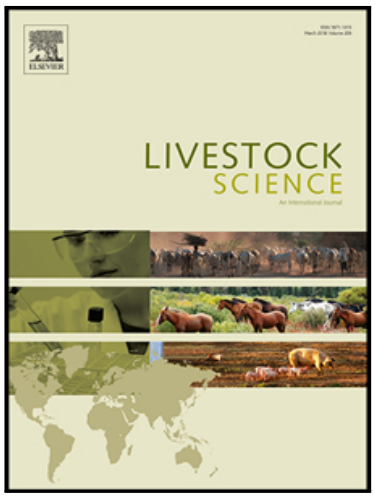

The time after feeding alters methane emission kinetics in Holstein dry cows fed with various restricted diets

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Highlights

- Cattle CH_4 production dynamics are continuously characterized by the exhaled CO_2 : CH4 ratio.
- CH_4:CO_2 ratio in breath is used to investigate the kinetics of CH_4 production.
- Diets composition influences daily CH_4 emission and eructation frequency.
- Post-feeding time induces differences as high as 100% in the CH_4 emission rates.
- Eating behavior has an impact on the CH_4 emission.

The time after feeding alters methane emission kinetics in Holstein dry cows fed with various restricted diets

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ABSTRACT

This study aims to investigate shifts in methane (CH_4) emission in cattle in relation to the time after feeding, diet composition, and feed allowance. Four non-cannulated dry Holstein cows were equipped with activity and infrared sensors to monitor feeding behavior and CH_4 and carbon dioxide (CO_2) levels in the breath, continuously and at a frequency of 4 Hz. The second goal pursued, was to assess the methane emission estimation (CH_4, L/h) by the CO_2-method based on the ratio between CH_4 and CO_2 in the exhaled air, using metabolic CO_2 as a marker. All cows were fed twice a day at 12 h intervals with contrasting isoenergy diets in a cross-over design: LIN100 diet (5562 VEM, i.e. Voedereenheid Melk, Dutch energy unit for milk production, 1 VEM = 6.9 kJ net energy for lactation) composed of haylage, linseed and wheat, and HAY100 (5367 VEM) diet containing only haylage. After a 2 week adaptation period to the diets, 3 days were required for the measurements and immediately after, two additional experimental treatments were applied by reducing the feed allowance to 70% with the same diets to evaluate the impact of the dry matter intake, yielding the two additional treatments HAY70 and LIN70. In addition, two other rumen-cannulated cows were used to monitor time after feeding short-chain fatty acid concentrations in the rumen. On a daily basis, all indicators (daily CH_4:CO_2 ratio, eructation frequency and CH_4 emission) followed the same trend and showed that cows on a hay-based diet produced more CH_4 and feed restriction induced different production levels for the same type of diet. The average CH_4 emission for the different diets were 6.86 L/h for HAY100 > 6.25 L/h for HAY70 > 4.26 L/h for LIN100 > 3.97 L/h LIN70 ($P < 0.001$). The LIN100 diet produced 38% lower daily CH_4 emissions than HAY100 and reduced the eructation frequency by 44%. During feeding, the eructation frequency was higher ($P<0.001$) for HAY than LIN diets.

This work underlines the daily CH_4 emission dynamics observed using the CH_4:CO_2 ratio in the cow's exhaled air. Methane emissions (L/h) are strongly influenced by the time after

feeding time ($P < 0.001$). They increased for up to 2 hours after the distribution of the meal, and then decreased until the next meal, with shifts between the maximum and the minimum emission of more than 100% for LIN100 and 22% for HAY100. Consistently, the acetate:proprionate ratio was smaller for the LIN100 diet between 2 to 5 hours after the meal ($P < 0.001$).

Keywords: methane, carbon dioxide method, time after feeding, long term measurement, methane kinetic

1. Introduction

In ruminants, CH_4 is generated in the rumen and in the hindgut when microbes ferment feed components, mainly carbohydrates. Most of the CH_4 is eructed, although 2% is eliminated via flatus and 11% is absorbed into the blood and exhaled via the lungs (Ricci et al., 2014). Besides dry matter intake (DMI) and diet composition, many factors are likely to influence CH_4 emission, in particular the time after feeding and the behavioral phase such as eating, grazing, or ruminating (Hammond et al., 2016; Knapp et al., 2014). To achieve further reduction in CH_4 production, it is important to capture the complexity and kinetics of feed fermentation and digestion and the related metabolism in ruminants. Few studies specifically investigate CH_4 kinetics over the course of a day (Cottle et al., 2015; Lockyer and Champion, 2001; Velazco et al., 2015). The reference method to quantify CH_4 emissions requires animals to be kept in respiration chambers. This technique offers a low variability between daily measurements conducted on the same animal but it is both time-consuming and expensive, and raises several welfare issues related to the housing conditions (Grainger et al., 2007; Hammond et al., 2016). To measure enteric CH_4 emissions from ruminants in their production environment, a tracer gas (SF_6) technique was designed (Johnson and Johnson, 1995). This is the predominant technique available to individually measure the daily CH_4 emissions of

