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Cover photo: Sofie Winding

Examensarbete / SLU, Institutionen för husdjurens utfodring och vård, **392**

Uppsala 2014

Degree project / Swedish University of Agricultural Sciences,
Department of Animal Nutrition and Management, **392**

Examensarbete, 15 hp
Kandidatarbete
Husdjursvetenskap
Degree project, 15 hp
Bachelor thesis
Animal Science

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Metanproduktion från nitratbehandlat tanninrikt foder till nötkreatur i Vietnam

Sofie Winding

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Omfattning:

Extent: 15 hp

Kurstitel:

Course title: Degree project

Kurskod:

Course code: EX0550

Program:

Programme: Animal Science, Bachelor thesis, Minor Field Study

Nivå:

Level: Basic G2E

Utgivningsort:

Place of publication: Uppsala

Utgivningsår:

Year of publication: 2014

Serienamn, delnr:

Series name, part No: Degree project / SLU, Department of Animal Nutrition and Management, 392

On-line publicering:

On-line published: http://epsilon.slu.se

Nyckelord:

Key words: Tropical, legume, feed, supplement, ruminants, tannins, nitrate

Sammanfattning

I utvecklingsländer likt Vietnam äter befolkningen mer animaliska produkter i form av mjölk och kött än tidigare vilket kräver mer animalieproduktion. Idisslare i sig bidrar till mer metanutsläpp vilket gör att en konflikt uppstår mellan produktion av livsmedel och dess miljöpåverkan. Genom att utfodra idisslare med näringsrika växter som människor inte kan tillgodogöra sig näring ifrån ökar djurens produktion genom bättre tillväxt och mer mjölk utan att inkräkta på tillgången av livsmedel som kan konsumeras direkt av människor. Genom ökad produktion minskas metanutsläppen per kg mjölk och kött där mängden metanutsläpp idag är högre i utvecklingsländer än i utvecklade länder. Syftet med denna studie var att undersöka tropiska baljväxter med högt tannininnehåll som ett potentiellt fodertillskott för idisslare genom *in vitroförsök*. Tanninrika baljväxter binder protein i våmmen, vilket ökar andelen bypass protein, samt reducerar metanproduktionen. Tillsats av nitrat reducerar metanproduktionen ytterligare samtidigt som andelen tillgängligt kväve ökar. Fyra baljväxter; Acacia mangium, Flemingia macrophylla, Leucaena leucocephala och Stylosanthes guianensis undersöktes tillsammans med en välstuderad tanninrik törelväxt; Manihot esculenta Crantz (Cassava) som referens. Baljväxterna torkades med fyra olika metoder; soltorkning, värmetorkning i ugn, frystorkning och torkning i rumstemperatur. Frystorkning gav signifikant ($P<0,05$) högre metangasproduktion än de andra torkmetoderna. Acacia mangium och Flemingia macrophylla producerade en signifikant ($P<0,05$) mindre mängd metan. Ingen signifikant skillnad kunde hittas i metanproduktion med eller utan tillsatts av kalciumnitrat till baljväxterna. Acacia mangium och Flemingia macrophylla torkade i sol, ugn eller rumstemperatur är potentiella fodertillskott för idisslare med egenskaperna att både minska metanproduktion i våmmen och öka andelen bypass protein i fodret.

Abstract

In developing countries such as Vietnam the population consumes more animal products for example milk and meat than before which requires greater livestock production. Ruminants contributes to more methane emission which creates a dilemma between food production and its environmental impact. By feeding ruminants with nutritive crops which humans can not assimilate the animal performance will increase in terms of better growth and milk production without inpinging on food that can be consumed directly by humans. Increasing animal performance reduces methane emission in terms of amount of methane in kg^{-1} milk and meat which today is much greater in developing countries than developed countries. The aim of the study was to investigate tropical tannin-rich legumes for their potential as a feed supplement for ruminants made *in vitro*. Tannin-rich legumes binds protein in the rumen, which increase the proportion of bypass protein in the feed, and reduces enteric methane emission. Addition of nitrate further reduces methane emission and available nitrogen increases. Four legumes; Acacia mangium, Flemingia macrophylla, Leucaena leucocephala and Stylosanthes guianensis was studied together with a well-studied tannin-rich euphorbiaceae; Manihot esculenta Crantz (Cassava) as a reference. Four different drying methods were used; sundried, dried in heat, freeze-dried and dried in room temperature. Freeze-drying showed significantly ($P<0.05$) higher production of methane than the other three methods. Acacia mangium and Flemingia macrophylla produced significantly ($P<0.05$) less amount of methane. No significant difference could be found on methane production with or without addition of calcium nitrate to the foliage. Acacia mangium and Flemingia macrophylla dried in the sun, dried in room temperature or heat-dried in oven are possible feed supplement for ruminants with the properties of both mitigating enteric methane and enhancing the amount of bypass protein in the feed.

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Introduction

Global warming has become a major issue for the society, politicians and scientists (FAO, 2009). One of the reasons to global warming are emission of greenhouse gases (GHG) such as carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) which agriculture contributes with a large part to (Weiske, 2005). Animal husbandry, especially ruminants, accounts for the largest part of GHG emissions within the agricultural sector, approximately 80 % (FAO, 2008a). In developed countries a lot of research has been made on feed together with long-term breeding strategy to improve the animal performance when it comes to milk production and growth (Idris *et al*., 2011). Ruminants in developing countries produce more methane kg^{-1} milk or meat than ruminants in developed countries. The amount of methane emisson kg^{-1} of milk or meat produced should be reduced by increasing the animals effectivity (Carlsson-Kanyama, 1998; Garnet 2009). A feed supplement with a high nutritional value for cattle affecting the animal performance at the same time as GHG emission is reduced will in the long run help people with nutritious food, increased living standard and improved economy (Goodland, 1997; Schils, 2007).

The large GHG emission and especially methane contributes to a climate change that will affect things such as rapid weather changes, increased temperature and worldwide water supplies (Moss *et al*., 2000). The weather and temperature will affect desert areas and wetland grounds, which in turn will increase the amount of pests. That can be a threat to health of both humans and animals. Increasing concentration of GHG in the atmosphere will decrease the thickness of the ozone layer and that contributes to increase the worldwide temperature (McMichael *et al*., 2007). All of this leads to a common goal; to reduce the emission. One study shows that food from livestock produces more GHG compared to other foodstuff and the largest amount of emission occurs at farm level (Foster *et al*., 2006). Products from dairy and meat food chain accounts for 18 % of the global GHG emission (FAO, 2006).

