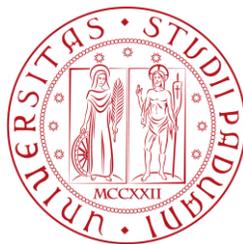


UNIVERSITÀ DEGLI STUDI DI PADOVA



DAFNAE

Corso di Laurea Magistrale in Scienze e Tecnologie per
l'Ambiente e il Territorio

*In vitro evaluation of methane production from rumen fluid and
faecal inoculum using an automatic gas production system*

Relatore
Dott. Tagliapietra Franco

Correlatori
Dott. Ramin Mohammad,
Dott. Cattani Mirko

Laureanda
Lerose Dora
Matricola n. 1034560

ANNO ACCADEMICO 2012-2013

Index

1. Abstract.....	5
2. Riassunto.....	7
3. Introduction.....	9
3.1 Ruminants' breeding's impact	9
3.2 Methane.....	9
3.2.1 How to decrease methane production	10
3.2.2 How to study methane production.....	11
3.2.3 Importance of inoculum for in vitro systems	12
4. Objectives	14
5. Materials and Methods.....	15
5.1 Materials	15
5.1.1 Incubator	15
5.1.2 Gas Cromatograph.....	16
5.2 Methods	17
5.2.1 Sample preparation	17
5.2.2 Buffered inocula preparation	17
5.2.3 Incubation	18
5.2.4 Gas sampling and methane measurement	19
5.2.5 VFAs sampling and determination	19
5.2.6 Original NDF determination for rumen-reticulum and faeces samples	20
5.2.7 In vitro true digestibility.....	21
Table 1. Menke and Steingass' buffered mineral solution for ruminal inoculum	23
Table 2: Akther's stock solution for faecal inoculum	24
6. Calculations and statistical analysis.....	25
6.1 Production of methane and total gas	25
6.2 Predicted methane production from VFA stoichiometric equation	27
6.3 Statistical analysis.....	27
7. Results and discussion	29

<i>7.1 Digestibility parameters</i>	29
<i>7.2 Volatile fatty acid production</i>	30
<i>7.3 Total gas and methane production</i>	32
8. Conclusions	36
Table 3: Chemical composition	37
Table 4: Digestibility values	38
Table 5: VFA.....	39
Table 6: GP and CH ₄	40
9. References	41

1. Abstract

The aim of the present study was to measure and compare methane (CH₄) production, digestibility and fermentation parameters from two different sources of inoculum (faecal and ruminal) from dairy cattle using a fully automated *in vitro* gas production system.

When CH₄ production is predicted based on stoichiometric relationships of volatile fatty acids (VFA) in the hind-gut, predicted CH₄ production can be higher than the corresponding value predicted from rumen VFA due to higher acetate to propionate ratio in the hind-gut. However, it is hypothesized that CH₄ production is lower when measured from hindgut fermentation. This discrepancy may be related to the effective role of acetogenesis in the hindgut of dairy cattle: here hydrogen is used by acetogens bacteria for acetic acid production (acetogenesis), whereas in the rumen hydrogen is used by methanogens to give methane (methanogenesis). Indeed, acetogens are present also in the rumen but they seem to grow as heterotrophs rather than autotrophs when methanogens are also present.

For the comparison of two different sources of inoculum, five different substrates were used in the current study; pooled samples after wet sieving from digesta collected from rumen and reticulum (RR), faecal particulate matter (FC), timothy hay (H), first cut grass silage (S) and a mixture of grass silage and barley (50:50; S:B). One gram of each substrate in three replicates was incubated either in 60 ml of buffered rumen inoculum or faecal inoculum for 48 h and the run was conducted on two different consecutive weeks.

Total gas production, CH₄ production, CH₄/total gas production and digestibility values (NDFD, TOMD) were greater for all substrates when rumen inoculum was used as compared to faecal inoculum.

Molar proportion of acetate among all feeds was not significantly different between the two sources of inoculum whereas propionate was higher ($P = < 0.01$) and that of butyrate was lower ($P = < 0.01$) for all feeds when incubated in faecal inoculum compared to rumen inoculum. When CH₄ production was predicted based on VFA

stoichiometry after 48 h of incubation from the faecal inoculum, the values were much greater ($P = <0.01$) as compared to the observed values measured from the *in vitro* gas system.

The ratio of CH_4 to total gas production was lower ($P = < 0.01$) for faecal vs. rumen inoculum. When expressed as total gas per TOMD, the values were significantly lower ($P = <0.01$) for faecal vs. rumen inoculum.

It can be concluded that when faecal inoculum was used CH_4 production was approximately half of the amount produced from rumen inoculum and that the greater values of predicted CH_4 production in the faecal inoculum from VFA stoichiometry calculations supports the presence of acetogenesis in the hindgut of ruminants.

2. Riassunto

L'obiettivo del presente studio è stato quello di misurare e comparare i parametri riguardanti la degradabilità, la produzione di metano (CH_4) e di altri prodotti della fermentazione ottenuti dall'incubazione di substrati con due diverse fonti di inoculo microbico (ruminale e fecale) prelevati da vacche da latte. Il lavoro è stato condotto in vitro utilizzando un sistema per la misurazione delle produzioni di gas completamente automatico.

Quando la produzione di metano viene predetta sulla base di relazioni stechiometriche sugli acidi grassi volatili nel cieco, il valore ottenuto può essere maggiore rispetto al corrispondente valore predetto sulla base degli acidi grassi volatili nel rumine. Questo fenomeno può essere spiegato dalla maggiore produzione di acido propionico rispetto all'acido acetato che si osserva nel cieco rispetto al rumine. È stato invece ipotizzato che il valore di produzione di metano, se effettivamente misurato dalle fermentazioni intestinali, sia minore rispetto al valore predetto sulla base del contenuto ruminale di acidi grassi volatili.

La discrepanza tra i due valori, quello predetto e quello misurato, potrebbe essere messa in relazione al ruolo dell'acetogenesi nel tratto intestinale delle vacche da latte; qui, infatti, l'idrogeno è usato dai batteri per produrre acido acetico (acetogenesi) mentre nel rumine l'idrogeno è usato dai batteri metanogeni per produrre metano (metanogenesi). Infatti i batteri acetogeni sono presenti anche nel rumine, ma sembrano comportarsi da eterotrofi in presenza di metanogeni.

Per comparare le due fonti di inoculo sono stati scelti cinque substrati: i) campioni di materiale ruminale prelevati da vacche da latte fistolate (RR), ii) campioni di materiale fecale prelevati da vacche da latte (FC), iii) fieno di graminacee (H), iv) insilato d'erba di primo taglio (S) e una miscela di orzo e insilato d'erba (50:50, SB). Un grammo di ogni substrato, in tre repliche, è stato incubato in 60 ml di inoculo ruminale o fecale per 48 ore, in due esperimenti identici condotti in due settimane consecutive.