grazing ruminants over a whole day or more (e.g. Savian et al., 2014). In stables, Lassey et al. (2011), adapted this method to study CH_4 emission dynamics through a succession of 20 min sampled breath accumulations at the individual level. They showed for instance that feeding is immediately followed by a CH_4 peak. The major disadvantages of the SF_6 technique are its high cost, the long time required to analyze the collected gas, the complexity of the method to measure short term changes in CH_4 emissions, the physical constraints due to the equipment carried by the animal that can reduce free movement in the yards or the stables and finally the fact that SF_6 itself has a global warming potential of 23,900 CO_2 equivalents (Machmüller and Hegarty, 2006). Recently sniffer-based CO_2 methods tackled some of these issues by performing a few short measurements in the barn or on pasture of the exhaled air of the cows, usually when the animals are fed supplements or milked. The air exhaled by individual animals is analyzed by infra-red sensors dedicated to measuring CH_4 and CO_2 concentrations in order to estimate the CH_4 production (Cottle et al., 2015; Madsen et al., 2010). This process ensures reasonable accuracy and precision in CH_4 estimation, compared to the respiration chamber (Haque et al., 2017). On groups of animals, CH_4 kinetics can be extrapolated from many short measurements but correlate poorly with post-feeding patterns (Cottle et al., 2015). Another drawback arises from the need for animals to come to the feeder to perform a measurement. Moreover, compared to respiration chamber measurements, emissions display some bias ascribed to animal behaviors and time after feeding CH_4 emission kinetics that skew the CH_4 estimation curves (Cottle et al., 2015; Garnsworthy et al., 2012; Velazco et al., 2015).

Therefore, the main objective of this study was to measure the within daily variation in CH_4 production of individual cows according to the sampling time elapsed after feeding in order to assess the extent of possible biases related to few short time measurements and to show a potential link between CH_4 and behavioral phases. For this purpose, a portable gas analyzer

similar to those used in sniffer-based methods was developed in order to allow a continuous and high frequency analysis of the CH_4 to CO_2 ratios in breath to be made, and to investigate the kinetics of methane production at the individual level. Moreover, the link with the fermentation patterns in the rumen as measured through volatile fatty acid (VFA) profiles was investigated due to the relationship between VFA production and CH_4 emissions (Sauvant et al., 2011).

2. Materials and methods

Two complementary experiments were conducted in the experimental center for animal production of Gembloux Agro-Bio Tech – University of Liège (Gembloux, Belgium) (50°33'54.6"N 4°42'04.6"E). Animal works were approved by the Animal Care Committee of the University of Liège [N° 12-1288 and 14-1627]. In the first experiment, the time after feeding CH_4 and CO_2 emission kinetics of dry cows fed two types of diet (haylage vs. linseed-supplemented haylage diet) and two forage allowance levels were compared: a forage allowance level providing 100% of the maintenance energy requirements and a forage allowance level providing only 70% of the maintenance energy requirements via a reduction in dry matter intake (DMI). Linseed was chosen because it decreases daily CH_4 emissions in cows (Martin et al., 2016). In the second experiment, the impact of the diets on the production kinetics and molar ratios of VFA in the rumen were assessed in cannulated cows.

2.1. Experiment 1: gas emission kinetics

2.1.1. Animals and diets

Four dry red-pied Holstein cows of 736.2 ± 44.0 kg initial and 740 ± 40.7 kg final body weights (BW) were used and placed in a tie-stall barn. Two diets were formulated in order to supply similar levels of fermentable organic matter to rumen bacteria in order to stress differences in fermentation kinetics and pathways between diets. In addition, both diets supplied similar levels of net energy for lactation (VEM) within the Dutch feed evaluation

system (Tamminga et al., 1994): Diet 1 was composed of haylage (HAY) and Diet 2 was composed of haylage supplemented with wheat and linseed (LIN) (Table 1). The net energy animal maintenance requirements per day were calculated as 42.4 VEM/kg $BW^{0.75}$ (Van Es, 1975). Water was always freely available to the animals.

2.1.2. Experimental set up

Two sets of conditions were used to induce variation in rumen fermentation: the type of diet (haylage (HAY) vs. linseed (LIN) diet) and the daily feed allowance (DFA) levels (100% vs. 70%): HAY100, HAY70, LIN100 and LIN70 (Table 1). The 100% DFA was designed to cover all nutritional requirements of the animals while the 70% was designed to assess the link between fermentable DM intake and CH4 production levels.

At the beginning of the measurement period, two cows were ascribed to HAY100 and two others to LIN100. After 2 weeks of adaptation to the diets, a period of 3 days of measurements was carried out. Subsequently, the cows were rationed to 70% of DFA on the same diet. The periods during which the animal received a diet under the required maintenance level was limited to 4 days for animal welfare reasons. Hence, one day after the reduction in DFA, measurements were performed for 3 additional days. Cows, received a quantity of food which corresponded to 70% energy requirements but were only under a limited energy deficit considering the short period of restriction. Finally, diets were swapped between cows and the experimental scheme was repeated for a second period so that both diets were tested on all cows. DFA was split into two equal meals fed at 08:30 AM and PM. All the feed was eaten at once. The average the time spent eating a meal was 61.6 ± 16.9 min for HAY100, 41.0 ± 8.5 min for HAY70, 22.6 ± 4.7 min for LIN100 and 21.1 ± 3.5 min for LIN 70. No refusals were observed.

2.1.3. Gas sensors

The developed gas measurement device uses two gas infra-red sensors, with the CH_4 sensor placed upstream of the CO_2 sensor (NG Gascard® 0-1 % CH_4 and Gascard® NG 0-10% CO_2, respectively; Edinburgh Sensors, Livingston, UK). Those sensors were calibrated by the Edinburgh Sensors each year. The exhaled gas is sucked (24V DC Pump Gascard NG Models) into the sensors directly from the nostrils via a 1.85 m polyethylene pipe (inner ø 4 mm) at a flow rate of 0.5 L/min. A 1 µm filter placed before the first sensor protects both sensors. All components were supplied by a 12V battery. In order to optimize continuous air sampling through the day, a nostril ring was specifically designed (Figure 1). It maintained the tube inlet at constant distance and orientation from the nostril. A microcontroller recorded data from both sensors at 4 Hz and continuously stored them on a SD-card over 24 hours.

