN*S) |
|---|---|---|---|---|---|---|---|---|---|---|
| **6h** | | | | | | | | | | |
| Gas production, ml | 852 | 960 | 14.8 | <0.001 | 970 | 874 | 874 | 18.2 | 0.002 | 0.10 |
| Methane, ml | 101 | 134 | 3.84 | <0.001 | 129 | 104 | 119 | 4.70 | <0.001 | 0.01 |
| Methane, % | 11.8 | 13.8 | 0.25 | <0.001 | 13.1 | 11.9 | 13.5 | 0.31 | 0.004 | 0.009 |
| Methane, ml/g substrate | 17.3 | 21.1 | 0.73 | <0.001 | 20.5 | 17.0 | 20.1 | 0.89 | 0.02 | 0.01 |
| **12h** | | | | | | | | | | |
| Gas production, ml | 1204 | 1457 | 20.6 | <0.001 | 1280 | 1366 | 1345 | 25.2 | 0.06 | 0.05 |
| Methane, ml | 156 | 272 | 4.38 | <0.001 | 200 | 219 | 222 | 5.36 | 0.02 | 0.15 |
| Methane, % | 12.9 | 18.7 | 0.24 | <0.001 | 15.3 | 15.7 | 16.4 | 0.30 | 0.04 | 0.68 |
| Methane, ml/g substrate | 23.2 | 36.9 | 0.63 | <0.001 | 27.3 | 30.9 | 32.1 | 0.77 | 0.001 | 0.08 |
| **18h** | | | | | | | | | | |
| Gas production, ml | 1879 | 2077 | 17.6 | <0.001 | 2048 | 1954 | 1933 | 21.58 | 0.003 | 0.06 |
| Methane, ml | 277 | 466 | 8.15 | <0.001 | 398 | 364 | 352. | 9.98 | 0.01 | 0.56 |
| Methane, % | 14.7 | 22.4 | 0.37 | <0.001 | 19.2 | 18.5 | 17.9 | 0.46 | 0.21 | 0.70 |
| methane, ml/g substrate | 38.7 | 61.4 | 1.33 | <0.001 | 52.5 | 48.3 | 49.4 | 1.63 | 0.18 | 0.22 |
| **24h** | | | | | | | | | | |
| Gas production, ml | 1796 | 2074 | 21.3 | <0.001 | 2023 | 1905 | 1878 | 26.1 | 0.002 | 0.53 |
| Methane, ml | 381 | 505 | 4.40 | <0.001 | 479.84 | 429.45 | 420 | 5.39 | <0.001 | 0.78 |
| Methane, % | 21.2 | 24.4 | 0.20 | <0.001 | 23.7 | 22.4 | 22.2 | 0.24 | 0.001 | 0.90 |
| Methane,, ml/g substrate | 52.9 | 65.5 | 0.98 | <0.001 | 60.9 | 57.6 | 59.1 | 1.20 | 0.18 | 0.34 |
| **48h** | | | | | | | | | | |
| Gas production, ml | 1889 | 2219 | 27.4 | <0.001 | 2217 | 1987 | 1957 | 33.5 | <0.001 | 0.40 |
| Methane, ml | 411 | 572 | 5.67 | <0.001 | 554 | 470 | 448 | 6.94 | <0.001 | 0.05 |
| Methane, % | 21.7 | 25.7 | 0.19 | <0.001 | 24.9 | 23.4 | 22.7 | 0.23 | <0.001 | 0.02 |
| methane, ml/g substrate | 50.5 | 65.9 | 0.96 | <0.001 | 64.6 | 55.6 | 54.6 | 1.17 | <0.001 | 0.02 |
| DM solubilized, % | 73.5 | 74.8 | 0.61 | 0.145 | 74.3 | 74.4 | 73.7 | 0.74 | 0.76 | 0.34 |

