# Effects of high starch-high fat and roughage diets of lambs on methane production using in-vitro gas production techniques

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**Abstract**—The climate has been warming with 0.74 °C and the global average temperature has been increasing during the last century due to accumulation of greenhouse gases in the atmosphere from different sources. Enteric methane (CH4), one of the greenhouse gases, is produced through anaerobic fermentation of feed components in the rumen. Beside its contribution to global warming which is one of the challenge human beings are facing, CH4 emission from ruminants represent loss of energy. In order to develop appropriate mitigation strategies for enteric CH4 emission, measurement of CH4 produced from a ruminant fed a given type of diet is essential. In-vitro gas production technique was applied to evaluate CH4 production from three experimental feeds: high starch-high fat (CC), hay (HY), and standard hay (SH) with rumen fluid from lambs fed either hay (rumen fluid A) or high starch-high fat (rumen fluid B). The effects of these rumen fluids on in-vitro CH4 production were assessed. About 0.5 gram of each experimental feed type, with additional 2ml of cream sample to each high starch feed sample, was incubated with each inoculum rumen fluid. The volumes of CH4 produced by experimental feeds were calculated relative to dry matter incubated and relative to degraded dry matter. Roughage feed produced more CH4 relative to degraded dry matter (ml/g dDM) except within inoculum rumen fluid B in which standard hay produced insignificantly lower CH4 than high starch-high fat because of rumen fluid (diet of donor lambs) effect on fibrous feed. A rank of the experimental feeds on CH4 production depends on whether the CH4 output was expressed relative to dry matter incubated or degraded dry matter.

**Key words:** Enteric methane, In-vitro gas production, Rumen fluid, high startch-high fat, hay.



## 1. INTRODUCTION

The increasing of greenhouse gases' (GHGs) concentration in the atmosphere have contributed to increasing of global warming, which is one of the challenge facing human beings [14]. In addition to adverse effects on human-beings, the consequences of increasing greenhouse gases in the atmosphere alter the viability of plants, animals and microbes [13].

Methane (CH4) is an important greenhouse gas emitted into the atmosphere from different sources, among agricultural sector contributes most of it. Currently, the concentration of CH4 is more than double compared to that of pre-industrial time and it has global warming potential of 23 times that of carbon dioxide (CO2) [14, 12]. The high growth rate concentration and potential effect of CH4 on global warming induce international concern on its sources and mitigations. About 70% of CH4 emission is linked to human activities (anthropogenic) and the remaining 30% is from natural sources [7, 9]. Human activities include energy utilization, transport, industry and agriculture.

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The majority of anthropogenic CH4 emission from agricultural sector is released by ruminants. The growth of human population and demand for livestock products can be the major driving forces behind increasing livestock production. Livestock sector accounts for about 37% of anthropogenic CH4 emission [14]. Although prevention of CH4 emission from agriculture (e.g. livestock production) is impossible, it is possible to reduce CH4 emission from ruminant livestock by modifying their production systems.

Ruminant livestock cannot digest structural carbohydrates (cellulose and hemicelluloses) directly by their digestive enzyme instead they depend on rumen microfloras which degrade ingested plant polymers through fermentation before enzymatic digestion in the abomasums. Microorganisms in the ruminants have the ability to ferment feeds, in the absence of oxygen, into useful energy sources, volatile fatty acid. However, the microbial fermentation process in the rumen also generates waste products such as CO2 and CH4. The conversion of an ingested feed into CH4 in the rumen involves integrated activities of different species of rumen microbes (bacteria, protozoa, fungi and methanogens) in which methanogens, members of domain Archaea, accomplish the methanogenesis (methane production). Methane production in this way

not only contributes to environmental impact but also represents energy losses from ruminants. The average CH4 emission from sheep represents about 7.2% of gross energy intake or 22.15 g CH4 per day [10]. Thus, in general there is growing interests in reducing CH4 emission from ruminants.

Methane reduction would be advantageous both in terms of minimizing environmental impacts and energy loss from ruminants. One of the possible ways to reduce enteric CH4 production is through nutrition. Improvement of nutrition plays an important role in increasing productivity of the ruminants and at the same time in reducing the amount of CH4 production per unit of product [1]. Better nutrition increases digestibility of the diet which can be efficiently converted into products by ruminants. In other words, as productivity increases equivalent products can be maintained from less number of animals and as a result, the overall CH4 production is reduced [1].