I dati ottenuti per la produzione di metano, la produzione totale di gas, il rapporto fra i due valori precedenti e i valori di digeribilità (digeribilità vera della sostanza organica, TOMD; digeribilità della fibra al detergente neutro, NDFd) sono risultati, per tutti e cinque i substrati, sempre maggiori con l'inoculo ruminale rispetto a quelli ottenuti con l'inoculo fecale.

Per quanto riguarda gli acidi grassi volatili, il valore di proporzione molare di acetato è risultato non significativamente differente tra i due inoculi, al contrario dello stesso valore per propionato e butirato, risultati rispettivamente ($P = <0.01$) maggiore e minore per tutti i substrati quando incubati con l'inoculo ruminale rispetto a quello fecale. Quando il valore di metano prodotto dopo 48 ore con l'inoculo fecale è stato predetto sulla base delle relazioni stechiometriche sugli acidi grassi volatili, i valori sono risultati molto maggiori ($P=<0.01$) rispetto a quelli misurati nel sistema *in vitro*. Il rapporto produzione di metano/produzione di gas totale per l'inoculo fecale è risultato minore che per quello ruminale ($P=<0.01$). Quando espressi in rapporto al parametro TOMD, i valori di produzione di gas totale sono risultati ($P= <0.01$) minori per l'inoculo fecale rispetto a quello ruminale.

Si è quindi potuto concludere che le differenze nella produzione di metano e di acidi grassi volatili ottenute con le due fonti di inoculo microbico dipendono dal differente andamento dei processi fermentativi e supportano l'ipotesi della maggiore incidenza dell'acetogenesi nell'intestino rispetto al rumine.

3. Introduction

3.1 Ruminants' breeding's impact

Ruminants' breeding has a great impact on environment and it contributes to its pollution as it produces many waste products, mainly in two kind of emission, as nitrogen and as gasses.

Nitrogen emission is one of the causes of eutrophication of aquatic ecosystems. Indeed excessive nutritional elements in water bring to an overgrowth of vegetation, which gradually causes a consumption of all oxygen in water and so the death of many species, especially fishes. Gasses emissions are produced by ruminants as waste products of the digestion process and they are mainly nitrous oxide (N₂O), carbon dioxide (CO₂) and methane (CH₄). While N₂O and CO₂ animals' emissions are evaluated as limited, compared to other anthropogenic sources, methane produced by ruminants is approximately a quarter of all anthropogenic methane emissions (Beauchemin et al., 2008).

3.2 Methane

This gas is one of the most significant contributors to the greenhouse effect, having effect on climate change and global warming (Johnson and Johnson, 1995) by trapping the heat 20 times more effectively than carbon dioxide (Yan et al., 2010). Moreover, methane production in the rumen represents also a consistent dietary energy loss, about 0.04 to 0.12 of the gross energy intake (Johnson and Johnson, 1995), because ruminants are not able to recycle it in their metabolism.

Methane is produced by microbial population in both rumen and hindgut of ruminants (dairy cattle here) where methanogens bacteria reduce carbon dioxide to methane (Mills et al., 2001) by using hydrogen (H₂), in the methanogenesis process.

Another process is present in the hindgut, and it is called acetogenesis. Both processes need hydrogen to produce methane or VFA. According to some stoichiometric calculations based on VFA proportion, the predicted value of CH₄ production in the hindgut is higher than the value coming from CH₄ production measurements in the hindgut. This difference is probably due to the role of acetogenesis and methanogenesis: in the rumen H₂ is used most for methanogenesis, while in the hindgut more H₂ is used for acetogenesis.

The amount of produced methane can vary with the farming system, the animal species (Fonty et al., 2007), but the most important factor is feed, especially its nature and its digestibility (Ramin and Huhtanen, 2013).

3.2.1 How to decrease methane production

As soon as the importance of methane in environment pollution has been discovered, many attempts have been made to decrease its production, such as inducing rumen acetogenesis (as explained before) (Fievez et al., 1999), trying different diet compositions, adding feed additives to ordinary diets (Ramin et al., 2012) or using antibiotics and chemicals as methnogenesis inhibitors.

There are mainly two ways to decrease methane production:

- The indirect way to lower methane production is to improve animal's productivity. This method doesn't have direct effects on methane production, but brings to produce less methane per production unity. Indeed, animals with low productivity level give few products using most of the energy intake for their maintenance, so more productive animals produce less methane, compared to less productive animals. If the productivity is increased, then the number of animals can be decreased, with an additional reduction of methane produced.
- The direct way is to improve the animals' nutrition. Low quality food have low digestibility and so causes high excretion level per production unit or food intake

unit. Low quality forages are common in dry and also tropical or subtropical regions of the planet.

Here there are the main ways to reach this goal:

- Produce different diets according to the different needs of animals in different life's stages (as growth or lactation).
- Integrate the animals' diet with lipids, as rapeseed oil or linseed oil. Indeed, methane production has been found to be negatively related to fat concentration in the diet (Ramin and Huhtanen, 2013).
- Increase high fiber food digestibility by milling it or by adding urea.
- Use chemicals or antibiotica in order to limitate or inhibit microbial activity of methanogens bacteria. For instance, monensin and 2BES are respectively non-specific and specific inhibitors of methanogenesis (Fievez et al., 1999). Tannins are also object of study for the same reason (Puchala et al., 2005).

All these techniques could decrease methane production up to 25%.

3.2.2 *How to study methane production*

Since *in vivo* methods for measuring gas production are very expensive and laborious, many gas production (GP) techniques have been developed in order to mimic ruminal digestion and fermentation's processes. In these *in vitro* techniques, gas produced is measured as an indirect indicator of fermentation kinetics. At first, a system to measure gas produced by a batch culture was developed; later, gas syringes were used, both with rotating incubator or waterbath. Then, sealed vessels were also developed to measure fermentation kinetics and gas produced was measured with pressure transducer (Rymer et al., 2005).

The system used for the present study is a fully automated *in vitro* gas system, introduced by Cone et al. (1996) and it is described in the section *Materials and methods*. It consists in an automated apparatus made of bottles, in which fermentation

takes place, connected to TRG boxes, where fermentation data are registered. This system allows to measure total gas production, to take gas or liquid samples through a three-way valve during the incubations and not only at the end of them, but also to predict methane production in the *in vivo* systems. This is also more precise, as compared to others, in the determination of environmental conditions, mainly temperature and pressure, which must be controlled because they influence widely gas and methane production values. The system is finally able to monitor continuously the whole gas production kinetic while other systems were able to do it only at set time points.

3.2.3 Importance of inoculum for in vitro systems

Inoculum is the microbial population source and since it's the responsible of food degradation and fermentation it's essential to mimic the ruminal conditions. This is the most variable and less described part of the *in vitro* method in scientific papers, so slight variations in inoculum could have substantial effects on gas production. Then it seems to be pertinent, not only to permit comparison between studies but also to limit potential errors, to have a set of accepted guidelines (Mould et al., 2005).