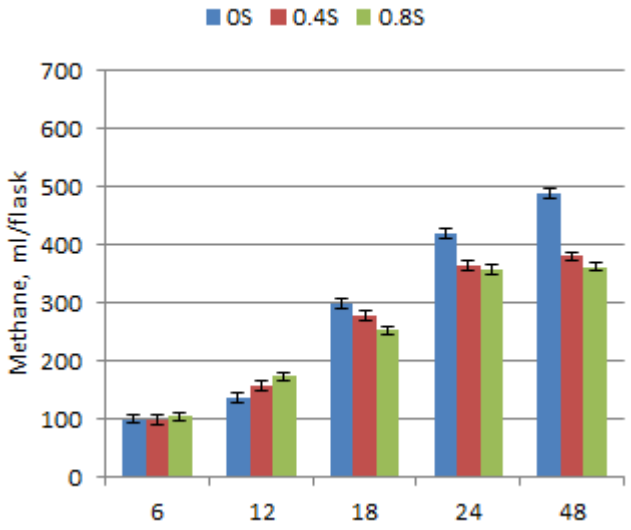



**Figure 1.** Effect of added sulphur on methane production for different lengths of incubation and substrates with potassium nitrate as NPN source

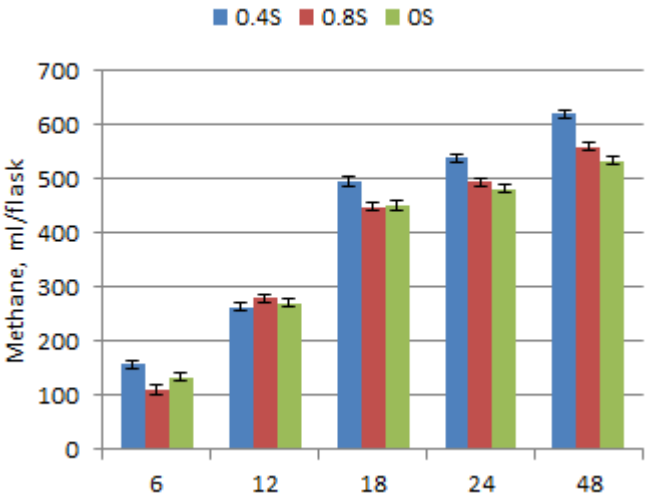



**Figure 2.** Effect of added sulphur on methane production for differe lengths of incubation and substrates with urea as NPN source

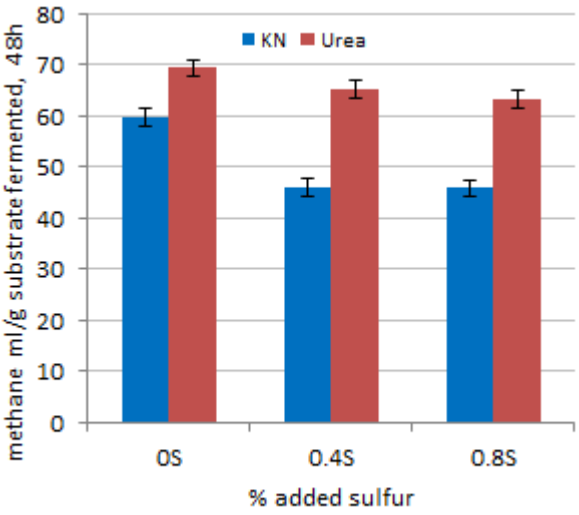



**Figure 3.** Effect of added sulphur on methane production in the presence of urea or sulphate after 48h incubation

The results of these studies indicate that nitrate-reducing organisms (NRB) are present in the rumen of cattle un-adapted to nitrate, as methane emissions were decreased with nitrate addition compared to that when urea was added. Apparently nitrate reduction takes precedence over sulphur reduction but the apparent effect of added sulphur in the late stage of the fermentation possibly indicates that nitrate N had been fully converted to ammonia and

then sulphur reduction commences indicating the presence of sulphur-reducing bacteria (SRB) in the medium. The lowering of methane production is consistent with higher affinity for hydrogen (or greater Gibbs Free energy change) from reduction of nitrate and sulphur in the rumen as compared to reduction of carbon dioxide. The Gibbs Free energy change of the reduction is greater for nitrate than sulphate which is greater than for carbon dioxide. SRB activity is likely to reflect both sulphur and nitrate reduction as these organisms have a wide range of substrates that they can use in their energy metabolism (Moura et al 2007).