Quantification of CH4 produced from a ruminant fed a given type of diet is essential to develop appropriate mitigation strategies for CH4 emission. Methane emissions can be measured using in-vivo and in-vitro methods. Compared to in-vivo, in-vitro technique is cheaper, rapid and simple to handle, which make it more attractive to use. The in-vitro method requires an inoculum rumen fluid to create similar environment for fermentation by rumen microbes so that it can simulate the in-vivo CH4 production. In this context, rumen fluid is also expected to be representative of rumen microbial composition to maintain their fermentation contribution similar to that of in-vivo. Diet of rumen fluid donor animal [3, 16] and experimental feed composition [2] can determine end-products of fermentation, for instance CH4 production.

The type of carbohydrate in the diet has impact on ruminal pH and microbial population [4] and influence the proportion of each volatile fatty acid (VFA) produced which in turn influence CH4 production [1]. Johnson and Johnson [13] found that fermentation of cell wall of carbohydrates produces higher ratio of acetic to propionic acid than its non-cell wall components (soluble carbohydrates or sugars) and thus, the digested cell wall of carbohydrates leads to higher CH4 production compared to the sugars. The effects of carbohydrate type on CH4 production has been assessed in previous studies. However, the effect of a high starch-high fat feed on CH4 production has not been assessed so far. Along with this, due to type of diets given to rumen fluid donor lambs the adaptation of the rumen microbial population can be vary. As a result, microbial activities in the in-vitro fermentation and hence, CH4 production might differ for experimental feeds. Therefore, the aims of this study were to investigate the effects of the experimental feeds as well as the effect of rumen fluids on CH4 production.

**Objectives**

- To investigate the effects of a high starch-high fat diet on CH4 production.
- To examine the effect of two distinct rumen fluids on CH4 production.

**Hypotheses**

Using high starch-high fat diet instead of roughage diets will reduce methane production per degraded dry matter.

The microbial population adapts depending on the diets given to the lambs, and the rumen fluid adapted to a high starch-high fat diet will be significantly different from the rumen fluid adapted to a roughage rich diet. In other words, CH4 production from these two rumen fluids is expected to be significantly different.

## 2. MATERIALS AND METHODS

### 2.1. Location

The study was conducted in the laboratory of Large Animal Science department, University of Copenhagen which was formerly known as the Royal Veterinary and Agricultural University.

### 2.2. Feed samples

Feed samples used in this study were sample of feeds fed to two groups of lambs, except standard hay which is a hay sample used in all in-vitro incubations at University of Copenhagen. The samples were taken from both roughage and concentrate feedstuffs. These included: a high starch-high fat diet (CC) consisting of 0.5 g flaked maize and 2 ml of cream, and a grass hay (HY) and standard hay (SH) from roughage feeds. All samples except cream were ground on a cyclone mill (Cyclotec 1093, Tecator, Höganäs, Sweden) with a 1 mm mesh before use and about 0.5g of each sample was added to F57 Fiber Filter Bags. For dry matter determination all samples were oven dried at 105 oC overnight. Additionally, Neutral Detergent Fiber (NDF) analyses were done for all samples using ANKOM200 Fiber Analyzer.

### 2.3. Rumen fluid and buffer preparation

Two different fresh rumen fluids from two pairs of lambs, kept on distinct diets: A, and B, were used. The rumen fluids were taken just after slaughter and named as rumen fluid A and rumen fluid B, based on the diet of donor lambs. The whole rumen contents were collected before feeding and filtered through a cheese cloth before added into pre-warmed insulated flasks separately and then immediately transferred to laboratory for incubation-medium preparation, see table below.

Table 1. Incubation-medium preparation components.

| Component solutions | Amounts | Component solutions | Amounts |
|---|---|---|---|
| Micro mineral solutions | 13 g CaCl2 * 2H2O | Macro mineral solutions | 5.7 g Na2HPO4 * 2 H2O |
| | 10 g MnCl2 * 4 H2O | | 6.2 g KH2PO4 |
| | 1 g CoCl2 * 6 H2O | | 0.6 g MgSO4 * 7 H2O |
| | 0.8 g FeCl3 * 6 H2O | | |
| Buffer solutions | 35 g NaHCO3 | Reducing agents | De-ionized water |
| | 4 g (NH4)HCO3 | | 1 M NaOH |
| Redox indicator | 100mg Resazurin | | Na2S.9 H2O |

The rumen fluids from the two different groups were separately mixed with solutions containing mixture of the same buffer and minerals (micro and macro). Medium preparation has been done as described by Menke and Steingass [6]. Continuous flushing with CO2 was done during preparation as well as during addition of the medium (90 ml) to each glass bottle containing feed sample.