A large part of the younger population in developing countries, including Vietnam, suffers from malnutrition due to poor access to sufficient quantity of nutritious food (Neumann et al., 2002). The cause of that are widespread poverty, lack of nutritive commodities and an almost fully vegetarian diet. The extreme weather in the tropics makes it sometimes hard to get a high yield from cultivating vegetables and grains. This in turn affects the people's possibility of having a high protein- and energy consisting diet through the year (Neumann *et al*., 2002).
In developing countries agriculture represent 80 % of the population's livelihood, which makes it even more important for agricultural development (McMichael *et al*., 2007). Keeping ruminants for milk and meat production is not yet very common in Vietnam and the access to nutrient feed all year round is also a difficulty due to the weather conditions. The seasonal shifting of nutrition in feed contributes to a hurdle concerning milk yield and animal growth. To increase the nutritional value of food, people in developing countries should increase their meat intake per capita (Garnet, 2009).

The prospective for year 2050 predict that people in developing countries will on the average eat 44 kg of meat and 78 kg of milk $person^{-1}$ and $year^{-1}$ and people in developed countries consume 227 kg milk and 103 kg meat $person^{-1}$ and $year^{-1}$ (FAO, 2006). It means developing countries need to increase their intake and developed countries need to decrease their intake compared to the current amounts. In East Asia the average intake of dairy products is 11 kg $year^{-1}$ and meat intake is 10 kg $person^{-1}$ and $year^{-1}$ (FAO, 2006).

It has been demonstrated that tropical plants such as legumes containing tannins are a possible source of feed to ruminants in the tropics (Puchala *et la.*, 2005). When tropical legumes were used as feed for ruminants CH_4 emission was reduced compared to a diet containing crabgrass. This is believed to be because of a mechanism the tannins perform in the rumen. Studies made on legumes both *in vitro* and *in vivo* have shown that CH_4 production can be reduced by tannin rich foliage and therefore the interest of using such plants has significantly increased (Kamra *et al*., 2006).

Nitrate is a multifunctional chemical, which can be used in feed instead of urea for additional contribution of nitrogen (N) (Leng *et al*., 2010; Sakthivel *et al*., 2012). By adding nitrate the methane emission can be reduced while the animal performance is maintained or even increased. Nitrate can be one factor helping to reduce the enteric GHG emission from ruminants.

The aim of this study was to investigate, using *in vitro* technique, a potential tropical feed supplement to cattle, which not only had a high crude protein value to increase the animal performance but also possibly reduces the enteric emission in ruminants. It was hypothesized that the legume with highest content of tannins; *Acacia mangium,* dried in room temperature with addition of nitrate will reduce the amount of methane emission most.

Background

Greenhouse emission in South-East Asia

Livestock production is increasing very quickly since the people all over the world are changing their diets, especially in developing countries such as Vietnam (FAO, 2006). Together with changed diet and enlarged livestock production environmental impact is also increasing. This dilemma of increased requirements of food and nutritional feed conflicts with the requirements developing countries have to reduce their GHG emission. Agriculture represent approximately 80 % GHG (McMichael *et al*., 2007) from the 80 % human affected GHG produced $year^{-1}$ (FAO, 2008b).

Given that ruminating livestock not only helps to supply humans with food but also with wool, leather, maintaining biodiversity, preserve nutritious soils and keep the landscape open the methane emission also has to be compared to what it brings back (Garnet, 2009). Vietnam is struggling to increase people's standards of living. Meanwhile the living standard is increasing so are also the GHG emission $capita^{-1}$ (Herrero *et al*., 2011). Vietnam along with other developing countries are getting requirements to reduce their GHG $year^{-1}$, which makes this to a dilemma and a long-term work (Committee on Climate Change, 2008).

Methane emission from cattle

Enteric methane is produced by ruminants in their naturally occuring rumen fermentation (Nilsson, 2009). Inside the rumen millions of anaerobic living microbes, bacteria, archea protozoas and fungis are fermentating the organic matter the ruminant is ingesting. Without the microbes the ruminants could not assimilate the nutrition in the feed. The bacteria and protozoa in the rumen are always trying to keep certain environment in the rumen to make the anaerobic fermentation even to work (Nilsson, 2009).

Mostly the ruminant's feed consists of grass and different types of plants which are composed of carbohydrates (Bannink *et al*, 2005). Other words for structural carbohydrates is fibre and neutral detergent fibre (NDF) which means they can not be dissolved in neutral detergent. The rumen's largest task is to ferment the fibre. It means the bacteria and fungi in the rumen needs to have good properties to fermentate fibres. Fermentation leads to degradation of carbohydrates to monomers which then will be used as a source of nutrition and building blocks to volatile fatty acids (VFA) and gases. Protein is also degraded in the rumen for building blocks to microbes and is also contributing to gas production (Sjaastad *et al*., 2003). The part of protein not degraded in the rumen is so called bypass protein. This protein will together with the protein from the microbes pass the rumen and enter the abomasum and the intestines. In the intestines the protein will be degraded and absorbed representing the animal's protein source (Sjaastad *et al*., 2003).

The ruminants energy source comes from the VFA's acetic acid, butyric acid and propionic acid as can be seen in figure 1 (Bannink *et al*, 2005). The VFA can not be produced without the gas production. The gas produced is CO_2 which uses hydrogen (H_2) to be reduced to CH_4 and is a natural part of the rumination. Studies have shown that there is a 6 – 8 % loss in gross energy intake from gases during fermentation in the rumen (Nilsson, 2009).