The stoichiometry of nitrate reduction to ammonia indicates that the reduction of 1 mole of nitrate results in the lowering of methane production by 1 mole. From the lowering of methane release measured it appears that the efficiency of nitrate reduction approaches 100% after 12-18h incubation period and then sulphide reduction over the next 30hours contributes further lowering methane production by about 10% over the next 12h.

## Conclusions

- Potassium nitrate reduced methane production compared with urea. This effect of nitrate was consistent over all incubations times from 6 to 48h.
- Increasing supplementary level of sulphur did not affect methane production in the primary fermentation (12h).
- From 18 through 48h incubation, supplementary sulphur reduced methane production but there was no difference between 0.4 and 0.8 % added sulphur.
- The reduction in methane production from combined nitrate and sulphur was greater than from nitrate alone.

## Acknowledgements

This research was done by the senior author with support from NUFU as part of the requirements for the MSc degree in Animal Production "Specialized in Response to Climate Change and Depletion of Non-renewable Resources" of Cantho University, Vietnam. The authors wish to thank SIDA-SAREC for funding this research through the regional MEKARN project. We also acknowledge the administration at Nong Lam University for support in providing the facilities to carry out the research.

# References

**AOAC 1990** Official methods of analysis. Association of official Analysis (15th edition). Washington, D.C, USA.

**Binh Phuong L T, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of supplementary sulphate and nitrate on methane production in an in vitro incubation using sugar cane stalk and cassava leaf meal as substrate. Livestock Research for Rural Development. Volume 23, Article #22. http://www.lrrd.org/lrrd23/2/phuo23022.htm

**Blakley R L and Coop L E 1949** The metabolism and toxicity of cyanide and cyanogenic glycosides in sheep. 11. Detoxification of hydrocyanic acid. New Zealand Journal of Science Technology 31 (3) A: 1 - 16

**Blaxter K L and Clapperton J L 1965** Prediction of the amount of methane produced by ruminants. British Journal of Nutrition 19 (1) pp 511-522

**Cheng K J, Fay J P, Howarth R E and Costerton J W 1980**. Sequence of events in the digestion of fresh legume leaves by rumen bacteria. Applied and Environmental Microbiology 40, 613–625.

**Cleasby T G 1963** Feeding values of molasses. Proceeding of The South African Sugar Technologist's Association.

**Ffoulkes D and Preston T R 1978** Cassava or sweet potato forage as combined sources of protein and roughage in molasses based diets: effect of supplementation with soybean meal. Tropical Animal Production 1978, Volume3, Number 3 http://www.utafoundation.org/TAP/TAP33/3_3_1.pdf

**Keo Sath, Borin K and Preston T R 2008:** Effect of levels of sun-dried cassava foliage on growth performance of cattle fed rice straw. Livestock Research for Rural Development. Volume 20, supplement.http://www.lrrd.org/lrrd20/supplement/sath2.htm

**Leng R A 2008** The potential of feeding nitrate to reduce enteric methane production in ruminants. A Report to The Department of Climate Change Commonwealth Government of Australia. ACT Canberra Australia For paper and PPT presentation see http://www.penambulbooks.com

**Leng R A and Preston T R 2010** Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. Livestock Research for Rural Development. Volume 22, Article #221. http://www.lrrd.org/lrrd22/12/leng22221.htm

**McAllister T A, Bae H D, Jones G A and Cheng K J 1994**. Microbial attachment and feed digestion in the rumen. Journal of Animal Science 72, 3004–3018

**Minitab 2000** Minitab Release 13.31 for windows, Windows* 95/98/2000/xp. Minitab Inc., State College Pennsylvania, USA.