### 2.4. Experimental design and in-vitro Gas Production Technique

The experiment was formulated with three different feeds and two distinct rumen fluids. Five replications of each experimental feed with each inoculum rumen fluid were used. Including blanks, totally 40 modules were randomly assigned numbers before divided into two equal numbers of modules and incubated into two incubators at 39 oC for 24 hr.

For in-vitro gas production, apparatus AnkomRF Gas Production System was used. The system is automatic and a wireless module fixed on each fermenting bottle communicates information with a computer. The fermenting bottles had been shaken by orbital shaking incubators in automate manner throughout the incubation period.

Gas produced during the incubation period is released from the system into a collecting bag when cumulative pressure in headspace of each bottle reaches pre-set threshold value (0.5psi). Release of the gas that exceeds a given threshold level throughout the incubation period maintains pressure in the fermenting bottles near atmospheric pressure. The cumulative pressure is recorded every 5 minutes in a computer spreadsheet. Finally, the gas samples were taken and analyzed for composition of CH4 using gas chromatography.

### 2.5. Data handling and statistical analysis

Collected data includes pH before and after fermentation, absolute pressure, cumulative pressure and composition of gas samples. Weight of feed samples before fermentation, DM of samples and DM weight after NDF had been taken for both raw feed as well as fermented feed samples. Before calculating the quantity of the total gas, the cumulative pressure of each feed sample was blank corrected, and the mean of the blank corrected cumulative pressure of each sample at 24 hours was calculated. To convert the cumulative pressure to volume of gas, the ideal gas law was applied. The coefficient of the linear regression of the standard CH4 was used to calculate the percentage composition of CH4 in the collected gas samples.

The effects of feeds and rumen fluids on CH4 production were modeled by a multiple linear regression and the effects were tested at significance level of 5%. All analyses were done with R, version

2.14.0 (www.r-project.org). The statistical model used for CH4 production is the following:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ij}$$

Where:

$y_{ij}$: Response variable (CH4 produced)

$\mu$: Mean value of response variable (overall mean)

$\alpha_i$: CH4production induced by feed type

$\beta_j$: CH4 production induced by rumen fluid type

$\alpha\beta_{ij}$: CH4 production induced by feed-inoculum rumen fluid interaction

$\varepsilon_{ij}$: Residual effect

## 3. RESULTS

### 3.1. Quantity of CH4 produced

Expressing CH4 outputs relative to dry matter incubated or relative to degraded dry matter determined the methane production ranking of experimental feed types. Therefore, the result will be shown in both calculations.

### 3.2. Quantity of CH4 produced relative to dry matter incubated

Amount of CH4 produced (ml/g DM) by each experimental feed with both rumen fluid A and B are shown in Fig. 1. The result showed that both experimental feed and rumen fluid types have high significant effect on CH4 production ($p<0.001$). Each feed type has significant effect on CH4 production in both rumen fluids and there was feed-rumen fluid interaction effect on CH4 production ($p < 0.001$). Volume of CH4 produced per dry matter incubated was lower for each roughage feed in both rumen fluids compared to a high starch-high fat feed. The amount of CH4 produced (ml/ g DM) by hay and high starch-high fat were 12.83 vs 15.27 ml/g DM within rumen fluid A and 3.14 vs 5.27 ml/g DM within rumen fluid B (Fig. 1). No significant difference was seen within roughage feeds in rumen fluid B ($p=1.00$) though hay produced slightly higher volume of CH4.