Usually, the inoculum used in *in vitro* incubations is rumen fluid, but other sources of microbial population, as fresh faeces, cell-free enzymes, culture effluent and bacterial cultures, are object of study in order to see if they can replace ruminal fluid as inoculum source. These solutions would be better not only because they would be easier to use than alive animals, but also because they would overcome the need for surgically modified animals (Rymer et al., 2005).

In this study two different inoculum sources have been used, ruminal fluid collected from fistulated animals (Ramin and Huhtanen, 2012) and fresh faeces (Akhter et al., 1999), collected from the same animals.

It is known that faeces as inoculum source give lower values for rate of total gas production (Cone et al., 2002) and for digestibility parameters because this inoculum has lower activity, due to smaller and different microbial populations, as compared to the ruminal ones, but already published studies are not in accordance about its use in *in vitro* studies as a substitute of ruminal fluid. Indeed, according to some study, as Akther et al.'s (1999), “bovine faeces showed potential as an alternative to rumen liquor [...] when estimating digestibility using the *in vitro* technique”, while according to other researchers, as Cone, faeces can replace rumen fluid only for determination of 48 h gas production, but not for 24 h gas production or gas production profiles, showing that faeces give differences in rate of fermentation but not in total fermentation (Cone et al., 2002).

Fresh faeces are anyway used as inoculum source when it is not possible to keep ruminally fistulated animals; it is also possible to use an oesophageal tube to obtain ruminal fluid (cannulated animals), but this procedure could be harmful to the animals.

4. Objectives

The objective of the present study was to measure and compare methane production from fecal inoculum (hindgut) and rumen inoculum from dairy cattle using a fully automated *in vitro* gas system and five different substrates.

Indeed, stoichiometry formulas predict that methane production is high in the hindgut of dairy cattle, but since acetogenesis seems to be predominant in the hindgut, methane production is hypothesized to be lower in the hindgut than in the rumen.

The specific objectives were in particular:

- To compare the differences between ruminal inoculum and fecal inoculum with different substrates on GP and methane production.
- To compare aNDFomD (neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash) and fermentation parameters from ruminal inoculum versus fecal inoculum.
- To compare the methane production predicted from stoichiometrical equations and the one measured.

5. Materials and Methods

5.1 Materials

5.1.1 Incubator

Both runs were conducted by using a fully automated *in vitro* gas production system, described by Cone et al. (1996). Since this is a completely automated system, the apparatus simplifies the study of fermentation kinetics in the rumen.

This system allows to record total GP (gas production) data, to collect gas and liquid samples but it also allows calculating methane concentration (by using a GC, as explained later) and predicting methane concentration in *in vivo* systems.

The system is made of bottles in which fermentation takes place and which have valves that are able to release a certain amount of gas at each opening, in order to avoid overpressure in the bottle. Indeed pressure in the vessels is one of the most important causes affecting the GP variation (Rymer et al., 2005). Bottles are connected to TRG (Time Related Gas recording) boxes, which collect all the data for GP.

Since we aimed to study methane production at different time points (8, 24, 48 h) and the collection of fluid from the fermentation unit over time for the VFAs determination, the modified tubes method described by Karlsson et al. (2009) was used (T-tube). It is a T-tube, which allows collecting liquid samples, and also has a three-way valve, to collect methane samples from the headspace through a rubber suba seal septa.

Here the main principles are described:

- The pressure in the bottle is measured by an electronic pressure transducer until the pressure's value is equal to a threshold pre-set upper value

(± 0.65 kPa); at this moment an electric gas valve opens in order to release the gas and to allow the pressure to fall back to a pre-set lower value (± 0.4 kPa)

- The valve closes again (the valve is opened for approximately ± 50 ms). Each valve opening represents about 0.7 ml of gas released. The number of gas openings is proportional to gas production
- After the termination, data can be transferred to a personal computer by a plug.

Bottles are placed in waterbath at 39 °C, which is continuously shaking during the incubations, in order to mimic the ruminal conditions.

5.1.2 Gas Chromatograph

Methane concentration was determined by injecting 0.2 mL of gas samples into a star 3400 (CX series) gas chromatograph (Varian Chromatography, USA) equipped with a thermal conductivity detector (TCD). Separation was achieved using a 1.8 long stainless steel column packed with Haysept T (80-100 mesh), argon as the carrier gas with a flow rate of 32mL/min and an isothermal oven temperature of 32°C. The injector and detector temperatures were set to 110°C and 135°C, respectively. Calibration gas was completed using a standard mixture of CO₂ and CH₄ (110mmol/mol) prepared by AGA Gas (AGA Gas AB, Sundbyberg, Sweden).

Peaks were identified by comparison with the standard gas. The gasses are released from the system by opening of the electric gas valve.

5.2 Methods

5.2.1 Sample preparation

Fresh feeds (silage, barley and hay), residues from already digested feeds (rumen, reticulum) and faecal particle matter were selected as substrates for the present experiment, to have a big variation in digestibility rate among feeds. Each food sample was sieved (rumen and reticulum were wet sieved), dried and milled through a 1 mm screen. The final substrates used were: rumen-reticulum digesta (RR), fecal particle matters (FC), silage (S), hay (H), silage-barley (SB, 50:50). Rumen-reticulum and faecal samples, since they were already partly or completely digested, were used to mimic the food as it is in the rumen and in the hindgut.

Prior the incubation, 1 g of each feed was weighed and put in standard glass bottle (rumen and reticulum samples were pulled together, assuming that there is no big difference between the two samples).

Each feed was present in three replicates for both inocula (30 bottles) and blanks (6 in 36 vessels, 3 for ruminal inoculum and 3 for faecal inoculum) were also included in the experiment as a control treatment without substrate. Blanks were used in order to investigate the real gas production value from the treatments with different substrates. Indeed, inoculum (without any substrate, the blank here) produces a limited fermentation which is due to feed particles already present in the rumen of the animal. After feeds were put in the bottles, they were put in waterbath incubators and kept at 39°C, in order to mimic the rumen temperature.

5.2.2 Buffered inocula preparation

Two inoculum sources were used in the present experiment, ruminal fluid and fresh faeces, both from two fistulated Swedish Red cows. Inocula sources were collected in the early morning (incubation day) two hours after morning feeding and kept in pre-

warmed thermos flasks that were previously flushed with CO₂. Both inocula were transported to the laboratory, pooled, filtered through four (for the ruminal fluid) and two (for the fresh faeces) layers of cheesecloth and flushed with CO₂ at 39°C. Flushing with CO₂ was repeated in every step of the experiment's preparation in order to keep always the anaerobic condition, which is essential to maintain the microbial population alive.

Filtered rumen fluid was then mixed with a buffered mineral solution introduced by Menke and Steingass (1988, see table n. 1) supplemented with 2 g peptone (pancreatic digested protein), with constant stirring and continuous flushing with CO₂. Fresh faeces inoculum was prepared mixing 500 g of fresh faeces with 1.5 liter of artificial saliva (Akther and al., 1999), with constant stirring and continuous flushing with CO₂. Artificial saliva was made mixing a stock solution (see composition in the table n. 2) with deionized water (100 mL stock : 400 mL water).