**Moura J G, Gonzales P, Moura I and Fauque G 2007** Dissimilatory nitrate and nitrite ammonification by sulphate – reducing eubacteria. Chapter 8 In: Sulphur-reducing bacteria. Environmental and Engineering Systems (editors Barton L L and Hamilton W A) Cambridge University Press

**Suttle 2010** Mineral nutrition of livestock
,4th edition. http://books.google.com/books?id=RwlJJbjATXEC&pg=PA207&dq=molasses+contain+sulphur&hl=vi&ei=3dNEToSvG6XomAWg9OnkBg&sa=X&oi=book_result&ct=result&resnum=3&ved=0CDQQ6

**Sangkhom Inthapanya, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of calcium nitrate as modifier of the fermentation in an *in vitro* incubation using cassava root as the energy source and leaves of cassava or *Mimosa pigra* as source of protein. Livestock Research for Rural Development. Volume 23, Article #021. Retrieved , from http://www.lrrd.org/lrrd23/2/sang23021.htm

**Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M and de Haan C 2006** Livestock's Long Shadow: Environmental Issues and Options. Rome: Food and Agriculture Organization of the United Nations; 2006.

**Tham H T, Man N V and Preston T R 2008** Performance of young cattle fed rice straw sprayed with mixture of urea and molasses supplemented with different levels of cassava leaf meal. Livestock Research for Rural Development. Volume 20, supplement. http://www.lrrd.org/lrrd20/supplement/tham1.htm

**Tilley J M A and Terry R A 1963** A two stage technique for the *in vitro* digestion of forage crops. Journal of the British Grassland Society 18 : 104.

**Wheeler J L, Hedges D A and Till A R 1975** A possible effect of cyanogenic glucoside in sorghum on animal requirements for sulphur. Journal of Agricultural Science 84:377379.

# Effect of NPN source, level of added sulphur and source of cassava leaves on growth performance and methane emissions in cattle fed a basal diet of molasses

**Le Thuy Binh Phuong**

## Abstract

The objective of this study was to determine the effect of potassium nitrate versus urea, and of supplementary sulphur, on growth performance of cattle fed molasses and cassava foliage. The experiment was designed as a 2*2*3 factorial with 4 replications. The factors were source of NPN (potassium nitrate: 6 % of diet DM basis or urea: 1.8 % of diet DM basis), level of added sulphur (0 or 0.8% S) and source of cassava foliage (fresh foliage or dried leaf meal).

DM intake was not affected by NPN source, but was depressed by adding 0.8% sulphur and was lower for the treatment with fresh cassava foliage compared with leaf meal. After correcting the data by covariance for differences in initial live weight, growth rate was depressed by adding 0.8% sulphur to the diet but was not affected by source of NPN or source of cassava foliage. The ratio of methane to carbon dioxide was reduced by feeding potassium nitrate rather than urea and by fresh cassava foliage compared with cassava leaf meal.

***Key words**: Feed conversion, foliage, leaf meal, potassium nitrate, urea*

## Introduction

Earlier studies in my laboratory showed that nitrate salts, replacing urea as the NPN source, and supplementary sulphur as sodium sulphate reduced methane production in an *in vitro* incubation with molasses and cassava leaf meal as the substrate (Papers 2 and 3).

The objective of the following experiment was to determine if these dietary modifications would result in improved performance of growing cattle fed a basal diet of molasses and cassava foliage, since it is known that enteric methane production results in 8-12% loss of the gross feed energy resulting from the ruminant digestion process (Blaxter and Clapperton 1965).

## Materials and methods

### Location and duration

The experiment was conducted at the cattle research station in Binh Duong province, Viet Nam, from November 2011 to January 2012.

### Experimental design

The experiment was designed as a 2*2*2 factorial with 2 replications using a total of 16 growing crossbred cattle.

The factors were:

*Source of NPN:*

- Potassium nitrate (6% of diet DM basis) or urea (1.8% of diet DM basis)

*Level of added sulphur:*

- 0 or 0.8% S

*Source of cassava foliage:*

- Fresh foliage or dried leaf meal

### Basal diet

Molasses derived from sugar cane was fed *ad libitum.* Fresh native grass was fed at 2 kg/day. Cassava foliage (fresh or as leaf meal) was supplied at 1% of LW (DM basis).

### Animals

Sixteen growing Laisind (Red Sindhi*local "Yellow" breed) with initial live weight of 187-234kg were tethered separately in a concrete-floor building. They had individual access to feed troughs containg the molasses and the forage. Water and salt were always available. The cattle were vaccinated against foot and mouth disease and were de-wormed before starting the experiment.