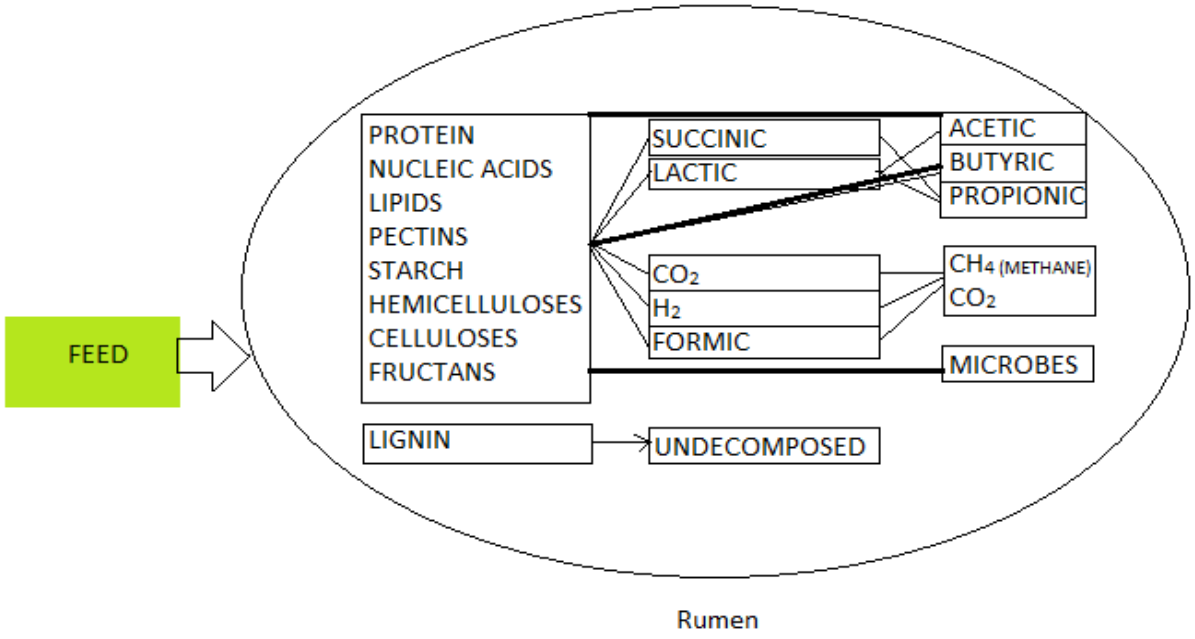

Figure 1. Schematic picture over degradation of feed inside a rumen and which components that are reconstructed. Figure adapted from text in Mjölkkor by Nilsson, 2009.

Ruminants can also get energy from starch degradation (Bannink *et al*, 2005). Starch is found in grains and tubers and not really a natural energy source for ruminants. A large part of a ruminants starch intake are passing the rumen and will be decomposed and absorbed in the small intestine as an energy source. This extra energy source is especially needed when animal performance needs to be increased for example milk production for dairy cows or growth for beef cattle. Since not all starch is fermentated in the rumen less gas will be produced when feeding with starch. Too much starch on the other hand can make the ruminant diseased because the rumen require straw to function properly. Starch makes the

rumen pH drop and if it drops below pH 5.5 the activity of the microbes stops. When feeding with grass or other carbohydrate consisting feed to ruminants the usual pH in the rumen is pH 7.0 ± 0.2 (Nilsson, 2009).

Tropical foliages

Already year 1977 a study were performed on cassava (*Manihot esculenta* Crantz) foliage as a potential feed for cattle because of its protein and starch content (Ffoulkes *et al*., 1977). Even though it is not a legume the protein content in cassava foliage is high, in average 210g kg^{-1} (Ravindran, 1993). But because of high levels of hydrogen cyanide (HCN), which is toxic for ruminants, it has limitations as a feed (Kumar, 1992). Other foliage high in protein is legumes, which have an average of 200-300 g protein/kg DM and also a high fiber content, 500-600g NDF/kg DM (McDonald *et al*., 2002). Studies made in the early 1990s discovered the lethal level of HCN in feed is 2-4mg HCN kg^{-1} for cattle and sheep but much lower for monogastric animals (Kumar, 1992). The toxic effect from HCN comes when it is released from its original place in the plant, for example when the plant tissue is damaged. Linamarin and lotaustralin, glucosides in cassava that are cyanogenic, are hydrolysed by linamarase in plant when the cell walls are broken and by β-glucosides in the intestinal tract (Ravindran, 1993). Linamarase and β-glucosides are only coming in contact with each other in wither plants and when plant tissue is damaged. The cyanide is detoxicated through reaction with thiosulphate to become thiocyanate, which is 200 times less toxic then cyanide. However, thiocyanate is not a desired substance because it can cause goiter to the animal. Other symptoms from HCN poisoning are reduced growth and trouble with the gastrointestinal (Ravindran, 1993).

Dried cassava root have been used for a long time in small amount because of its high proportion of starch (Geoffroy *et al*., 1983). A recent study on lambs tried to find strategies to avoid intoxication from HCN when feeding with fresh cassava (Hue *et al*., 2012). The results from that study showed that an adaption time for seven days with slowly increased level of cassava foliage is preferred. An involvement of maximum 2 % cassava foliage showed no negative effects on the rumen, respiration- or heart rate but increased live weight gain in response to increased daily intake (*Hue et al*., 2012).

The restricted use of cassava as a feed for cattle is due to its HCN content (Pham Ho *et al*., 2009). Every year a large amount of cassava leaf and root peal was left on the field in Vietnam after harvesting season of cassava root. Studies were made on those leftovers for a potential feed supplement for ruminants (Khang *et al*., 2000; Pham Ho *et al*., 2009). The result showed that sun-drying cassava foliage made 86 % of the amount of HCN disappear and even more if the leaves were cut into small pieces (Ravindran *et al*., 1987). Tropical foliage has a high content of condensed tannins, CT, which do not have a positive effect on the animal consuming the foliage (McDonald, *et al*., 2002). Legumes have an average of 108 kg N ha^{-1} and yr^{-1} depending on which kind of legume it is (Peoples *et al*., 2009). This is by itself a reasonably high protein value affecting the animal's productivity by providing them a large amount of nitrogen (N). This makes legumes to an even stronger candidate for being a feed supplement to ruminants. Legumes are easy to grow since they do not need any addition of fertilizer, exists in large parts of the tropics and grows wild (Peoples *et al*., 2009).

Tannins

Studies have been made on tropical legume as a potential feed for ruminants (Barbehenn *et al*., 2011). Legumes are living in symbioses with nitrogen fixating bacteria, which increase the amount of nitrogen in the foliage. It is also known legumes have the property of containing a substance called tannins. Tannins bind protein, which decrease the amount of free protein in the rumen and increase the amount of protein entering the small intestine (Wang *et al*., 1996). A change in the number of cellolytical bacteria in the rumen is also done by tannins, which lead to less number of microbes (Hess *et al*., 2003). However tannins have anti-nutritional effects on the rumen, which limits it usability as a feed supplement (Singh *et al*., 2003). A study from 2011 showed that the actual drying progress reduces the methane production during the fermentation and make the tannins in the legumes go in to an invertible form nontoxic to animals (Barbehenn *et al*., 2011).

A mechanism that tannins are involved in, which is not yet fully comprehended, creates a complex containing protein and tannins in the rumen (Hess *et al*., 2003). When this complex enters the abomasum and the pH drops to approximately 3.5 the protein is released and can be utilized by the ruminant (Kariuki, 2004; Mueller-Harvey, 2006; Lorenz, 2011). The methane is reduced since the fiber digestion is reduced. It results in reduced H_2-production, which in turn yields less substrate to create methane and the methanogenesis is slowed down (Tavendale *et al*., 2005). On the other hand tannins are also an anti-nutritional substance for ruminants, which can reduce feed intake, reduce growth, reduce digestion of protein and DM and reduce milk production (Makkar *et al*., 1995). In a study from 2011 it was found that the level of tannins was not linked to the decomposition of protein (Lorenz, 2011).