18 bottles were filled using an auto pipette with 60 mL of buffered rumen fluid and 18 with the same quantity of buffered faeces. According to Akther's procedure, 1 mL ammonia was also added to the faeces bottles, as a buffer.

pH in ruminal fluid and in both inocula was also measured.

5.2.3 Incubation

The 36 bottles were placed in three waterbaths at 39° C. Deionized water level in waterbath was checked before and during the running because of the evaporation. For the same reason, bottles were also covered with plastic material, in the waterbath. As soon as the bottles were connected to the fully automated system, incubation could start. Moreover, a solution against bacterial growth was added to the deionized water in the waterbath.

Checking the temperature of waterbath and the pressure in the bottles during the incubation is necessary (temperature must be constantly around 39°C); if the pressure's values are increasing, means that bottles are producing gas. Moreover,

during incubation bottles are constantly shaking, in order to mimic ruminal conditions.

The experiment was completed with two runs of 48 h incubation. In both runs samples were collected for methane production and VFA's analysis while the gas production measurement was conducted by the fully automated system. Bottles were connected to TRG boxes, which collect GP data; readings were done every 12 min and corrected to the normal air pressure (101.3 kPa) (Cone et al., 1996). For a complete description of the in vitro system, see the *Material and Methods* section.

5.2.4 Gas sampling and methane measurement

Gas samples were drawn from each bottle by a gas tight syringe at 8, 24, 48 h. of incubation through the rubber suba seal. Methane concentration was determined by injecting 0.2 mL of gas samples into a star 3400 (CX series) gas chromatograph (Varian Chromatography, USA) equipped with a thermal conductivity detector (TCD). For a complete description of the GC, see the Material section.

Before each injection (8, 24, 48 h), the GC was calibrated by using a standard gas mixture (see Material section). Peaks obtained from the samples were identified by comparison with the standard gas ones.

This method was used to determine methane concentration, but for methane production (mL/g DM) calculations were necessary (see formula and explanations in the *Calculations and statistical analysis* section).

5.2.5 VFAs sampling and determination

As the incubation was terminated (48 h), 0.5 mL of sample were collected from each bottle and pooled together with the sample from the same feed and inoculum (12

samples in total). In each tube 200 μ L of 24 % metaphosphoric acid was added in order to make sure the fermentation will be stopped. Samples were stored at -18°C until processed for VFAs determination. VFAs analysis was conducted by Kungsängen Research Centre (SE-753 23 Uppsala, Sweden).

At the end of incubation, GP, temperature and pressure data were collected. Also pH from each bottle was measured. Bottles were then put on ice in order to terminate the fermentation.

5.2.6 Original NDF determination for rumen-reticulum and faeces samples

NDF is the residue remaining after digesting food in a detergent solution. This value was already available for hay, silage and barley, but it wasn't for rumen-reticulum and faeces samples, so it was determined by using Ankom Technologies method. According to this method,

- Eleven filter bags were at first weighed without any sample (W_1).
- 0.5 g of 1 mm milled sample for both faeces and rumen-reticulum were weighed directly in eight filter bags, four for rumen-reticulum and four for faeces (W_2).
- The bags were sealed and placed into the Bag Suspender, together with three blanks bag. The Bag Suspender was then inserted in the fiber analyzer vessel with a weight on top of it to keep it submerged.
- 1500 mL of ND solution, 15 g of sodium sulfite and 4 mL of alpha-amylase were added to the vessel for the extraction of fiber. The bags were heated and agitated for 75 minutes.
- After that, the solution was exhausted and 1900 mL of rinse water ($70-90^{\circ}\text{C}$) with 4 mL of amylase were added for rinsing twice. Then a third rinse was made with hot water.

- Bags were removed and covered with acetone in a beaker for 5 minutes. They were then dried both with air and in oven at 102 °C.
- Bags were finally weighed (W_3).
- NDF was finally calculated using the following formula:

$$\% \text{ NDF} = (W_3 - (W_1 * C_1)) / W_2 * 100$$

Where

W_1 = bag tare weight

W_2 = sample weight

W_3 = dried weight of bag with fiber after extraction process.

C_1 = blank bag correction (running average of final over-dried weight divided by the original blank bag weight). If the value is larger than 1.00, sample particles were lost from filter bags. Any fiber particle loss from the filter bags generates erroneous results.

5.2.7 In vitro true digestibility

aNDFomD is the value of neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash digestibility and it was determined using the oven method.

- At the end of the 48 h incubation the bottles were put on ice in order to terminate the fermentation.
- All samples were transferred in crucibles, after measured their first weight (W_1), and 50 ml of ND solution were added to each of them. Crucibles were then put in oven at 85 °C
- After 16 h, sodium sulfite and 0.1 mL amylase were added in each crucible in order to remove starch; they were put again in the oven for two hours and then emptied.

- 50 ml of amylase solution (2 L hot water : 4 mL amylase) were added again to each crucible. Crucibles were then emptied and rinsed three times with hot water and once with acetone.
- Crucibles were put again in the oven for 16 h at 105°C
- Crucibles were weighed (W_2) and put in the ash oven for 3 h at 550 °C
- Crucibles were weighed (W_3).

NDF true digestibility was calculated using the NDF residues after the 48 h in vitro incubation, according to the following formulas.

Because of the failure of some crucible (one sample from hay and three samples from forage-barley, from the first run) NDFD for these crucibles was predicted by using the regression equation obtained by plotting gas data at 48 h and NDFD of all samples. This could be possible since the correlation between samples was good (R^2 value was 0.92).

TOMD was also calculated according to the following formula:

$$\text{TOMD} = 1 - \text{ash free NDFin residue (mg) / OM in sample (mg)}$$

Where

OM = organic matter content in the samples.

Table 1. Menke and Steingass' buffered mineral solution for ruminal inoculum (1988).

Solution elements	Quantity for 24 bottles
Microminerals solution 13.2 g CaCl ₂ 2 H ₂ O 10 g MnCl ₂ 4 H ₂ O 1 g CoCl ₂ 6 H ₂ O 8 g FeCl ₃ 6 H ₂ O Make up to 100 mL distilled water	0.16 mL
Macrominerals solution 5.7 g Na ₂ HPO ₄ 6.2 g KH ₂ PO ₄ 0.6 g MgSO ₄ 7 H ₂ O Make up to 1000 mL distilled water	316 mL
Buffering solution 35 g NaHCO ₃ 4 g ((NH ₄)HCO ₃) Make up to 1000 mL distilled water	316 mL
Resazurin 100 mg Resazurin Make up to 100 mL distilled water	1.63 mL
Rumen fluid	666.7 mL
Deionized water	632 mL
Total	1932.5 mL
Need	1440 mL

Table 2: Akther's stock solution for faecal inoculum (1999).