### Feeding system

The additives (potassium nitrate, uurea and sodium sulphate) were dissolved in the molasses. The grass was harvested in the morning and chopped by machine prior to feeding it at 15.30. Fresh cassava foliage was also harvested in the morning, from plants of 4 to 5 months

maturity, and was offered in the fresh state at 07.15. Cassava leaf meal, purchased from a local feed company, was given at the same time.

Prior to starting the experiment, the cattle were adapted gradually over a 2-week period to the NPN source and the sodium sulphate. Fresh molasses was offered 3 times every day (07.15, 11.00 and 15.30). The prescribed quantities of fermentable N sources (urea, nitrate) and the sodium sulphate were dissolved in the molasses offered at 07.15 and 11.00). Before each morning feeding, the feed residues were removed from the troughs and weighed to determine feed intake.

**Data collection and measurements**

The cattle were weighed every 14 days using an electronic balance. Feed samples were collected every two weeks for DM and N analysis. Live weight gain was calculated from the luinear regression of weight (Y) on days of experiment (X).

**Chemical analysis**

The DM and N contents of the feeds were analyzed according to AOAC (1990). Samples of eructed gas were collected and analyzed by GASMET infra-red analyser (Gasmet Company, Finland) for methane and carbon dioxide using the method described by Phonevilay Silivong et al (2011).

**Statistical analysis**

The data were analyzed by the General Linear Model option in the ANOVA program of Minitab (2000). Sources of variation were: NPN source, Level of sulphur, Source of cassava foliage, interaction NPN*sulphur level and error.

## Results and discussion

As expected the crude protein content of the leaf meal was slightly higher than in the fresh foliage (Table 1).

**Table 1.** Composition of dietary ingredient

| | DM % | Crude protein, % in DM | Sulphur, g/kg DM |
|---|---|---|---|
| Molasses | 62 | 1.28 | 2.53 |
| Cassava leaf meal | 86.3 | 24.1 | |
| | | | |
| Cassava foliage# | | | |
| Stem | 23.8 | 9.50 | |
| Leaves | 30.7 | 28.1 | |
| Grass | 20.9 | 8.93 | |

*#Ratio on fresh basis was 60.2% leaf and 39.8% stem; calculated average % crude protein in DM of foliage was 21.8%*

Molasses represented 53-57% of the DM intake (Figure 1) with cassava foliage providing 34%.

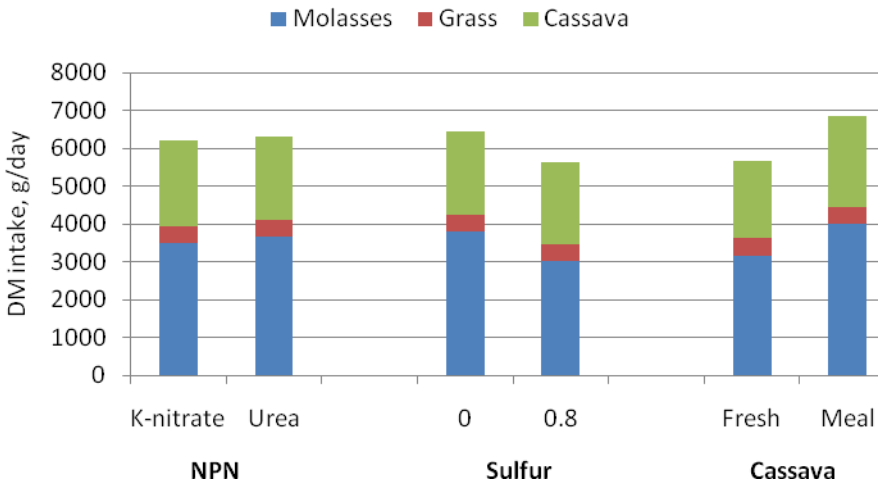



**Figure 1.** DM intakes of dietary components (excluding NPN sources and sulphate)

DM intake was not affected by NPN source, but was depressed by adding 0.8% sulphur and was lower for the treatment with fresh cassava foliage compared with leaf meal (Table 2).