Tannins affect several enzymes in the intestinal tract and growth hormones for ruminants (Hess et al., 2003). High concentration of tannins in feed can cause gastric ulceration and enhanced secretion of the mucosa (Mitjavila *et al*., 1977). Tannins in too high concentrations can give high levels of methaemoglobin in the blood, which leads to impaired ability of oxygen absorption, which affects the whole animal (Zhu *et al*., 1995). Tannins are found in two forms, condensed tannins (CT) and hydrolysable tannins (HT) where HT is the toxic version and is rare in legumes (Waghorn *et al*., 2003; Animut *et al*., 2008). The kidney and liver can be damaged from HT because it is causing necrosis in the tissue-cells or even cause death to the animal (Zhu *et al*., 1995). Diets containing tannins can reduce the methane emission, which is expressed in lower DM intake day^{-1} (Puchala *et al*., 2005). By conserving tropical tannin-containing foliage the amount of soluble N is reduced which increase the nutritive value of the feed (Albrecht *et al*., 1991). One way to dilute the effect of tannins is to mix feed and ad an energy source in terms of a starch-containing feed (Mueller-Harvey, 2006).

The use of tannin containing plants in the tropics is increasing because of is positive effect of animal performance (Makkar *et al*., 1995). The level of tannins in the plants also has an important role in the reducing effects on CH_4 (Kongvongxay *et al*., 2001). A study from 2008 showed similar properties as a bypass protein in some foliage (Tran, 2008). A negative correlation was found between tannin content and amount produced methane gas, i.e. the more tannin the plant contained the less methane was produced. The correlation was $r = -0,76$ (Hariadi *et al*., 2010). If the level of CT represents 4 % of the DM in the plant it will increase the nutrient content in the feed by binding to proteins. This keeps the protein safe from degradation in the rumen and increase amount of protein that enters the intestines (Barry *et al*., 1999).

With CT present in the feed, it helps binding proteins to themselves creating a kind of protein precipitate, avoiding degradation in the rumen (McDonald, *et al*., 2002). If there is too much CT it binds to the protein to hard so it will not let go of the protein in the small intestinal. Foliage with a content above 100g CT/kg DM feed is considered to have a high content of CT. When the level of CT is high, the digestibility is usually low too as well as the palatability (McDonald, *et al*., 2002).

Results from Drobnic-Kosorok's (1995) study showed that depending on the level of protein in the tested substrate, tannins reduced the amount of protein degradable in the rumen and converting them to bypass protein which lead to reduced production of methane emission (Drobnic-Kosorok, 1995).

Conservation of legumes

To be able to store feed during periods of drought in countries resemble to Vietnam, drying is a possible conservation method. Only a few studies are made on drying tropical legumes in different ways but studies of freeze-drying tropical legumes showed results of a very low conservation of the nutrition (Tiemann *et al*., 2009). Conservation of feed in the tropics is desirable since the supply is varied through the year in due to heavy rain and dry seasons. One way of feed conservation is to dry it in a way that keeps the nutrition value nearly intact and makes it possible to store for a long time in hot temperature. Sun-dried feed is usually applied but only a few studies have been made on other kinds of drying methods such as drying in room temperature or in oven. Year 2010 a study made on fresh and sun-dried cassava was made in Vietnam by Bounthavone *et al*., which showed that the DM intake was higher when the animals were fed fresh feed and not sun-dried.

Nitrate

Nitrate reduces the amount of available hydrogen in the rumen, which affects the production of methane (Leng, 2008). Two studies have shown reduction in methane emission when nitrate was added to the ruminants feed but with no changes in feed intake (Le Thi Ngoc *et al*., 2010; Sakthivel *et al*., 2012). A hydrogen sink reduces the substrate to produce methane (Le Thuy Binh *et al*., 2011). The chemical nitrate has been highly questionable about being fed to animals because of its toxic effects. Nitrate itself is not toxic but when nitrate is reduced to ammonia the toxic intermediate nitrite is produced and can cause death to the animal (McDonald *et al*., 2002; Leng, 2008). Nitrite binds to haemoglobin and becomes methaemoglobinaemia, which reduces the capacity of transporting oxygen in the blood (McDonald *et al*., 2002). Depending on what kind of feed the animal is given, nitrate can replace carbon dioxide in the methanogenesis and become an electron acceptor, which reduces the amount of methane (Sakthivel *et al*., 2012). It has though been discovered that ruminants can be safely fed with feed containing nitrate because the microbes can use it for nitrogen requirements during the fermentation without producing nitrite (Leng, 2008). If the cattle are fed with soluble carbohydrates at the same time as nitrate, it can be less toxic (McDonald *et al*., 2002). The toxicity level by nitrate is 0.7 g nitrate-N kg^{-1} DM for cattle but the lethal level is 2.2 g nitrate-N kg^{-1} DM, which means animals are showing symptoms of intoxication at reasonably low levels of nitrate in the feed. The level of nitrate in feed varies a lot but is correlated to the amount of crude protein (McDonald *et al*., 2002).

Material and methods

The experiment took place at the Livestock Feed Research and Trial Station at the National Institute of Animal Science, (NIAS), Hanoi in Vietnam. Five foliages were chosen, four legumes; *Leucaena leucocephala* (Leu), *Stylosanthes guianensis* (Sty), *Acacia mangium* (Aca) and *Flemingia macrophylla* (Fle) and one euphorbiaceae; *Manihot esculenta Crantz* (Man) was used as a reference. The legumes Leu and Man was collected at the farming land of the Forage and Pasture Department owned by NIAS. The other three foliage, Sty, Aca and Fle, was collected at the Goat and Rabbit Research Department part of in the province of BaVi, 60 km northwest of Hanoi belonging to NIAS. All samples were randomly collected from five different places on the field and later on mixed and pooled into one sample.

Drying methods

Each foliage was separated to four bags representing one of four ways of drying methods. The drying methods chosen were sundried (SD), dried in room temperature (DRT), heat dried in oven (HD) and freeze dried (FD). During sun drying samples from all five foliage were placed outside, figure 2, in the sun on sheets of newspaper placed on wooden tables for three days when the average outside temperature was 38° C. The samples were turned and mixed twice a day during the drying period. After three days the samples were put back in small marked bags.

Figure 2. Recently collected foliage samples placed outside to dry in the sun. Photo: Sofie Winding

The average room temperature in the laboratory where samples were dried was 28° C. Samples were spread on sheets of newspaper (figure 3) on the floor in the laboratory and dried for seven days and twice a day turned and mixed.

Figure 3. Foliage sample placed on newspaper in the laboratory to dry in room temperature. Photo: Sofie Winding

Samples dried in hot temperature were placed in metal buckets and put in a 100° C oven for three hours. After one and a half hour in the oven samples were taken out, (figure 4) mixed and turned.