2.1.4. Activity sensors

Simultaneous to gas production kinetic measurements, cows were equipped with an iPhone 4S (Apple Inc., Cupertino, CA, USA) attached to the neck (Figure 1), whose built-in inertial measurement unit (IMU) was used to record head and jaw movements. An open-source algorithm analyzed IMU signals to differentiate eating and ruminating behaviors and convert them into a behavior matrix (Andriamandroso et al., 2017).

2.2. Experiment 2: in vivo volatile fatty acid (VFA) kinetics

The objective of Experiment 2 was to acquire dynamics in rumen VFA production, to support observed differences in CH_4 emissions between HAY100 and LIN100.

2.2.1. Animals and diet

In Experiment 2, two others cows were used. The two dry red-pied Holstein cannulated in the rumen were kept in pens (25 m²) and fed with HAY 100 and LIN100.

2.2.2. Experimental set up

Cows were used in a 2 x 2 Latin square design. After a 2-week adaptation to the diets, they were fed at a 12-hour interval on HAY100 or LIN100 diets. Ruminal fluid was then collected for 3 consecutive days just before the meal (0 h) and 1, 2.5, 4.5, 7, 9.5 and 12 h after the morning feed. Diets were then swapped between cows and the procedure was repeated to yield a total of 6 samples per sampling time after feeding (N = 6; 2 cows × 3 days). A total of 100 mL of rumen fluid was collected using a pump with a probe covered by a fine metal mesh (Benchaar et al., 2015). The pH was immediately measured and an aliquot of 2 mL was centrifuged, diluted, acidified (pH < 3) using H_2SO_4, filtered (0.45 µm) and frozen at –20°C until further determination of VFA concentrations.

2.3. Measurements and chemical analyses

Every time a new haylage bale was opened, one sample was taken for analysis yielding a total of six samples (N = 6). For wheat and linseed three samples were taken, at the beginning, in the middle and at the end of the trial (N = 3). All feed samples were dried (60°C, 48 h) and ground in a Cyclotec mill (1 mm screen FOSS Electric, Hillerød, Denmark) before being analyzed for their chemical composition. Samples were analyzed for dry matter (DM) by drying at 105°C for 24 h (method 967.03; AOAC, 1995), organic matter (OM) by burning at 550 °C for 8 h (method 923.03; AOAC, 1995), crude protein (CP) using the Kjeldahl method (CP = N × 6.25; method 981.10; AOAC, 1995), ether-extract content (EE) with the Soxhlet method by using diethyl ether (method 920.29; AOAC, 1995), and gross energy using an adiabatic oxygen bomb 107 calorimeter (1241 Adiabatic Calorimeter, PARR Instrument Co., Illinois, USA). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were also measured according to Van Soest et al. (1991) and corrected for their ash content. In addition, diet samples were also analyzed for their nutritive values using a near infrared spectroscopy

(NIRS) system 5000 monochromator spectrometer (XDS Rapid Content Analyzer XM-1100 Serie, FOSS Electric, Hillerød, Denmark) to predict fermentable (FOM) and digestible organic matter (DOM), the truly digested protein in the small intestine (DVE), degraded protein balance (OED), metabolic energy (ME) and net energy (VEM). The absorption spectrum of each sample was recorded as log 1/*R* for wavelengths ranging from 1100 to 2498 nm, every 2 nm (WINISI 1.5, FOSS Tecator Infrasoft International LCC, Hillerød, Denmark). Prediction equations used (Decruyenaere et al., 2009) to convert spectral data were provided by the Reference Laboratory Network REQUASUD (Gembloux, Belgium).

VFA concentrations were analyzed using a Waters 2690 high performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) fitted with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) combined with a UV detector (210 nm; Waters, Milford, MA, USA) as described by Poelaert et al. (2017).

2.4. Data processing and analyses

Using the open-source algorithm developed by Andriamandroso et al. (2017), data from the IMU was used to classify the cows' behavior by time windows of 300 seconds in MatLab R2014a (MathWorks, Natick, MA, USA). The algorithm detected eating and ruminating behaviors by steps of 1-second, and probability of appearance of each behavior was calculated over time windows of 300 seconds. MatLab R2014a was also used to visualize CO_2 and CH_4 concentrations and process the raw results. Eructations were detected visually on the CH_4 signal. Carbon dioxide and CH_4 concentrations were averaged over time windows of 300 seconds. Background concentrations were subtracted, calculated as the minimum observed values over the studied time windows. After subtraction of the background noise corresponding to the natural concentration of gases in the environment of the stables, all values below 400 ppm of CO_2 were discarded to avoid samples with a very low concentration

of breath (Haque et al., 2014). Such rejection of data was mainly ascribed to clogging of the pipe with food or water. Moreover, time windows for which no behavior data could be recorded because of a failure of the IMU were also discarded. Following this process, 74% of the whole observation data was kept.

Carbon dioxide was used as a natural marker and the ppm ratio (on a volume basis) between CH_4 and CO_2 was used to estimate CH_4 emission (CH_4 (L/h)) as detailed by Madsen and Bertelsen (2012) and Madsen et al. (2010). In this method, the CO_2 production was calculated from the daily heat production by individual cows and assuming a value for the energy equivalent of CO_2 (Equation 1) (Haque et al., 2014).

$$HP = 5.6 \times BW^{0.75} + [(Y \times 22) + (1.6 \times 10^{-5} \times P^3)] \quad (1)$$

where:

HP is the heat production, watt (W)

BW is the body weight of the animals, kilograms (kg)

Y is the milk production of the cow, liter (L)

P is the number of days pregnant, day.