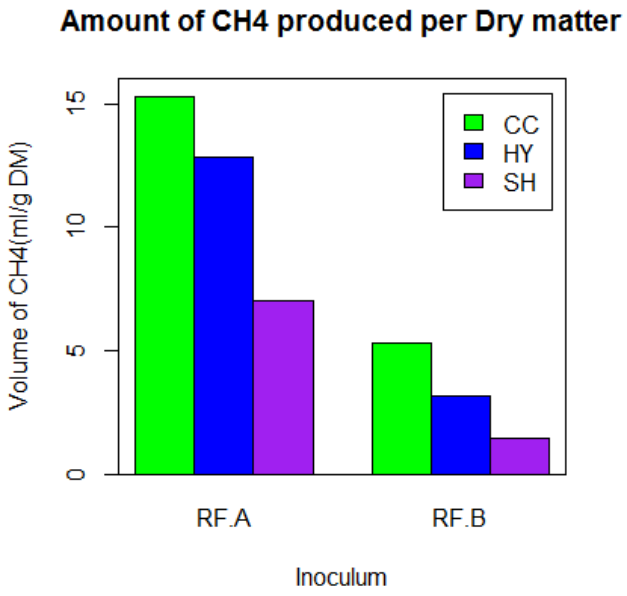


Fig. 1: Amount of CH4 produced (in ml) per dry matter of feed types within each rumen fluid for 24 hours (CC=high starch-high fat; HY=hay; SH=standard hay; RF.A=rumen fluid A; RF.B=rumen fluid B; n=5)

Similar to experimental feeds, rumen fluids also showed significant effect on CH4 production. For each experimental feed, the amount of CH4 produced (ml/g DM) was higher in rumen fluid A than in rumen fluid B (Fig. 1). There was highly significant difference ($p<0.001$) between rumen fluid A and rumen fluid B on amount of CH4 production for each feed type.

### 3.3. Quantity of CH4 produced relative to degraded dry matter

The volume of CH4 produced relative to degraded dry matter (ml/g dDM) for all experimental feeds are depicted in Fig. 2. High starch-high fat with rumen fluid A produced lower volume of CH4 than hay (15.6 vs 23.1 ml/g dDM) and than standard hay (15.6 vs 18.4 ml/g dDM). However, the difference was only significant between high starch-high fat and hay with inoculum A and there was no significant differences among feeds within rumen fluid B. Standard hay produced insignificantly lower CH4 than high starch-high fat in rumen fluid B ($p=0.675$).

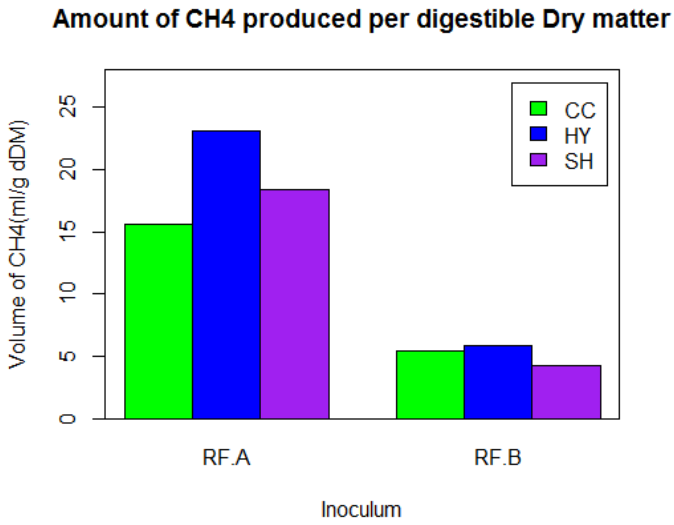


Fig. 2: Amount of CH4 produced (ml) per gram degraded dry matter of feed types within each rumen fluid for 24 hours (CC=high starch-high fat; HY=hay; SH=standard hay; RF.A=rumen fluid A; RF.B=rumen fluid B; n=5)

Each experimental feed produced highly significant different quantity of CH4 within rumen fluid A compared to within rumen fluid B and the difference was higher for hay (17.3 ml/g dDM) than the others and lower for high starch-high fat (10.2 ml/g dDM).

## 4. DISCUSSION

The amount of methane produced within 24 hours was calculated relative to dry matter incubated (Fig. 1) and degraded dry matter of feed types (Fig. 2). The rank of experimental feed types on total methane production depends on whether the methane outputs are expressed relative to gram dry matter incubated (ml/g DM) or gram degraded dry matter (ml/g dDM), and therefore it will be discussed in both ways of calculations.