Chemicals	Quantity
NaHCO ₃	49 g
Na ₂ HPO ₄	23.183 g
NaCl	2.35 g
KCl	2.85 g
MgCl ₂ 6 H ₂ O	0.6 g
CaCl ₂	0.2 g

6. Calculations and statistical analysis

Methane and total gas calculations were made following the method set by Ramin and Huhtanen (2012). The procedure and formulas used are described as below:

6.1 Production of methane and total gas

Total gas was recorded automatically from the in vitro gas system every 0.2 h and the total volume of gas produced from each bottle were reported after 48 h of incubation. Gas samples were withdrawn from each bottle at time points of 8, 24 and 48 h, injected into the gas chromatography (GC) in order to determine the concentration of methane in each bottle. Methane concentration values were identified by comparing the peaks from the sample with the peaks of the standard gas (known concentration of methane, 10%).

The general formula to calculate methane production was as below:

$$\text{Total CH}_4 \text{ production (ml)} = \text{HS volume (ml)} \times \text{HS CH}_4 \text{ concentration} + \text{GP (ml)} \times A \times \text{HS CH}_4 \text{ concentration}^{(1)}$$

where

HS = headspace;

GP = gas production;

A = ratio of methane concentration in outflow gas to HS.

HS volume in the system is 265 mL (volume for bottles and pressure tubes connected to the gas reader box) and the ratio A value is 0.55.

Since the gas in vitro system used in the current study doesn't allow collecting the outflow of gas, the ratio of the methane concentration in the outflow (measured GP) to the methane concentration in the HS (A) was predicted using a mechanistic model

(for more details about the modeling procedure please refer to Ramin and Huhtanen (2012)).

Methane concentration at time intervals of 0.2 h was estimated by a logarithmic model of time (using values from 8, 24 and 48 h) versus methane concentration. The logarithmic model for each bottle was used in order to estimate methane production at each time intervals of 0.2 h.

The equation had a reasonable fit of approximately 0.99 values as R^2 .

Total gas production and methane production values at time intervals of 0.2 h was then used to estimate the kinetic parameters of fermentation using a two pool Gompertz model, in which the gas and methane production curve can be divided into two pools, a rapid and a slow one. Data were fitted to the two pools Gompertz function (Schofield et al., 1994) as follows:

$$V_t = V_1 \times \exp(-\exp(1 + k_1 \times e \times (L_1 - t))) + V_2 \times \exp(-\exp(1 + k_2 \times e \times (L_2 - t))) \quad (2)$$

where

V_t = measured total gas or methane volume at time t ;

V_1 = asymptotic cumulative gas volume (mL/g DM) for the first pool (rapid pool);

k_1 = rate (/h) for the first pool (rapid pool);

L_1 = lag (h) for the first pool (rapid pool);

V_2 = asymptotic cumulative gas volume (mL/g DM) for the second pool (slow pool);

k_2 = rate (/h) for the second pool (slow pool);

L_2 = lag (h) for the second pool (slow pool);

t = incubation time.

This model fits the data better than the one – pool models and it also predicts *in vivo* data accurately (Huhtanen et al., 2008). In order to predict methane production *in vivo* these parameters were then used in a dynamic, mechanistic two-compartment model, described by Huhtanen (Huhtanen et al., 2008). This model was originally used to predict pdNDF digestibility from gas kinetic data., but here the model was

used to estimate the proportion of asymptotic methane production at infinitive time ($V_1 + V_2$), produced by the residence of substrates in the rumen.

Predicted in vivo methane production (mL/g DM) was calculated as = proportion \times asymptotic methane production (mL/g DM).

The effective first-order methane production rate was estimated by solving Allen and Mertens' two-compartment equation (1988) for k_d when kinetic parameters and digestibility are known. The mean retention time used in the model was 50 h.

6.2 Predicted methane production from VFA stoichiometric equation

Methane production was then predicted according to Wolin (1960) VFA stoichiometry equation:

$$\text{Predicted CH}_4 \text{ (mL)} = 22.4 \times (0.5 \times \text{AA} - 0.25 \times \text{PA} + 0.50 \times \text{BA} - 0.25 \text{VA})^{(3)}$$

where

22.4 = gas volume (mL/mmol gas);

AA, PA, BA, VA = acetate, propionate, butyrate and valerate production (mmol).

6.3 Statistical analysis

The data were analyzed using the GLM procedure of SAS (SAS Inc. 2002-2003, Release 9.2; SAS Inst., Inc., Cary, NC) by applying the following model:

$$Y_{ijk} = \mu + I_i + S_j + (IS)_{ij} + R_k + e_{ijk},$$

where Y_{ijk} = dependent variable, μ = overall mean, I_i = inoculum source i , S_j = substrate j , $(IS)_{ij}$ = interaction between inoculum i and substrate j , R_k = run k and $e_{ijk} \sim N(0,)$ is the random residual error. Least square means are reported and mean separation was done by least significant difference to test differences between treatments.

7. Results and discussion

7.1 Digestibility parameters

As presented in table n. 3, aNDFomD values were significantly greater ($P = < 0.01$) for all feeds when rumen inoculum was used as compared to faecal inoculum and this difference was greater for lower digestible substrates, as RR with faecal inoculum value is around 50 % of RR with rumen inoculum, while for S:B with faecal inoculum the value was around 80 % of rumen inoculum value.

The values between substrates were significantly different as well ($P = < 0.01$), with already digested feeds having a fiber content around a half of the other feeds.

aNDFomD results show that fiber digestibility is higher in the rumen than in the hindgut, for all feeds, and this is probably due to the lack of cell wall degrading enzymes in the small intestine and to the short retention time of ingesta in the hindgut as compared to the rumen (Varadyova et al., 1999).

TOMD values were significantly different among both inocula ($P = < 0.01$), with a greater difference for low quality feeds (TOMD value for RR with faecal inoculum was 25 % of the same substrate with rumen inoculum, while for S:B the same difference was around 11 %).

The difference was significative between feeds as well, as low quality feeds had a TOMD value around a half of the value for high quality feeds, for both ruminal and faecal inoculum: RR and FC values were around 0.460 and 0.320 respectively with rumen and faecal inoculum, while the same values for S were 0.840 and 0.730

These data show the difference in organic matter between already digested samples (RR and FC) and normal feeds.

RR and FC values were expected to be lower for both aNDFD and TOMD because these substrates are already digested samples and so they represent what is left from the feeds after the ruminal digestion.

7.2 Volatile fatty acid production

As showed in table n. 4, total VFA production was not significantly different between the two inocula, but it was different between substrates ($P = < 0.01$), with a higher production from high quality ones, as S:B and S gave twice the RR value and three times the FC one with rumen inoculum, and a bigger difference, between substrates, with faecal inoculum. Values were, respectively for RR and FC, 4.25 and 4.41 mmol as compared to 1.96 (RR) and 1.33 (FC).