During both experiments, the cows were dry and not pregnant. Hence, Y and P were both equal to zero. The HP is then expressed in kJ per day; as one HP (1 watt) is equal to 1 J/sec or 86.4 kJ/day.

For a ruminant fed at the maintenance level heat production is equal to the ME intake, and for a dry feed-restricted animal, i.e. for a DFA of 70%, the heat production is equal to metabolizable energy (ME) intake + mobilized energy – energy in milk (Madsen et al., 2010). Firstly, ME intake was calculated using a CVB standard (Table 1). The proportion of CO_2 produced by ME intake is then calculated. For animals at maintenance a value of 24 kJ/L CO2 is used, which corresponds to a respiratory quotient of 0.85 to 0.90. Table 2 displays the calculated daily CO_2 production per diets.

For animals fed below maintenance, the energy mobilized from body reserves was calculated as the difference between animal HP needed and ME intake (Table 1). The CO_2 from mobilized energy was estimated according to Madsen et al. (2010) who quote 28 kJ of fat metabolized per 1 liter of CO_2 produced. It was assumed that only the fat is mobilized because cows in negative energy balance mobilize body fat while the energy mobilized from muscle protein is limited (Komaragiri et al., 1998). Finally, the total CO_2 emitted was the sum of CO_2 due to ME intake and mobilized energy (Table 2).

The methane emission CH_4 (L/h) was then calculated as:

$$\mathrm{CH_4\,(L/h)} = [\text{tot daily } \mathrm{CO_2} \times \text{ a/b}]/24 \qquad (2)$$

where:

a is the [CH_4] in air mix minus the minimum [CH_4] in the time-windows studied, ppm

b is the [CO_2] in air mix minus the minimum [CO_2] in the time-windows studied, ppm

tot daily CO_2 is the volume of CO_2 emitted by the cow

24 is to express CH_4 emission per hour

2.5. Statistical analyses

For Experiment 1, responses to diets or behaviors were compared using a PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, NC, USA). The response variables were CH_4 and CO_2 concentrations in breath (ppm), CH_4:CO_2 ratio on a volume basis, CH_4 (L/h), and eructation frequency. Diets combined with DFA (HAY100, HAY70, LIN100, LIN70) were used as fixed effects while cows (1, 2, 3, 4) and periods were used as random variables the model, as suggested for studies involving large variability between individuals (Festing and Altman, 2002). Measurements performed on each cow during one measurement period were used as the experimental unit.

The impact of time after feeding time (0 to 144 time windows of five minutes) for the four diets (HAY100, HAY70, LIN100, LIN70) on CH_4 (L/h) was also studied. Cows (1, 2, 3, 4)

and measurement periods were used as random variables. For this purpose, different models including time as continuous variable and diet, cow and period as class variables were tested as well as their first order interactions. Those models differed according to the power to which time was raised (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} power) and the model with the best fitting performances was selected. The resulting model that was used was as follows, with time to the 4^{th} power: $y_{ij\alpha} = \mu + m \times time_i + n \times time_i^2 + o \times time_i^3 + p \times time_i^4 + diet_j + q \times time_i \times diet_j + r \times time_i \times cow_\alpha + s \times time_i \times cow_\alpha \times period_\varphi$ (3)

where:

$y_{ij\alpha}$ is the studied trait for time i, diet j and cow α;

$time_i$ is the covariate for the time after feeding time (i.e. 0 to 12 h);

$diet_j$ is the diet fixed effect (4 levels);

cow_α is the cow random effect (4 levels);

$period_\varphi$ is the period random effect (2 levels);

Methane kinetics for each cow and for each diet were modeled and parameters describing the kinetic response were calculated using the curvefit function in MatLab R2014a: the time when the maximum of CH_4 (L/h) is reached (max time), the maximum emission obtained between two meals ($maxCH_4$ (L/h)), the minimum emission obtained between two meals ($minCH_4$ (L/h)) and the time needed between two meals to reach the half of the total emission ($t_{1/2}$). The three kinetics response parameters were in turn compared for the diets and DFA levels using a PROC MIXED procedure in SAS where diets were fixed effects while cows and periods were used as random variables.

In experiment 2, the VFA concentrations and acetate:propionate molar ratios at the different sampling time-points after feeding were compared using the fixed linear models in the MIXED procedure in SAS with diets (HAY100, LIN100) as a fixed factor and cows as a random factor.

3. Results

3.1. Gas emission kinetics

In Figure 2, distinctive CH_4 and CO_2 signals recorded using the developed instruments are displayed. Each maximum in the CO_2 signal corresponds to exhalation and each minimum to inhalation. An eructation consists of a rapid rise in CH_4 followed by an exponential decrease convoluted to the specific breathing pattern. In Figure 2, 3 eructation peaks are displayed. This pulse-release of CH_4 by the cow during eructation is highly specific. In Figure 2, just before the first eructation, the cow holds her breath for a few seconds. This specific pattern often occurs during rumination.

3.2. Effect of diet and feed allowance on average daily methane emission

All CH_4 production indicators (i.e. CH_4 concentration, CH_4:CO_2 ratio, and eructation frequency) were consistently lower for both linseed-based diets (LIN100 and LIN70) than for haylage-based diets (HAY100 and HAY70) (Table 3) ($P < 0.001$). The average CO_2 concentrations followed the same trend. CH_4 (L/h) ranged from 6.86 L/h for HAY100 to 3.97 L/h for LIN70 and the intermediate values are 6.25 L/h for HAY70 and 4.26 L/h for LIN100 ($P < 0.001$). All these values ranked as follows: HAY100 > HAY70 > LIN100 > LIN70. Within a same diet, reducing DFA to 70% decreased CH_4, CO_2, CH_4:CO_2 ratio, and eructation frequency.