Growth rates of the cattle were uniform throughout the experiment (Figure 2). There were differences in initial live weight between NPN sources and source of cassava foliage. After correcting the data by covariance for differences in initial live weight: (i) adding 0.8% sulphur

to the diet reduced the final weight and the live weight gain (Figure 3); and (ii) sources of NPN and cassava foliage had no effect on final live weight or live weight gain. DM feed conversion was poorer when 0.8% sulphur was added to the diet but was not affected by source of NPN or source of cassava foliage.

**Table 2.** Mean values for changes in live weight, DM intake and DM feed conversion for cattle fed molasses supplemented with fresh cassava foliage or cassava leaf meal, with NPN from K-nitrate or urea and with or without added sulphur

| | NPN | | | Added sulphur, % | | | Cassava leaves | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | KN | Urea | P | 0 | 0.8 | P | Fresh | Meal | P | SEM |
| Live weight, kg | | | | | | | | | | |
| Initial | 214 | 208 | | 211 | 210 | | 196.1 | 225.4 | | 2.70 |
| Final | 246 | 239 | 0.20 | 245 | 239 | 0.29 | 226 | 259 | 0.001 | 3.50 |
| Final# | 242 | 243 | 0.91 | 245 | 240 | 0.051 | 243 | 242 | 0.78 | 1.58 |
| Daily gain, g | 420 | 409 | | 448 | 381 | | 384 | 444 | | 17.0 |
| Daily gain, g# | 413 | 416 | 0.92 | 447 | 382 | 0.013 | 419 | 409 | 0.86 | 20 |
| DMI, g | 6591 | 6416 | 0.48 | 6724 | 6283 | 0.09 | 5877 | 7130 | 0.001 | 168 |
| DM FCR | 15.8 | 15.8 | 0.90 | 15.1 | 16.5 | 0.015 | 15.4 | 16.2 | 0.176 | 0.35 |

*# Corrected by covariance for differences in initial live weight*

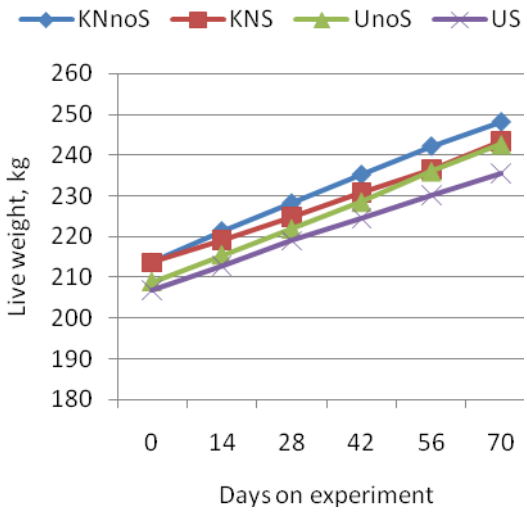



**Figure 2.** Growth curves of the cattle fed molasses and cassava foliage and supplemented with potassium nitrate or urea and zero or 0.8% added sulphur

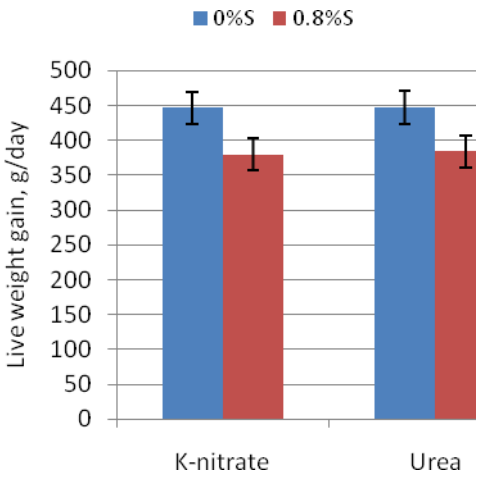



**Figure 3.** Effect of added sulphur and source of NPN on live weight gain of cattle fed molasses and cassava foliage

The ratio of methane to carbon dioxide ratios was reduced by feeding potassium nitrate rather than urea and by fresh cassava foliage compared with cassava leaf meal (Table 3; Figure 4).