Values were blank corrected.

VFA concentration values (not blank corrected) were significantly higher ($P = < 0.01$) for all feeds incubated with the rumen inoculum compared to the faecal one and the difference was also significant among substrates ($P = < 0.01$), as high quality ones gave higher values (FC's value with rumen inoculum was around 50 % of S:B's, and around 40% with faecal inoculum).

Acetate molar proportion among all feeds was not significantly different between the two sources of inoculum, with an average value of 620 mmol/L from rumen incubation and 624.8 from faecal incubation.

Propionate molar proportion values were significantly higher (239 mmol/mol to 194, on average, $P = < 0.01$) and butyrate's were significantly lower (90.4 mmol/mol to 128.2, on average, $P = < 0.01$) for all feeds when incubated in faecal inoculum compared to rumen inoculum. Valerate and isovalerate molar proportion values were significantly greater among feeds incubated with rumen inoculum compared to faecal inoculum.

Similar results were reached by El-Meadaway (El-Meadaway et al., 1998), who obtained higher values for total VFA and butyrate concentration when incubated with rumen fluid as compared to faeces.

Even if acetic acid's production was not significantly different between the two inocula, it is possible to see a trend of increasing acetate production in the hindgut compared to the rumen (especially from low digestible feeds, RR, FC and H). These results are consistent with Demeyer and De Graeve's (1991), as they found that more short chain fat acids per unit of organic matter fermented are produced in the hindgut than in the rumen.

The discrepancy between CH₄ production predicted *in vivo* and CH₄ production predicted on VFA stoichiometric equations can be explained with the presence of acetogenesis process. This process takes place in the hindgut, but not in the rumen (Immig, 1996). Indeed, even if acetogens bacteria are present in small populations in the rumen, they seem to grow as heterotrophs rather than autotrophs when methanogens are also present (Joblin, 1999), so methanogens bacteria can use all hydrogen in there to reduce CO₂ to CH₄.

In the hindgut, on the contrary, acetogens bacteria can live as autotrophs and use hydrogen to produce VFA.

This is the reason why many studies have been conducted (Fievez et al., 1999, Joblin, 1999) to try to inhibit methanogenesis and at the same time to induce acetogenesis in the rumen: if this could be possible, two main goals can be reached, to increase the efficiency of energy conversion and also to decrease the impact of ruminants on greenhouse effect.

Values for pH were not significantly different between the two inocula, with 6.35 and 6.36 as average values, respectively for rumen and faecal inoculum. This data shows that the method used was correct and the buffers chosen were effective, so it was possible to compare two different inoculum sources.

7.3 Total gas and methane production

As presented in table n. 6, values for CH₄ production, total gas production and ratio between CH₄ and total gas production were higher when rumen inoculum was used as compared to faecal inoculum (approximately half production) from all substrate.

Asymptotic gas (corresponding to V₁+V₂ in the two pool Gompertz's model) values for substrates incubated with rumen fluid were around 250-280 ml/g DM for high digestibility substrates (S, S:B) and decreasing to 150 mL/g for the low digestibility ones (FC,RR). The difference was also greater ($P < 0.01$) with substrates incubated with faecal inoculum, where FC's value was around 30% of S:B's value, respectively 68 mL/ g DM to 225 mL.

The rate of degradation (for total GP) for all feeds was significantly higher ($P < 0.01$) when they were incubated with faecal inoculum compared to the ruminal inoculum, with average values of 0,062 and 0.742 for rumen and faecal inoculum. RR had the same degradation rate with both inocula, 0.029.

Anyway, greater values were obtained from high quality substrates as compared to low quality ones.

Rate GP results from faecal inoculum are greater ($P < 0.01$) for all substrates except RR. Since faecal rate should be similar to the ruminal one, we can suppose that faecal is faster because from this inoculum the gas produced is less, so the incubation with faecal inoculum reaches the asymptotic production in less time as compared to the one with ruminal inoculum. We can hypothesize the values between the two inocula to be more similar if the incubation would have last longer.

Gas 48h/TOMD values were significantly different ($P < 0.01$) between the two inocula, and the values obtained with the same substrate and both inocula were more different in low digestibility substrates (FC, faecal value was 27% lower than the rumen) than in high digestibility ones (S:B, the difference was about 12%).

Indeed, an interaction between inoculum and substrate has been observed ($P < 0.01$).

The significant difference in the interaction between inoculum and substrate for the gas₄₈ h/TOMD parameter means that each inoculum gave different results with each substrates, so substrates with high fermentability gave high gas production level and small difference between inocula, while substrates with low fermentability gave less gas and bigger difference between inocula.

Asymptotic CH₄ values (corresponding to V₁+V₂ in the two pool Gompertz's model) were also significantly different ($P < 0.01$) between the two inocula (faecal inoculum value about 50 % lower than from rumen one) and between substrates ($P < 0.01$), with greater values for the high digestibility ones and lower values for the low digestibility ones. The biggest difference was between RR and S:B, where RR had, respectively with rumen and faecal inoculum, 18.5 and 7 mL/g DM as results, while S:B had 43 and 22.3 mL/g DM.

CH₄ rate production was not significantly different between inocula and substrates, with an average value of 45 % for rumen inoculum and 47 % for faecal.

All values for the ratio CH₄/gas were similar (even if significantly different, $P < 0.01$) among substrates: ruminal inoculum gave a ratio around 13 % and faecal inoculum gave values around 8-9 %, for each substrate.

The CH₄/total gas parameter results show that the amount of methane produced in the rumen is always constant, even if the substrates present in there have different digestibility and fiber content, and the rate for CH₄ confirms that there is no difference between feeds.

This result is not in contrast with the predicted *in vivo* methane production values: with different substrates different amounts of gas are produced, due to the different degradability of substrates, but the methane produced is always around the same % value.

The significant difference observed is due to the small standard error obtained (0.004).

On the other hand total gas production is different among feeds, as it is possible to see a difference in total gas production between high and low quality feeds (rate/h GP).

Predicted *in vivo* CH₄ production was significantly higher ($P = < 0.01$) from ruminal inoculum (50 % more than faecal inoculum) and there was also a significant difference between substrates, where low digestibility ones gave 1/3 of the value obtained from high digestibility ones (S:B, B), with both inocula.

Values obtained in methane production are consistent with previous studies, as Ramin and Huhtanen's (2013), where methane production was found to be positively related to diet digestibility.

When CH₄ production was predicted based on VFA production after 48 h of incubation from the faecal inoculum, the values were much greater, from 30 to 50 %, as compared to the values measured with the *in vitro* gas system. The average for CH₄ production predicted from VFA and incubated with faecal inoculum was 18.69 mL/g DM while the average value for the measured CH₄ was 11.23 mL.

High quality feeds gave significantly higher ($P = < 0.01$) values compared to low quality feeds: S:B value was 29.5 mL while RR value was 9.07 mL.