3.3. Daily patterns of gas emissions

Differences in the daily patterns were observed between treatments for eructation frequency, CH_4 concentration, CH_4:CO_2 ratio and CH_4 (L/h) (Figure 3). Just after the meal, a rapid rise in these values was observed, followed by a slow decrease until the next meal. CO_2 concentrations for the different meals did not show such a distinctive and clear pattern.

The modeling of the CH_4 (L/h) curves by the polynomial Equation 3 ($R^2 = 0.39$) showed that both time after feeding and diets (including forage allowance) as well as the respective interactions and the random factor "cow", influenced the CH_4 (L/h) ($P < 0.001$). The calculation of kinetics parameters from the modeled curves showed that the maximum production (max time) was reached approximately 2 hours after the distribution of the meal (Table 4). With lower $t_{1/2}$ values, the concentrate-based treatments (LIN100) fermented earlier than the forage-based treatments (HAY100), respectively, 4.75 and 5.44 hours after the beginning of the meal. The CH_4 emission peak ($maxCH_4$) was at 5.77 L/h for LIN100 and the minimum baseline ($minCH_4$) was at 2.61 L/h. Such values are less intense and lower compared to the HAY100 diet ($maxCH_4$: 7.98 L/h and $minCH_4$: 6.19 L/h). As expected, for a given diet, a reduction in feed allowance reduced $maxCH_4$ (Table 4).

Specific unitary behaviors, especially rumination phases after a meal, were not distinctively associated with particular CH_4 emission dynamics (Table 5). However, eructation frequency was higher during eating than during the other behaviors but this phenomenon was observed for the haylage-based diets only ($P < 0.001$).

3.4. In vivo *volatile fatty acid (VFA) kinetics*

For LIN100, VFA concentrations increased right after the meal then decreased after 4.5 h. VFA concentrations evolved differently ($P < 0.001$) for HAY100: one hour after the beginning of the meal, a decrease in VFA concentration was observed which was followed by an increase up to 4.5 h after feeding (Figure 4). Total VFA concentrations did not differ between treatments. Only acetate concentrations differed for some time-points, since HAY100 concentrations were consistently higher than LIN100. These differences induced significant changes ($P < 0.001$) in the acetate:propionate ratio which remained more constant and higher with HAY100 than with LIN100 at time 0, 2.5, 4.5, 7, and 9.5 hours.

4. Discussion

The continuous monitoring of CH_4 emission (L/h) provided information on CH_4 production and kinetics for stable-fed cattle with a restricted feeding ration and according to a fixed timetable. It revealed a relationship between the different kinds of diets and forage allowances, on the one hand, and CH_4 (L/h), eructation frequency, time after feeding and acetate:propionate ruminal ratio, on the other hand. It also showed that a within daily variation exists for the CH_4:CO_2 ratio, the eructation frequency and the CH_4 emission (L/h).

The technique to evaluate CH_4 was based on a method by Madsen et al. (2010) which uses metabolic CO_2 as a natural marker, which raises some methodological issues since a good estimation of CH_4 (L/h) depends on the accuracy of the daily metabolic CO_2 production estimation and on the constant emission of this marker gas. The method is based on average CO_2 emission per day and assumes constant efficiency of energy utilization, whereas studies in metabolic chambers show that these factors vary with the animal, level of feeding, and diet composition (Bell et al., 2014; Yan et al., 2010). So, the use of this marker possibly leads to biases which are unavoidable. Moreover, ruminal CO_2 production is not taken into account. This source of CO_2 emission is, on average, 11 times lower than the metabolic CO_2 (Madsen et al., 2010; Martin et al., 2016). To limit changes in CO_2 emissions, physical effort was limited with the cows housed in a stanchion-tied stable. On pasture, cows would be grazing and have more physical activity. Therefore, the application of this method on pasture should include better monitoring of the CO_2-entry rate due to higher physical activity. The use of heart rate belts is one possible solution (Blaise et al., 2016) although dynamic body acceleration might be more appropriate to grasp short term variations in energy expenditure (Miwa et al., 2017). Finally, this study assumed that only fat is mobilized for cows in negative energy balance to estimate the CO_2 produced, although a proper validation of this hypothesis would require the respiration quotient of the animals to be measured (Komaragiri et al., 1998).

Concerning the sampling conditions, the average CO_2 concentrations over time windows of 300 seconds were around 10,000 ppm, and peaks during exhalation reached 50,000 ppm. In breath, CO_2 concentrations range between 30,000 and 50,000 ppm (Haque et al., 2014; Smith et al., 2009). This confirms that the majority of the air that was sampled comes from the breath and only slightly diluted by atmospheric air. Hence, variation due to the position of the nose in relation to the inlet of the sensor was strongly reduced as recommended by Haque et al. (2014). Moreover, according to Madsen et al. (2010) as long as 2 to 3% of breath is present in the air sample, it is sufficient to calculate relevant CH_4 and CO_2 concentration ratios.

The breathing frequency approximately reaches 0.5 Hz, so, the sensors have to record at least at 1 Hz according to the Shannon theorem (1949). Thanks to a high rate of sampling (4 Hz), the actual pattern of breathing and eructation could be properly captured (Figure 2).