**Table 3.** Mean values for methane to carbon dioxide in eructed gas from cattle fed molasses supplemented with fresh cassava foliage or cassava leaf meal, with NPN from K-nitrate or urea and with or without added sulphur

| | NPN | | | Added sulphur, % | | | Cassava | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | KN | Urea | P | 0 | 0.8 | P | Fresh Foliage | Leaf meal | P | SEM |
| $CH_4/CO_2$ | 0.0295 | 0.0353 | 0.002 | 0.032 | 0.0324 | 0.97 | 0.0302 | 0.0347 | 0.016 | 0.0013 |

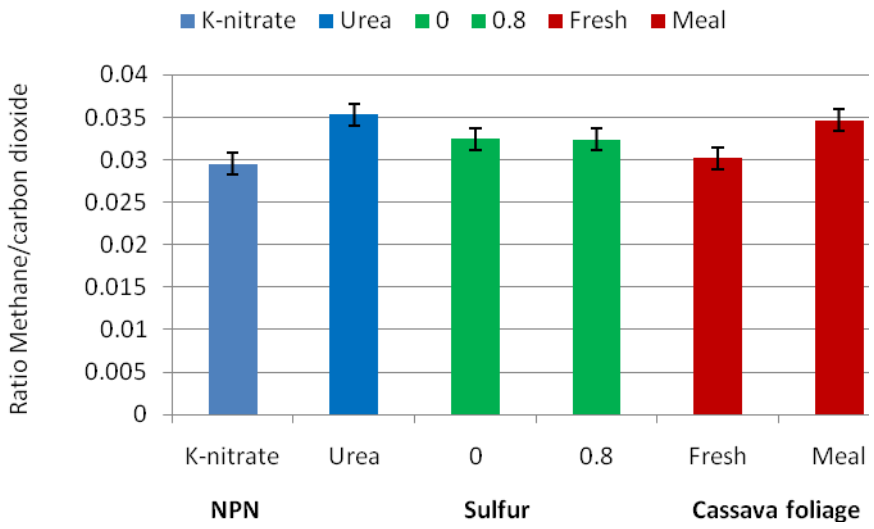



**Figure 4.** Effect of NPN source, level of sulphur and source of cassava foliage on the ratio of methane to carbon dioxide in eructed gas from cattle fed molasses and cassava foliage.

It appeared that the effect of nitrate in reducing methane production was greater when 0.8% sulphur was added to the diet (Figure 5).

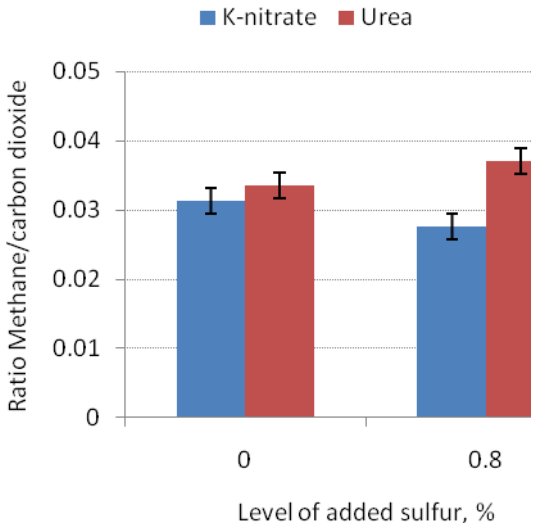



**Figure 5.** Effect of NPN source and level of sulphur on the ratio of methane to carbon dioxide in eructed gas from cattle fed molasses and cassava foliage

There are many reports showing reductions in methane production when nitrate salts replace urea in *in vitro* incubations with a range of substrates (Binh Phuong et al 2011; Du Thuy Thanh et al 2011; Inthapanya et al 2011). Reports from *in vivo* experiments show similar responses in cattle (Van Zijderveld et al 2010), in goats (Nguyen Ngoc Anh et al 2010) and in sheep (Nolan et al 2010).