The evident difference in total gas production and methane production values for substrates incubated with ruminal inoculum and faecal inoculum can be explained with the different quantity and quality of bacterial population respectively present in rumen and hindgut. Many earlier studies, as Fon and Nsahlai (2012), showed this difference between ruminal and faecal inoculum gas production.

According to the present study's results, faecal inoculum cannot substitute the ruminal one for *in vitro* feed evaluation, but it can be used for ranking the feeds because even if values are different, the patterns obtained from the two inocula are the same. The correlations between rumen and faecal inoculum for almost all variables were high, indicating that the ranking of feeds could be well established with both inocula (e.g. $R^2 = 0.99$ for predicted *in vivo* CH₄ production).

A further study should be done, as in Akhter et al.'s study (1999), in order to investigate the regression between ruminal and faecal inoculum results and to see if faecal inoculum can

substitute the ruminal one for feed evaluation. In this study the authors suggest to use a bigger quantity of faeces in order to have a comparable activity between ruminal and faecal inoculum. Mathematically corrected gas production profiles have been studied to make gas production profiles from fecal inoculum look like the reference profiles from ruminal liquor (Dhanao et al., 2004).

These methods would allow to use only faecal inoculum, and avoid to use fistulated animals.

8. Conclusions

Consistently with previous studies, total gas, CH₄ production, CH₄/total GP ratio and digestibility values were lower with faecal than rumen inoculum, due to the lower activity of faecal inoculum as compared to the ruminal one. Methane production was different among substrates because of their different fermentability, but the rate of production was found to be similar among all substrates. As it has been hypothesized, methane produced in the hindgut is lower than the value predicted by Wolin (1960). The difference between predicted and observed methane production with faecal inoculum suggests the use of hydrogen in other process instead of methanogenesis, such as acetogenesis in ruminant hindgut.

Table 3: Chemical composition. Chemical composition of the different feeds used in the gas in vitro incubations.

Feed	DM, g/kg	OM, g/kg DM	NDF, g/kg DM
RR ¹	963	962	810
FC ²	953	954	800
Silage ³	931	919	552
Hay	956	932	570
Barley	953	971	239

¹ RR: wet sieved digesta from rumen and reticulum (pooled). ² FC: fecal particle matter. ³ First cut silage.

Table 4: Digestibility values. Least square means of neutral detergent fibre digestibility (aNDFomD, g/g) and true organic matter digestibility (TOMD, g/g) from feed samples incubated in rumen inoculum or faecal inoculum in the gas in vitro system (n = 6).

Item	1 g sample (per 60 ml culture) ¹					SE ³	P-value ²		
	RR	FC	S	H	S:B		Inoculum	Substrate	I × S
aNDFomD, g/g									
Rumen I ⁴	0.368	0.351	0.736	0.519	0.686	0.0137	<0.01	<0.01	0.081
Faecal I ⁵	0.193	0.196	0.554	0.371	0.569				
TOMD, g/g									
Rumen I	0.468	0.456	0.841	0.706	0.869	0.0146	<0.01	<0.01	0.085
Faecal I	0.321	0.325	0.732	0.599	0.773				

¹ RR: wet sieved digesta from rumen and reticulum; FC: faecal particle matter; S: grass silage; S:B: silage/barley. ² Probability of a significant effect of inoculum, substrate, and interaction inoculum × substrate (I × S). ³ SE: standard error of mean. ⁴ Rumen I: rumen inoculum. ⁵ Faecal I: faecal inoculum.

Table 5: VFA.Total volatile fatty acids (VFA) production (mmol), concentration (mmol/L) and molar proportions of net VFA production (mmol/mol) after 48 h incubation from feed samples incubated in rumen inoculum or faecal inoculum (n = 6).

Item	1 g sample (per 60 ml culture) ¹					SE ³	P-value ²			
	RR	FC	S	H	S:B		Inoculum	Substrate	I × S	
Total VFA production, mmol										
Rumen I ⁴	1.96	1.33	4.41	2.89	4.25	0.420	0.63	<0.01	0.60	
Faecal I ⁵	1.44	1.19	4.48	3.60	4.78					
Total VFA concentration, mmol/L										
Rumen I	76.6	65.9	117.0	92.2	115.0	5.48	<0.01	<0.01	0.42	
Faecal I	43.2	38.6	93.5	78.8	98.5					
Molar proportion, mmol/mol										
Rumen I	Acetate	632	630	619	625	596	4.1	0.13	<0.01	<0.01
	Propionate	192	165	209	205	199	2.0	<0.01	<0.01	<0.01
	Butyrate	115	141	118	114	153	4.1	<0.01	<0.01	<0.01
	Isovalerate	38.4	39.9	29.7	31.7	27.3	1.6	<0.01	<0.01	0.05
	Valerate	22.7	23.6	23.1	23.2	24.2	0.7	<0.01	<0.01	<0.01
Faecal I	Acetate	659	635	610	661	559				
	Propionate	217	190	285	245	258				
	Butyrate	65.7	108	71.1	55.2	152				
	Isovalerate	34.9	41.1	21.7	22	20.9				
	Valerate	23.1	25.9	12.3	16.6	10.2				
pH										
Rumen I	6.57	6.60	6.22	6.36	6.01	0.022	0.10	<0.01	<0.01	
Faecal I	6.64	6.64	6.18	6.33	6.04					

¹RR: wet sieved digesta from rumen and reticulum; FC: faecal particle matter; S: grass silage; S:B: silage/barley. ²Probability of a significant effect of inoculum, substrate, and interaction inoculum × substrate (I × S). ³SE: standard error of mean. ⁴Rumen I: rumen inoculum. ⁵Faecal I: faecal inoculum.

Table 6: GP and CH₄. Effects of different source of inoculum (rumen vs. faecal) on total GP, predicted *in vivo* CH₄ production, CH₄ predicted based on stoichiometric relationship with volatile fatty acids (VFA) and their kinetic parameters (n = 6).