Although the results obtained here must be taken with some caution because of the methodological issues detailed above, the daily continuous monitoring of CH_4 and CO_2 has shown an important within daily variation in the CH_4:CO_2 ratio and CH_4 (L/h) (Figure 3). Even after the meal (approx. 60 min for HAY100, 40 min for HAY70, 23 min for LIN100 and 21 min for LIN70), CH_4 (L/h) continued to increase. HAY diets reached their maximum after LIN diets ($P < 0.001$) (Table 4). This is probably explained by the longer intake time required for a more fibrous diet. For LIN100, the minimum and maximum of CH_4 (L/h) recorded during the time after feeding phase were 2.61 and 5.77 L/day, respectively. This means that, depending on the measurement time, sniffer-based CH_4 estimates could double for a same individual fed on the same diet. For HAY100 the difference between the maximum and the minimum value for CH_4 reached 22.4%. Studies in chambers with the SF_6 method and with infra-red short spot measurements have noted that daily patterns were influenced by feeding events but have never characterized the time after feeding pattern so accurately (Cottle et al., 2015; Grainger et al., 2007; Hegarty, 2013; Lassey et al., 2011; Nolan et al., 2010).

Although CH_4 kinetics were clearly linked to the time after feeding, they did not appear to be associated with the onset of rumination phases. During the meal, the eructation frequency was higher for the HAY100 and HAY70 diets, but this was not observed for LIN100 and LIN70. In the present work the time taken for the meal was very short due to the limited daily feed allowances. In the literature, there is no clear answer for the relationship between CH_4 emissions and a specific behavior either. For Lockyer and Champion (2001), CH_4 emissions for gazing ruminants followed a behavioral pattern with peak emissions corresponding to feeding activity whereas emission rates dropped during rumination. Yet, for Dorich et al. (2015) and Hegarty (2013) CH_4 emissions were also higher during rumination. Whereas, for McCauley and Dziuk (1965) short sporadic variations in the CH_4 released were observed, but this was not explained by animal behavior.

With diets providing similar levels of net energy, cows produced 20% more CH_4 per kg DM consumed for HAY100 than for LIN100. Absolute values obtained here were consistent with data reported by Madsen et al. (2010) (1.342 L $CH_4 \times h^{-1} \times kg^{-1}$ DMI), or those presented by Martin et al. (2016) who quantified 1.363 L $CH_4 \times h^{-1} \times kg^{-1}$ DMI for a diet devoid of linseeds and 0.850 L $CH_4 \times h^{-1} \times kg^{-1}$ DMI for a diet with 15% linseed. In this study, incorporating 38% linseed and 19% wheat in the diet decreased the CH_4 emission by 38% (6.86 L/h vs. 4.26 L/h) and eructation frequency by 44% (0.462 eructation/min vs. 0.260 eructation/min). It is well documented that incorporating increasing levels of extruded linseed into the diet of cows reduces enteric CH_4 emissions linearly (Martin et al., 2016). When DFA was reduced by 30%, CH_4 emission only decreased by 8.9% and 6.8% and eructation frequency by 15.3% and 12.3%, for HAY and LIN diets respectively. The lower magnitude in the reduction in CH_4 emissions compared to the reduction in DFA is a consequence of the slower rumen passage rate, leaving a longer time for fermentation of the feed (Demeyer and Fievez, 2000).

Compared to LIN, HAY produced more CH_4 consistent with the VFA profile in the rumen with more acetate and a higher acetate:propionate ratio (Figure 4). The production of acetate and butyrate releases H_2, whereas propionate requires H_2, acting as a H_2 sink. Hydrogen released in excess must be used by methanogenic archaea to reduce CO_2 into CH_4. So a high acetate:propionate ratio leads to more CH_4 produced per mole of VFA (Benchaar et al., 2015; Sauvant et al., 2011). Lassen et al. (2012) explained that a high proportion of concentrates with limited physical structure in the diet decreases acetic acid. It was also reported that dietary polyunsaturated fatty acids, such as those prevalent in linseed, are extensively metabolized mainly through hydrogenation and this affects rumen methanogenic populations such as archaea (Doreau et al., 2012; Machmüller, 2006; Plascencia et al., 1999). Another feature is a reduction of the DFA by complementing the diet with lipids which leads to less fermentation material (Benchaar et al., 2015). Finally, the bypass effects of concentrate feeds in the rumen reduces CH_4 production by reducing fermentation (Knapp et al., 2014).

5. Conclusion

CH_4 emission dynamics was observed using the CH_4:CO_2 ratio in the cow's exhaled air. Moreover, the continuous monitoring of CH_4 and CO_2 in breath allowed the magnitude of changes in daily calculated CH_4 emission (CH_4 (L/h)) to be estimated according to the diet composition, the daily forage allowance and, most importantly, the time after feeding. This approach showed that a concentrate-based diet with linseed considerably reduces the eructation frequency and CH_4 (L/h) emissions. The same phenomenon was observed with a reduction of the DFA to 70%. Moreover, over the course of a day, the differences between the maximal and the minimal emissions varied by a factor of 2 according to the time after feeding, which was consistent with differences in acetate:propionate ratio dynamics. Hence, the variability of the CH_4 (L/h) suggests that an extrapolation of short term measurements could lead to up to 100% error in the estimation of CH_4 daily emissions. Finally, a better

knowledge of CH_4 emission patterns provided by a continuous high-rate measurement technique could lead to innovative management practices to limit CH_4 emissions.