However, the hypothesis that the reduction in methane would be accompanied by better animal performance was not proven in the present study, nor was such an effect observed in the reported experiments in the literature research (eg: Van Zijderveld et al 2010; Nguyen Ngoc Anh et al 2010; Nolan et al 2010).

## Conclusions

- The ratio of methane to carbon dioxide ratios was reduced by feeding potassium nitrate rather than urea and by fresh cassava foliage compared with cassava leaf meal.
- Live weight gain was not affected by replacing urea with potassium nitrate as NPN source, nor by the source of cassava foliage.
- Adding 0.8% sulphur to the diet reduced the live weight gain and resulted in poorer feed conversion.

## Overall conclusions

- Compared with urea, nitrate reduced methane production and gas production in all *in vitro* incubations but there were no differences among the three nitrate salts (potassium nitrate, calcium nitrate and sodium nitrate). This effect of nitrate on reducing methane was consistent over successive periods in the 48h incubation.
- The methane production increased linearly with the time of the incubation. It indicated that an *in vitro* incubation shoule be stopped after primary fermentation (12h-24h) to match the fermentation to the natural ecology of rumen.
- In the combination of sulphate and nitrate, the lowering in methane production was greater than on nitrate or sulphur alone. However, the presence of molasses as readily soluble carbonhydrate, increasing supplementary level of sulphur, did not affect methane production in the primary fermentation (12h).
- In cattle, the ratio of methane to carbon dioxide ratios was reduced by feeding potassium nitrate rather than urea and by fresh cassava foliage compared with cassava leaf meal. However, feeding nitrate rather than urea, and fresh rather than dried (leaf meal) cassava foliage, did not affect live weight gain. Adding 0.8% sulphur to the diet reduced the live weight gain and resulted in poorer feed conversion.

## References

**AOAC 1990** Official methods of analysis. Association of official Analysis (15th edition). Washington, D.C, USA.

**Binh Phuong L T, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of supplementary sulphate and nitrate on methane production in an in vitro incubation using sugar cane stalk and cassava leaf meal as substrate. Livestock Research for Rural Development. Volume 23, Article #22. http://www.lrrd.org/lrrd23/2/phuo23022.htm

**Blaxter K L and Clapperton J L 1965** Prediction of the amount of methane produced by ruminants. British Journal of Nutrition. **19**: 511–522.

**Du Thuy Thanh, Preston T R and Leng R A 2011** Effect on methane production of supplementing a basal substrate of molasses and cassava leaf meal with mangosteen peel (Garcinia mangostana) and urea or nitrate in an in vitro incubation. Livestock Research for Rural Development. Volume 23, Article #98. http://www.lrrd.org/lrrd23/4/than23098.htm

**Inthapanya S, Preston T R and Leng R A 2011** Mitigating methane production from ruminants; effect of calcium nitrate as modifier of the fermentation in an in vitro incubation using cassava root as the energy source

and leaves of cassava or Mimosa pigra as source of protein. Livestock Research for Rural Development. Volume 23, Article #21. http://www.lrrd.org/lrrd23/2/sang23021.htm

**Nguyen Ngoc Anh, Khuc Thi Hue, Duong Nguyen Khang and Preston T R 2010** Effect of calcium nitrate as NPN source on growth performance and methane emissions of goats fed sugar cane supplemented with cassava foliage. MEKARN Conference on Live stock production, climate change and resource depletion (Editors: Reg Preston and Brian Ogle). Pakse, Laos, November 7-9 2010.http://www.mekarn.org/workshops/pakse/abstracts/anh_grrc.htm

**Nolan J V, Hegarty R S, Hegarty J, Godwin I R and Woodgate R 2010** Effects of dietary nitrate on rumen fermentation, methane production and digesta kinetics in sheep. Animal Production Science 50 (8) 801–806

**Silivong P, Preston T R and Leng R A 2011**: Effect of sulphur and calcium nitrate on methane production by goats fed a basal diet of molasses supplemented with Mimosa (Mimosa pigra) foliage. Livestock Research for Rural Development. Volume 23, Article #58. http://www.lrrd.org/lrrd23/3/sili23058.htm