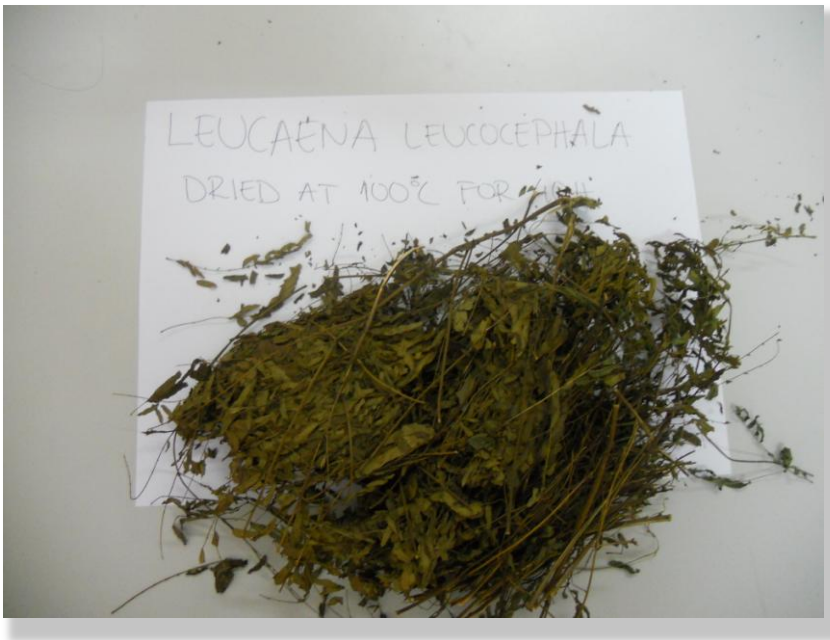

Figure 4. Foliage sample after dried in oven at 100° C for three hours. Photo: Sofie Winding

In the freeze dry method small glass bottles were filled with samples and attached to the vacuum- and freeze machine. Ice were filled in large Styrofoam boxes which the bottles were placed in to help keep them cold while the machine made the samples -86° C and the vacuum sucked all the liquid out as showed in figure 5. Four days was needed to freeze dry the samples. The machine was only used during daytime, which means over night the bottles were placed in a freezer with the temperature of -50° C without any suction.

Figure 5. Freeze and vacuum machine in which samples from the foliage are placed in glass bottles. Underneath and around glass bottles ice is placed to help keep the samples cool. Photo: Sofie Winding

Analyzing foliage content

Samples from the different plants were sent together with cassava root meal to the laboratory for analyzing the content according to the standard methods of AOAC (1990). For the CP content, 984.13 and NDF (aNDF) according to Van Soest *et al* (1991). The amount of total tannins, TT, was analyzed according to AOAC (1975) 30.018 and expressed in g kg^{-1} DM. The rest of the samples were chopped into small pieces, 3 to 4 cm^2.

Dry matter content

When all the samples were dried in the four different methods the foliage were placed in small marked resealable plastic bags before analyzing the DM. Three replicates of each foliage and each drying method were made to measure the DM which equals to the total of 60 Petri bowls. The empty Petri was weighted before adding any samples, the scale was then tared and one gram of sample was added and both weights were recorded. Later on they were put in a 100° C oven for 24 hours to dry and after cooling down for two hours the total weight for the Petri and samples were measured and written down in order to calculate the DM. All samples were then put in a grinder to make meal of it to get a homogenous mixture (figure 6).

By knowing the DM and the percentage of the N content, the amount of N could be calculated for each foliage and compared to one another. Given that the amount of N should be the same in all mixes, urea which is rich in N, had to be added to some samples to increase the level of N. Calculations for all samples were made on top of content-calculations for cassava root meal and calcium nitrate.

Figure 6. Grinder where all the samples were shopped into meal. Photo: Sofie Winding

A mix of 11 to 12 g for each sample was needed and therefore recipes with addition of the specific foliage, cassava root meal, calcium nitrate and urea were made for all ten different substrate mixes. Cassava root meal was added to be an energy source under the fermentation. Seven days before the collection of rumen fluid the adaption period started. The cattle was given calcium-nitrate, $Ca(NO_3)_2$, in small amounts increasing to 66 g day^{-1} split on two feeding occasions to prepare the microbes with the substance.

In vitro technique

By using in *vitro gas* production instead of animals, less animal experiments is needed (Soliva *et al*., 2008). By simulating the rumen in glass syringes a result close to the true value will be achieved (Menke *et al*., 1979). With this method, major part of the experiments can be done in advance without any animals involved. Experiments with inclusion of animals can be done from only a few of the in vitro experiments only to avoid animal experiments in a larger extent. To determine the methane concentration the gas was analyzed with a portable GASMET DX4030 gear using the CO_2 Technique, which measure the CO_2 content and then calculate the ration CH_4/CO_2. (Gasmet, 2012).

Experimental design

An in vitro experiment was performed to test the foliage mixes with the technique presented by Menke and Steingass (1988). In brief; the experiment was done in four steps, one for each drying method, with all five mixes with and without addition of calcium nitrate. All of them performed in triplicates which resulted in 5 x 2 x 3 samples plus three blanks which equals to 33 x 100 ml calibrated glass syringes. A milligram scale were used to measure 500 mg substrate to add to each syringe that was provided with a Vaseline lubricated piston, to make it slide in to the syringe, a piece of rubber tube and rubber bands on its tip to make the syringe airtight. The rubber tubes and bands were used to open and remove the produced gas from the calibrated glass syringes. All syringes were put in a 39° C incubator over night to give the substrate the same temperature as in the rumen and to make sure all the syringes were airtight (figure 7).

Figure 7. Glass syringe prepared with mealed and mixed foliage sample before putting in oven overnight. Photo: Sofie Winding

The next day 500 ml rumen fluid was collected from one selected adult female local Yellow Cattle with the age of seven years old. A vacuum pump and a rubber tube rubbed with vegetable oil were used to stick down the throat of the cattle to reach the rumen (figure 8).x

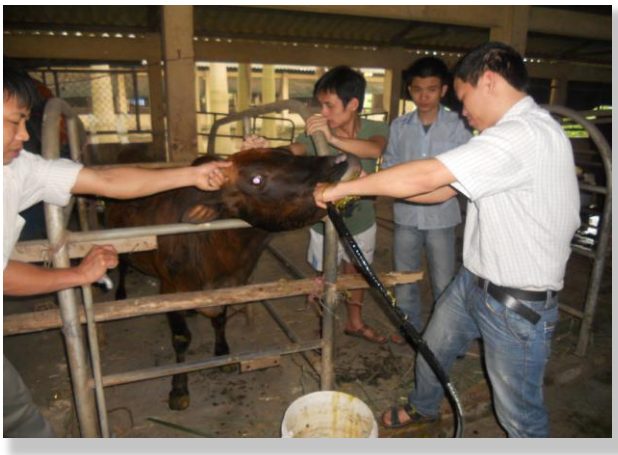

Figure 7. Collecting rumen fluid from a selected adult local Yellow Cattle. Photo: Sofie Winding