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TABLES

Table 1. Composition and analysis of the tested diets, haylage diet (HAY100), the haylage diet reduced to 70% (HAY70), concentrate diet with wheat and linseed (LIN100), and the LIN diet reduced to 70% (LIN70) (N = 6, samples of 6 bales of haylage and 6 samples of LIN and wheat)

Daily feed intake per cow, /kg of DM	HAY100	LIN100	HAY70	LIN70
Haylage	6.34	2.05	4.438	1.435
Extruded Linseed[1]	-	1.8	-	1.26
Rolled Wheat	-	0.8	-	0.56
Vitamin Mineral Premix[2]	0.05	0.05	0.05	0.05
Chemical composition, g/kg of DM				
CP [3]	109.5 ± 24.6	132.3 ± 11.7	109.1 ± 24.5	131.7 ± 11.7
DVE[4*]	54.2 ± 2.4	64.1 ± 1.4	54.0 ± 2.4	63.8 ± 1.4
OEB[5*]	–11.8 ± 21.7	15.3 ± 10.1	–11.7 ± 21.5	15.2 ± 10.0
VEM[6*]	839.9 ± 31.1	1183.3 ± 15.7	830.5 ± 30.8	1177.9 ± 16.0
EE[7]	29.3 ± 0.4	126.5 ± 4.5	29.0 ± 0.4	125.9 ± 4.5
DOM[8*]	654.3 ± 20.6	720.0 ± 10.0	647.0 ± 20.3	716.7 ± 9.9
FOM[9*]	566.5 ± 24.9	528.4 ± 11.5	560.2 ± 24.7	526.0 ± 11.4
NDF[10]	556.6 ± 35.5	277.4 ± 17.5	550.4 ± 35.1	276.1 ± 17.4
ADF[11]	312.8 ± 2.8	179.3 ± 2.2	309.3 ± 2.8	178.5 ± 2.2
GE[12] (kJ/kg DM)	16135 ± 613	18024 ± 553	16082 ± 612	17942 ± 551
ME[13*] (kJ/kg DM)	9911 ± 337	13717 ± 138	9877 ± 336	13655 ± 138
Daily supply, g/day				
CP	699 ± 157	622 ± 55	490 ± 110	435 ± 39

GE (kJ/day)	102297 ± 3886	84713 ±2600	71607.7 ± 2720	59299 ± 1792
ME (kJ/day)	63330 ± 1410	64471 ± 649	44330 ± 1406	45130 ± 452
DVE	346 ± 15	301 ± 6	242 ± 11	211 ± 5
OEB	–75 ± 139	72 ± 47	–52 ± 96	50 ± 33
VEM	5367 ± 199	5562 ± 74	3727 ± 138	3893 ± 53
EE	187 ± 3	595 ± 21	130 ± 2	416 ± 15
DOM	4181 ± 131	3384 ± 47	2904 ± 91	2369 ± 33
FOM	3620 ± 159	2483 ± 54	2514 ± 111	1738 ± 38
NDF	3557 ± 227	1304 ± 82	2470 ± 158	913 ± 58
ADF	1999 ± 18	843 ± 11	1388 ± 12	590 ± 7

[1] Extruded commercial concentrate (Nutex 68; Dumoulin, Seilles, Belgium) made of linseed, wheat, sunflower cake, field beans, peas, and salt.

[2] Declared contents 12% Ca, 4% Mg, 4% P, 38% Na, 6,000 mg of Zn/kg, 4,000 mg of Mn/kg, 1,750 mg of Cu/kg, 150 mg of I/kg, 100 mg of Co/kg, 40 mg of Se/kg, 750,000 IU of vitamin A/kg, 75,000 IU of vitamin D3/kg, 1,000 mg of vitamin E/kg, 30 mg of vitamin B1/kg, 80 mg of vitamin B2/kg, 20 mg of vitamin B6/kg, 0.3 mg of vitamin B12/kg, 4 mg/kg of vitamin K3, Biotine 0.1 mg/kg , Niacinamide 160 mg/kg (MATH'S PRESTA P, Bauwen Benoit SPRL, Sombreffe, Belgium).

[3]CP = crude protein.

According to the Dutch Feed Evaluation Scheme (Van Es, 1975; Tamminga et al., 1994):

[4]DVE = truly digested protein in the small intestine;

[5]OEB = degraded protein balance;

[6]VEM = Dutch standard for NEL (1 VEM = 6.9 kJ of NEL).

[7]EE = ether extract.

[8]DOM = digested organic matter.

[9]FOM = fermented organic matter in the rumen.

[10]ADF = acid detergent fiber.

[11]NDF = Neutral detergent fiber.

[12]GE = gross energy.

According to the "centraal Veevoeder bureau" evaluation (CVB, 2007)

[13]ME = metabolizale energy

* predicted values from NIRS analyzes

Table 2. For each cow and each diet, a description of the daily metabolic energy intake and mobilized energy (kJ/d) in order to calculate the daily CO_2 production.

BW[1] (kg)	HP needed (kJ/day) [2]	diet	ME intake (kJ/day) [3]	CO_2 due to ME intake (L)[4]	Energy mobilized (kJ/day) [5]	CO_2 due to energy mobilized (L) [6]	Daily volume of CO_2[7]
740.5 ± 40.7	68667 ± 2843	HAY100	63330	2639	5337 ± 2843	191 ± 102	2829 ± 102
		LIN100	64471	2686	4196 ± 2843	150 ± 102	2836 ± 102
		HAY70	44330	1847	24337 ± 2843	869 ± 102	2716 ± 102
		LIN70	45130	1880	23537 ±2843	841 ± 102	2721 ± 102

[1]Average body weight of the animal measured at the beginning and at the end of the trial

[2] Heat production as calculated by Haque et al., 2014 (Equation 1).