Item	1 gr sample (per 60 ml culture) ¹					SE ³	P-value ²		
	RR	FC	S	H	S:B		Inoculum	Substrate	I × S
Asymptotic gas, mL/g DM									
Rumen I ⁴	162	145	250	217	285	4.6	<0.01	<0.01	0.25
Faecal I ⁵	103	68	183	160	225				
Rate (/h) total GP									
Rumen I	0.029	0.036	0.071	0.057	0.108	0.0040	<0.01	<0.01	<0.01
Faecal I	0.029	0.063	0.094	0.069	0.116				
Gas 48 h/TOMD									
Rumen I	276	265	286	286	324	5.5	<0.01	<0.01	<0.01
Faecal I	233	195	252	262	287				
Asymptotic CH ₄ , mL/g DM									
Rumen I	18.5	19.9	38.5	31.1	43.0	0.79	<0.01	<0.01	<0.01
Faecal I	7.00	7.38	17.9	16.4	22.3				
Rate (/h) CH ₄									
Rumen I	0.030	0.038	0.048	0.045	0.064	0.0035	0.09	0.01	0.02
Faecal I	0.034	0.050	0.051	0.043	0.060				
CH ₄ /total gas									
Rumen I	0.12	0.14	0.13	0.13	0.13	0.004	<0.01	<0.01	0.21
Faecal I	0.08	0.10	0.08	0.09	0.09				
Predicted <i>in vivo</i> CH ₄ from CH ₄ , mL/g DM ⁶									
Rumen I	12.3	14.6	30.3	24.2	36.3	0.53	<0.01	<0.01	<0.01
Faecal I	4.94	5.90	14.4	12.5	18.4				
Predicted <i>in vivo</i> CH ₄ from VFA, mL/g DM ⁷									
Rumen I	13.0	10.2	29.3	18.9	29.5	2.96	0.44	<0.01	0.67
Faecal I	9.07	7.81	24.8	22.3	29.5				

¹ RR: wet sieved digesta from rumen and reticulum; FC: faecal particle matter; S: grass silage; S:B: silage/barley. ² Probability of a significant effect of inoculum, substrate, and interaction inoculum × substrate (I × S). ³ SE: standard error of mean. ⁴ Rumen I: rumen inoculum. ⁵ Faecal I: faecal inoculum; ⁶ CH₄ was predicted *in vivo* using a 50 h rumen retention time in the mechanistic rumen model. ⁷ CH₄ predicted from VFA (mmol): $22.4 \times (0.5 \times \text{acetate} - 0.25 \times \text{propionate} + 0.5 \times \text{butyrate} + 0.25 \times \text{valerate})$.

9. References

- Akhter S., Owen E., Theodorou M. K., Butler E. A. and Minson D. J., 1999. Bovine faeces as a source of micro – organisms for the in vitro digestibility assay of forages. *Grass and Forage Science*, 54, 219-226.
- Beauchemin K. A., Kreuzer M., O'Mara F. and McAllister T. A., 2008. Nutritional management for enteric methane abatement: a review. *Aust. J. Exp. Agric.* 48, 21-27.
- Cone J., van Gelder A. H. and Bachmann H., 2002. Influence of inoculum source on gas production profiles. *Anim. Feed Sci. Technol.*, 99, 221-231.
- Cone J., van Gelder A.H., Visscher G.J.W. and Oudshoorn, 1995. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. *Anim. Feed Sci. Technol.*, 61, 113-128.
- Dhanao M. S., France J., Crompton L. A., Mauricio R. M., Kebreab E., Mills J. A. N, Sanderson R., Dijkstra J. and S. Lopez, 2004. Technical note: A proposed method to determine the extent of degradation of a feed in the rumen from the degradation profile obtained with the in vitro gas production technique using feces as the inoculum. *J. Anim. Sci.* 82, 733-746.
- El-Meadaway, A., Z. Mir, P. S. Mir, M. S. Zaman, and L. J. Yanke, 1998. Relative efficacy of inocula from rumen liquor or fecal solution for determining in vitro digestibility and gas production. *Can. J. Anim. Sci.* 78:673–679.
- Fievez V., Piattoni F., Mbanzamihiho and Demeyer D., 1999. Reductive Acetogenesis in the Hindgut and Attempts to its induction in the Rumen – A Review. *Journal of Applied Animal Research*, 16:1, 1-22.

Fon F. N. and Nsahlai V., 2012. Laboratory cultured faecal inoculum as a substitute for fresh rumen inoculum for *in vitro* feed evaluation. *Afr. J. Agric. Res.* 7, 6595-6604.

Fonty G., Joblin K., Chavarot M., Roux R., Naylor G. and Michallon F., 2007. Establishment and Development of Ruminal Hydrogenotrophs in Methanogen-Free Lambs. *Appl. Environ. Microbiol.*, 73(20):6391.

Getachew G., Robinson P. H., DePeters E.J., Taylor S.J., Gisi D. D., Higginbotham G.E. and Riordan T. J., 2005. Methane production from commercial dairy rations estimated using an *in vitro* gas technique. *Anim. Feed Sci. Technol.*, 123-124, 391-402.

Immig I., 1996. The rumen and hindgut as source of ruminant methanogenesis. *Environmental Monitoring and Assessment* 42:57-72.

Joblin K.N., 1999. Ruminal acetogens and their potential to lower ruminant methane emissions. *Aust. J. Agric. Res.*, 50, 1307-13.

Johnson K. A. and Johnson D. E., 1995. Methane emissions from cattle. *J. Anim. Sci.* 73, 2483-2492.

Mills J. A., Dijkstra J., Bannink A., Cammell S. B., Kebreab E. and France J., 2001. A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: Model development, evaluation and application. *Anim. Sci.* 79, 1584-1597.

Mould F. L., Kliem K. E., Morgan R. and Mauricio R. M., 2005. *In vitro* microbial inoculum: A review of its function and properties. *Anim. Feed Sci. Technol.*, 123-124 31-50.

Puchala R., Min B. R., Goetsch A. L. and Sahlu T., 2005. the effect of a condensed tannin-containing forage on methane emission by goat. *J. Anim. Sci.* 83, 182-186.

Ramin M., Hetta M. and Huhtanen P., 2012. Effect of dwarf birch leaves (*Betula nana*) on in vitro methane production using a gas production technique. Proceedings of the 3rd Nordic feed Science Conference Uppsala Sweden, 98-101.

Ramin M. and Huhtanen P., 2012. Development of an in vitro method for determination of methane production kinetics using a fully automated in vitro gas system – A modeling approach. *Anim. Feed Sci. Technol.*, 174 190-200.

Ramin M. and Huhtanen P., 2013. development of equations for predicting methane emissions from ruminants. *J. Dairy Sci.* 96, 2476-2493.

Rymer C., Huntington J. A., Williams B. A. and Givens D.I., 2005. In vitro cumulative gas production techniques: History, methodological considerations and challenges. *Anim. Feed Sci. Technol.*, 123-124, 9-30.

Varadyova Z., Baran M. and Zelenak I., 2005. Comparison of two in vitro fermentation gas production methods using both rumen fluid and faecal inoculum from sheep. *Anim. Feed Sci. Technol.*, 123-124, 81-94.

Varadyova Z., Zelenak I. And Siroka P., 1999. in vitro study of the rumen and hindgut fermentation of fibrous materials (meadow hay, beech sawdust, wheat straw) in sheep. *Anim. Feed Sci. Technol.*, 83, 127-138.

Wolin M. J., 1960. A theoretical rumen fermentation balance. *J. Dairy Sci.* 40, 1452-1459.

The work has been accepted and published in the proceedings of the 4th Nordic Feed Science Conference.

Publications:

M. Ramin, D. Lerose and P. Huhtanen. Comparison of rumen fluid inoculum vs. faecal inoculum on methane production in vitro. Proceedings of the 4th Nordic Feed Science Conference, Uppsala, Sweden, 12-13 June 2013.