Rumen fluid was collected from one specific cow throughout the whole study to avoid individual differences. The diet the specific cattle where given was feed containing 50 % rice straw and 50 % elephant grass (*Pennisetum purpureum*) with the total DM of 8 kg day^{-1}. The cattle was placed inside a stable and tied in an individual pen and was manually fed twice a day, in the morning (07.30) and in the afternoon (16.30) and had free access to fresh water every day. The rumen fluid was filtered thru three layers of mesh fabric into a conical flask surrounded by 39° C water to keep the same temperature as in the rumen and with a lid to keep the environment anaerobic as much as possible (figure 9).

Figure 8. Filtrating rumen fluid through three layers of mesh fabric directly after collecting the fluid from the cattle. Photo: Sofie Winding

The pH in the rumen liquid was measured three times with a digital pH meter to get a more accurate value before it all started. The substrate-prepared glass syringes was filled with 30 ± 1.0 mL of buffer- and rumen fluid mix, air was removed and the exact volume was recorded and the syringes put back in the 39° C incubator (figure 10).

Figure 9. Glass syringe prepared with foliage mix, nitrate and rumen fluid. Photo: Sofie Winding

Data collection and sampling procedure

After three hours of incubation the volume of gas in each glass syringe was recorded and the gas was removed from the glass syringe and readjusted to 30 mL. The gas was transferred to plastic syringes filled with some water to turn upside down to avoid gas from leaking out and this was repeated after 3, 6, 9, 12, 24 and 48 hours of incubation (figure 11). After the final reading pH was measured in all samples with the pH meter and then all replicates of the same sample were pooled and put in the freezer for later analyzing. This was repeated four times,

one for each drying method.

All the plastic syringes was taken to the laboratory for storing, as in figure 12, and after collecting all volume data the gas was analyzed to find out the concentration of CH_4 since the gas contained both CH_4 and CO_2.

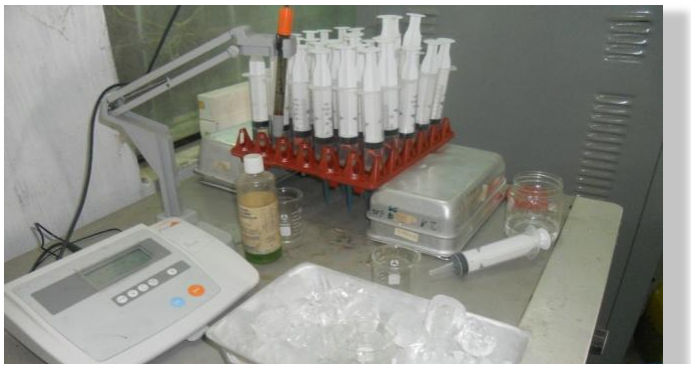

Figure 10. Transfer of produced methane from foliage sample mixed with rumen fluid, buffer and nitrate into plastic syringes for storing of methane gas. Photo: Sofie Winding

Figure 11. Plastic syringes for storing of methane gas. Photo: Sofie Winding

Statistical analyze

Data from 5 legumes x 2 treatments x 4 drying methods were analyzed with the General Linear Model in the ANOVA program using Minitab Software (version 16.0) with the model:

$$Y_{ijk} = \mu + L_i + T_j + D_k + LT_{ij} + LD_{ik} + TD_{jk} + LTD_{ijk} + e_{ijk}$$

Where Y_{ijk} is the dependent variable; μ is the overall mean, L_i the effect of legumes, T_j the effect of treatments, D_k the effect of drying method. LT_{ij}, LD_{ik}, TD_{jk}, LTD_{ijk} are legume x treatment, legume x drying method, treatment x drying method and legume x treatment x drying method interaction, respectively; e_{ijk} is the random error effect.

Results

Table 1 show the average of total DM from three samples for each foliage after being dried. When dried in room temperature DM varies between 87.0 % to 93.6 %. Heat-dried samples varies between 85.5 % to 95.1 % of the DM. Samples dried in the sun varies from 84.2 % to 92.9 % of DM and for freeze-dry the DM was ranging between 88.1 % to 92.4 %. The foliage chemical composition are shown in table 2. *Acacia mangium* had the largest content of tannins and *Stylosanthes guianensis* had the leastconten of tannins in comparision with the other foliages. The reference *Manihot esculenta* Crantz had the lowest tannin content of these five foliage.

Table 1. Dry matter (DM) for plant samples dried in room temperature, heat dried in oven, sundried and freeze-dried.

Plant species	Room	Heat	Sun	Freeze
	% in DM			
Acacia mangium	90.3	89.5	92.1	89.2
Flemingia macrophylla	93.9	95.1	92.0	90.0
Leucaena leucocephala	87.0	93.6	92.4	92.4
Manihot esculenta Crantz	88.9	85.5	84.2	88.9
Stylosanthes guianensis	92.5	94.7	92.9	88.1

Table 2. The chemical composition of crude protein (CP), nitrogen (N), total tannins (TT) and neutral detergent fibre (NDF) in plant samples.

Plant	CP	N	TT	NDF
	g/kg DM	g/kg DM	g/kg DM	g/kg DM
Acacia mangium	135,3	22,1	54,0	46,4
Flemingia macrophylla	189,5	30,3	46,7	57,0
Leucaena leucocephala	260,3	41,7	34,8	32,0
Manihot esculenta Crantz	155,4	24,9	18,9	43,0
Stylosanthes guianensis	157,8	25,3	23,9	57,1

The LS means for the amount methane produced during 48 hours of incubation with and without addition of calcium nitrate are shown in figure 13. There were no significant differences between the treatments addition of calcium nitrate and no addition of calcium nitrate.