### 4.1. Quantity of CH4 produced relative to dry matter incubated

The quantity of CH4 produced relative to gram dry matter of incubated samples in both rumen fluids were higher for a concentrate feed than roughage feeds and was ranked as: high starch-high fat>roughage: hay>standard hay (Fig. 1). More CH4 production (ml CH4/g DM) in a concentrate than roughage feeds was also found by Navarro-Villa et al [8], who compared CH4 production of three different feeds: grass silage, barley grain and barley straw relative to DM of the feeds incubated and degraded DM. These authors ranked from fibrous feed (barley straw) to concentrate feed (barley grain) with increasing order of CH4 production and reasoned out that CH4 production increases with amount of fermentable feed component.

The amount of CH4 produced by high starch-high fat was higher when the output is expressed per DM incubated due to more fermentable components and lower fiber content in high starch-high fat compared to roughage feeds. In fact, not only the amount of fermentable components of feed determine the amount of CH4 produced but also the proportion of VFA produced as end-product of fermentation. Fermentation of roughage feeds produce higher acetate than starch rich feed and as acetate proportion increases CH4 production per fermentable organic matter also increases [4]. To compare CH4 production in relation to level of digestibility of experimental feeds, expressing CH4 per degraded dry matter seems more preferable than expressing relative to dry matter incubated.

### 4.2. Quantity of CH4 produced relative to degraded dry matter

Roughage feeds produced more quantity of CH4 per degraded dry matter within rumen fluid A (Fig. 2) and the rank of feed types was: hay>standard hay>high starch-high fat. However, within inoculum B the standard hay produced insignificantly lower CH4 (ml CH4/g dDM) than high starch-high fat which could be due to the microbial activity of rumen fluids from concentrate-fed donor lambs affected in-vitro digestibility of fibrous feed. Standard hay contains highest neutral detergent fiber percentage (68.9%). The effect of microbial activity can be seen in that roughage feeds did not significantly produce different amount of CH4 from high starch-high fat within rumen fluid B but did so in rumen fluid A. It can be recognized that when CH4 calculated per degraded dry matter the in-vitro digestibility of roughage feeds within the rumen fluid B were more likely affected than that of high starch-high fat. Horton et al [3] and Tejido et al [16] reported that the in-vitro digestibility was reduced within rumen fluid from concentrate-fed donor animals. Addition of high level of a concentrate in the diets of donor animal has negative impact on microbial degradation [16]. According to these authors, addition of 80g concentrate reduced the pH of the rumen than addition of 20g of concentrate (pH: 6.2 vs 5.79). It has long been recognized that low pH of the rumen reduces the number of cellulolytic microorganisms, which consequently influences the in-vitro microbial degradation of roughage feeds [15]. Similarly, Ramos et al [11] reported that shifting of diet from forage to concentrate decreased the rumen pH. As a result, the

number of methanogens was reduced but the ruminal pH of cows fed on a forage diet relatively remained constant, 6.7-6.9 [5]. In the current study, the average final pHs were about 6.5 and 6.7 for a high starch-high fat and roughage feeds, respectively. Thus, it can be presumed that microbial activities in the rumen fluid from hay fed lambs were more due to optimal rumen environments for microbial growth. This could be the main reason for higher CH4 production within rumen fluid A. In generally, each experimental feed produced more CH4 within rumen fluid A as compared to that of rumen fluid B (Fig. 1 and Fig. 2), and the result showed that not only type of experimental feeds can determine total CH4 production but also type of rumen fluids used for incubation.

## 5. CONCLUSION

Both type of feed and rumen fluid have a significant effect on CH4 production. The rank of feed types on total gas and CH4 productions depends on whether the outputs were expressed relative to dry matter incubated or degraded dry matter. Roughage feeds produce more CH4 ml/g dDM than high starch-high fat. Standard hay produced insignificantly lower CH4 than high starch-high fat which could be due the effect of rumen fluids from concentrate-fed donor lambs on fibrous feed. The diet of the donor animal is the most important source of variation in CH4 production between rumen fluids. Methane productions of roughage feeds were more likely affected than a high starch-high fat feed by type of rumen fluids.

## ACKNOWLEDGMENT

I would like to express my deepest gratitude to my supervisors Hanne Helene Hansen and Ida Marie Lindhardt Drejer Storm for their advices and valuable comments.

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