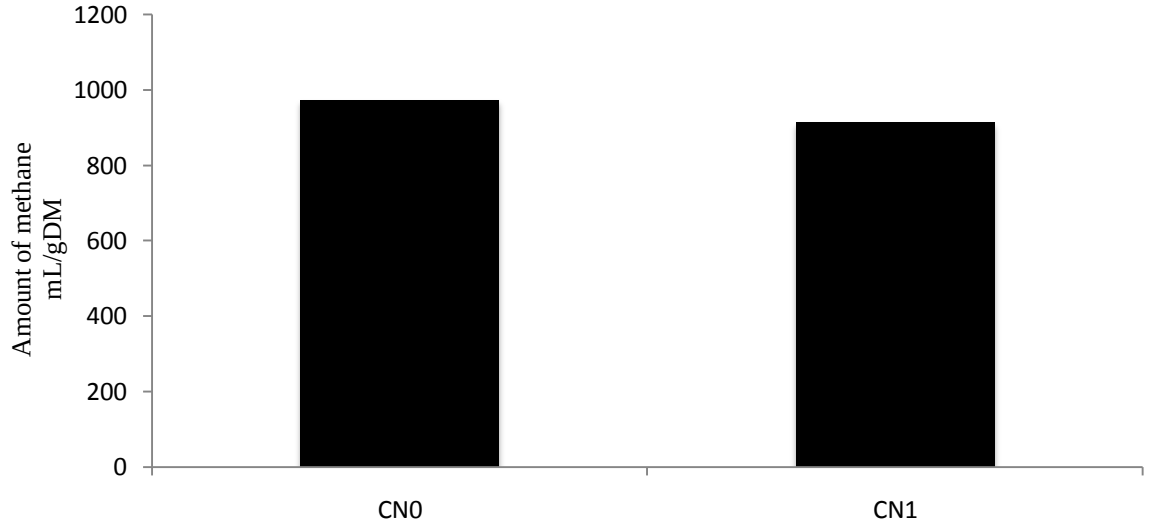

Figure 13. LS means with SE (23.5) in methane production in mL/g DM during 48h of incubation for the legumes Aca, Fle, Leu, Sty and the reference Man dried in room temperature, freeze-dried, sundried and heat-dried with addition of calcium nitrate (CN1) and without addition of calcium nitrate (CN0).

In figure 14 results can be seen for the four drying methods, dried in room temperature, heat-dried in the oven, sundried or freeze-dried. Each column represents the LS means for the amount of methane produced (mL/g DM) after 48 h of incubation from one of the drying methods where all legyms are pooled together with and without addition of calcium nitrate. There were a significant ($P<0.05$) difference between the method freeze-dry and the other three methods which reduced the amount of methane the least. Samples dried in room temperature reduced methane emission the most but there is no significant difference compared to heat-dried and sundried.

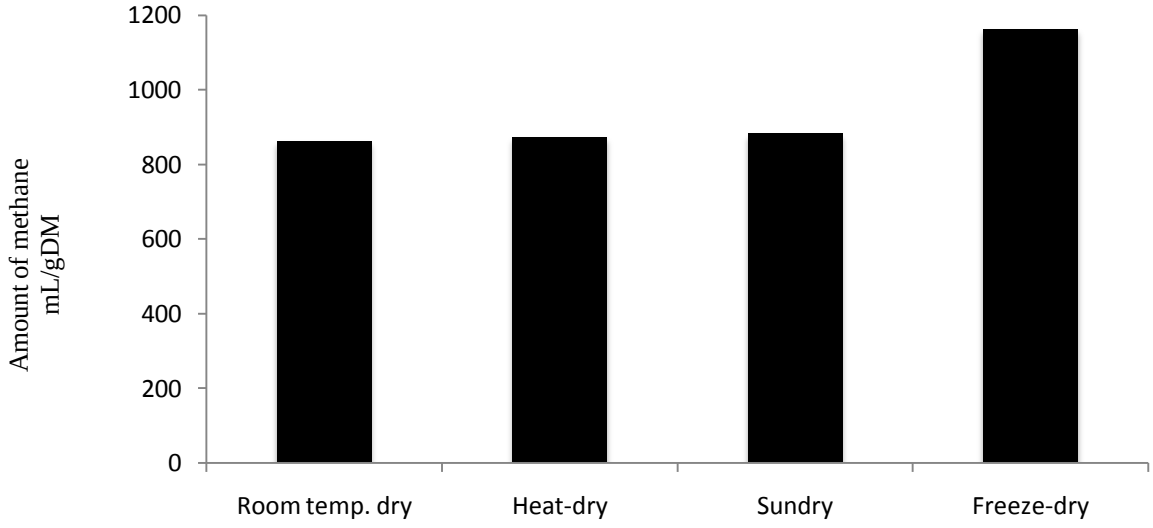

Figure 14. LS means with SE (33.2) in methane production in mL/g DM during 48h of incubation for the legumes Aca, Fle, Leu, Sty and the reference Man with and without addition of calcium nitrate dried in room temperature, heat-dried, sundried and freeze-dried.

Figure 15 shows the LS means for produced methane emission after 48 h of incubation for all four legumes and the reference in the four drying methods and for all with and without addition of calcium nitrate. After 48h of incubation Aca and Fle reduced methane emission significantly ($P<0.05$) more than the Leu and Sty.

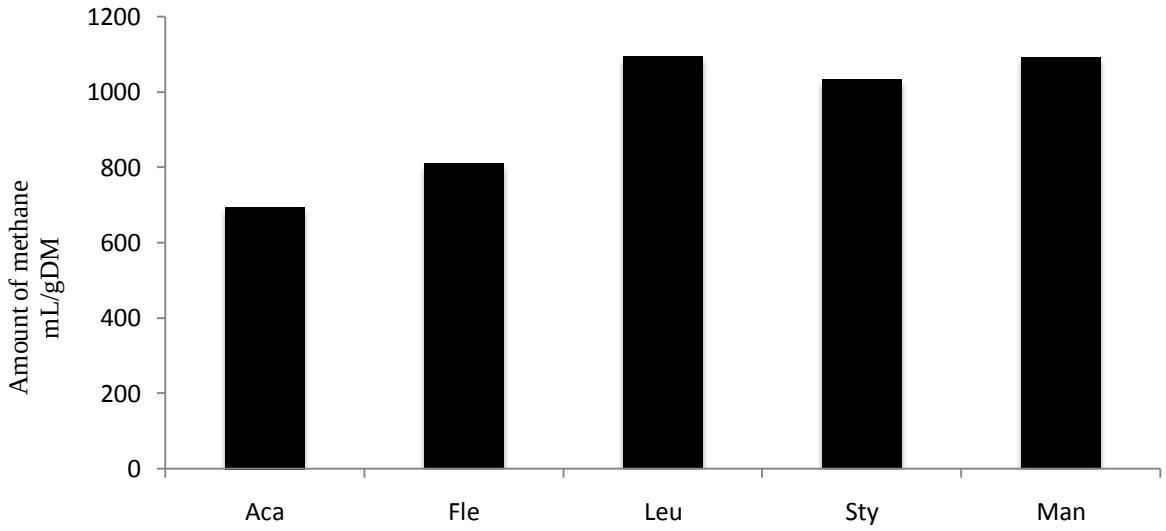

Figure 15. LS means with SE (37.2) for the amount of methane gas produced from samples with and without addition of calcium nitrate, dried in the four methods freeze-dried, sundried, heat-dried and dried in room temperature for the legumes Aca, Fle, Leu, Sty and the reference Man.

Discussion

The hypothesis of this study was that samples dried in room temperature would reduce methane emission the most since the time of drying foliage to a desired DM is the longest for the drying methods tested. However, the results showed that oven-dried samples reduces methane minimal more compared with sun-dried samples. Freeze-dried samples reduced methane the least and according to the hypothesis it should be oven-dried since those samples only dried for three hours and freeze-dry samples for 36 hours. This shows that time for drying is not the only factor when it comes to drying feed foliage in order to reduce methane production in the rumen. Other aspects are clearly needed to be considered.

One thing worth considering is what happens to the foliage chemical components during the drying. Since oven-dried samples reduce more methane *in vitro* compared to sun-and freeze-dried samples the 100^0 C heat must do something to the component in the foliage, which affects the emission. Protein degradation in oven-dried samples must be significant because of the high temperature and affect the nutritive value. This affects the possibilities of using this method when producing a nutritious feed. A feed with high nutrient content needs high levels of bypass protein to affect the animal performance for ruminants (Hess *et al*., 2003). The question is if oven-dried samples help increasing the amount of bypass protein in the foliage or not. High temperature for a short time in contrast with low temperature for a long time; the impact on the foliage must be different.

One important question to consider is if the emission itself can be target for the reduction interventions or if it can be combined with increased animal performance and food consumption (Garnett, 2009). The best way to mitigate the GHG is to increase productivity

and decrease number of animals (Garnet, 2009; Hensen *et al*., 2006). That will indeed affect the GHG emissions and that is why it makes it even more important to improve the animal performance and increase the number of animals. The emission will definitely increase when more concentrates are produced as feed for dairy and beef cattle but that increase in GHG is much less than the decrease in reduced methane emissions (Williams *et al*., 2006). The result will be decreased amount of CH_4 kg^{-1} meat or milk produced.

To obtain a sustainable livestock production in developing countries a long-term breeding strategy is needed together with nutritive feed or feed supplement with methane reducing properties (Lascano *et al*., 2010). In order to mitigate methane, the substrate H_2 and CO_2 also needs to be reduced. The substrates also have environmental impact and a great loss of energy for the ruminant (Tavendale, 2005).

A study showed that energy loss in terms of CH_4 is higher from cattle in the tropics fed on tropical plants than for cattle fed on plants not growing in the tropics (Kurihara *et al*., 1999). The reason for that is tropical plants contains much lignin and fiber and the cattle in the tropics are not fed with a lot of concentrates. Concentrates contain high levels of non-fiber carbohydrate, i.e. starch, which givs the cattle energy-rich feed.

Samples dried in room temperature had the longest drying period and had the lowest value of produced methane. One thing to reflect is that room temperature in this study was set to 28° C which is above the usual inside temperature in a lot of other countries. If a lower room temperature was used the results may turned out differently with even lower methane production since an even longer drying time would be needed to reach the same DM. Luckily freeze-drying samples are not only mitigating methane production the least of these four methods but also a very expensive and hard method to practice on farm-level. The method of sun-dry foliage goes way back in time and is not only an easy way to dry but also a cheap method since the foliage only have to be cut and let be on the field for a couple of days and only flipped ones or twice during the drying period before collected when the drying is finished. Drying in room temperature would mean two transports of the foliage instead of one; from the field to inside and dry, and one from the drying room to the storing area.

The fact that addition of calcium nitrate did not affect the methane emission in the extent to be statistical significant in this study can be a result from very low amounts of substrates and few replicates. For definitely reliable result larger amounts of mixed substrates would be one way continue in this field of research. This result is not matching the result from the study made with buffalo rumen liquid (Sakthivel *et al*., 2012). In that study the nitrate had a positive effect on the animal when it were not given a too large amount of nitrate at the same time. Some small trends were shown in the samples with nitrate addition which had lower values of methane production compared to samples with no nitrate added. Several studies have showed nitrates potential as a supplement in feed for reducing methane and to be an N source and that is why more research need to be made on nitrate as a reliable feed supplement.

The explanation why Aca and Fle were the two foliage with least methane production can be their tannin content, which is not what Lorenz (2011) found in his study. It was discovered that the amount of tannins were not connected to utilization of protein. Those two were the ones with the highest tannin content of the foliage tested in this study. This means tannins are not only enhancing the amount of bypass protein in the feed by binding to proteins, which escapes the rumen degradation, but also mitigate methane gas production. Legumes foliage is definitely needed in the world wide battle against methane emission. This result is going

hand-in-hand with the result in a study made by Mueller-Harvey in 2006. Tannins have a positive effect on the animal in small amount. Too large amount of it will decrease the palatability and make it toxic. If a standard way of tropical feed conservations is found legumes should be included. It is argued that a large investment on making farmers in tropical countries feed their ruminants with tannin-rich foliage should be made (Animut *et al*., 2008). It would provide the animals with nutrient feed with high amounts of bypass protein which would improve the animal performance and give the farmer a more nutrient food source and more economical benefits (FAO, 2008a).

Conclusion

Results from addition of calcium nitrate or not was not significant but a minimal trend was observed in all samples that addition of calcium nitrate might affect the methane production. Further, greater studies need to investigate nitrates reducing effects with several different amounts of nitrate and not only two. Samples dried in the sun, heat dried in an oven or in room temperature reduced methane emission better than freeze-dried samples. *Acacia mangium* and *Flemingia macrophylla* turned out to be the two legumes that reduced methane the most. Those were the foliages with highest content of tannins in this study. A greater *in vitro* study of Aca and Fle is needed before applying these on *in situ* experiments. *Acacia mangium* and *Flemingia macrophylla* are potential feed supplements for ruminants in the tropics. An interesting future study would be a deeper comparison of the chemical composition in legumes dried in the sun, in room temperatur and in the oven.

Acknowledgments

I would like to specially thank:

My supervisor in Sweden, **Dr. Ewa Wredle**, for all the guidance and help I got from her. I would also like to thank my supervisor in Vietnam, **Dr. Chu Manh Thang,** for all guidance and the warm welcoming I got and the great hospitality Dr. Thang showed me. I would also like to show my gratitude to **miss Hang** for helping me during my experimental work and **mr Thang** for helping me during the whole experiment.

A special thanks to all the obliging students at the National Institute for Animal Husbandry in Hanoi, Vietnam, for making my stay in Vietnam to an unforgettable memory. I would like to give the greatest thanks off all to Swedish Internatinal Development Cooperation Agency, SIDA, for making this Minor Field Study real to me by financial support.

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