[3] Metabolic energy measured in the diet.

[4] Volume of metabolic CO_2 produced with a coefficient of 24 kJ/L of CO_2 for a normal diet .

[5] Difference between net energy intake and energy required.

[6] Co-efficient of 28 kJ/L of CO_2 for fat mobilization.

[7] Sum of CO_2 due to ME intake and mobilized energy.

Table 3. Average and Standard Deviation of CH_4, CO_2 concentrations, CH_4:CO_2 ratio, CH_4 eructation frequency, and CH_4 emission (L/h) of cows fed with the 4 different diets (HAY100, HAY70, LIN100, LIN70).

Measurement	**Diet**[1]				**P-value**	**SEM**
	HAY100	HAY70	LIN100	LIN70		
Number of time windows (N)	2822	2997	3006	2826		
CH_4 (ppm)	607 ± 36[a]	540 ± 33[b]	298 ± 212[c]	297 ± 220[c]	<.0001	2.96
CO_2 (ppm)	10414 ± 4702 [a]	9710 ± 4465[b]	8713 ± 4860[c]	8620 ± 4597[c]	<.0001	43.7
Ratio	0.058 ± 0.019[a]	0.056 ± 0.020[b]	0.037 ± 0.019[c]	0.035 ± 0.018[d]	<.0001	2.14e-4
Eructation/min	0.462 ± 0.220[a]	0.391 ± 0.191[b]	0.260 ± 0.187[c]	0.228 ± 0.170[d]	<.0001	0.002
CH_4 (L/h) [2]	6.86 ± 2.25[a]	6.25± 2.22[b]	4.26 ± 2.18[c]	3.97 ± 1.94[d]	<.0001	0.023

[a-d] Means within a line with superscript letters differ significantly ($P < 0.001$)

[1] diets: HAY100 = diet exclusively composed of haylage; LIN100 = diet composed of haylage, wheat, and linseed; HAY70 = HAY100 reduced to 70% of the DMI; LIN70 = LIN100 reduced to 70% of the DMI.

[2] CH_4 (L/h) is calculated from CH_4:CO_2 ratio and the daily CO2 production displayed in Table 2.

Table 4. Kinetic parameters of the CH_4 emission (L/h), the time when the maximum values are reached (max time), the value of this maximum ratio ($maxCH_4$), the value of the minimum ratio ($minCH_4$) over 12 hours and the time needed between two meals to reach the half of the total emission ($t_{1/2}$).

	Diet[1]				
Item	HAY100	HAY70	LIN100	LIN70	P-value
max time (h)	2.03 ± 0.05[a]	2.02 ± 0.05[a]	1.86 ± 0.04[c]	1.95 ± 0.05[b]	<.0001
$maxCH_4$ (L/h)	7.98± 0.58[a]	7.37 ± 0.58[b]	5.77 ± 0.58[c]	5.17 ± 0.58[d]	<.0001
$minCH_4$ (L/h)	6.19± 0.50[a]	5.58 ± 0.50[a]	2.61± 0.63[b]	3.14 ± 0.50[b]	<.0001
$t_{1/2}$ (h)	5.44 ± 0.06[a]	5.39 ± 0.07[a]	4.75 ± 0.14[b]	4.83 ± 0.14[b]	<.0001

[a - d] Means within a line with superscript letters differ significantly ($P < 0.001$).

[1] diets: HAY100 = diet exclusively composed of haylage; LIN100 = diet composed of haylage, wheat, and linseed; HAY70 = HAY100 reduced to 70% of the DMI; LIN70 = LIN100 reduced to 70% of the DMI.

Table 5. Average eructation frequency and CH_4 emission (L/h) for the 4 different diets focusing on behaviors calculated for the four different diet × daily forage allowance combinations using 300-s time windows.

Diet[1]	Measure	N	eating	N	ruminating	N	Other	P-value
HAY100	Eructation/min	227	0.616 ± 0.243[a]	366	0.428 ± 0.200[b]	2090	0.452 ± 0.218[b]	<.001
	CH_4 (L/h)		7.05 ± 2.10		6.89 ± 2.13		6.84 ± 2.31	0.775
HAY70	Eructation/min	119	0.540 ± 0.257[a]	238	0.392 ± 0.195[b]	2091	0.352 ± 0.182[b]	<.001
	CH_4 (L/h)		6.39 ± 1.89		6.41 ± 2.25		6.40 ± 2.27	0.636
LIN100	Eructation/min	67	0.246 ± 0.184	89	0.261 ± 0.216	2299	0.258 ± 0.190	0.913
	CH_4 (L/h)		4.06 ± 1.88		4.03 ± 1.67		4.49 ± 2.25	0.636
LIN70	Eructation/min	64	0.166 ± 0.177[a]	95	0.223 ± 0.180[ab]	2589	0.232 ± 0.169[b]	0.001
	CH_4 (L/h)		3.67 ± 1.74		3.80 ± 2.03		4.00± 1.94	0.811

[a b] Means within a line with superscript letters differ significantly ($P < 0.001$).

[1] diets: HAY100 = diet exclusively composed of haylage; LIN100 = diet composed of haylage, wheat and linseed; HAY70 = HAY100 reduced to 70% of the DMI; LIN70 = LIN100 reduced to 70% of the DMI.

FIGURES

Figure 1. Equipment installed on a cow housed in stanchion-tied stable. Left: the nostril ring (A) and the fixed pipe (B) in front the nostril. Right: The box with the iPhone (C) attached to the halter (D) on the top of the neck.

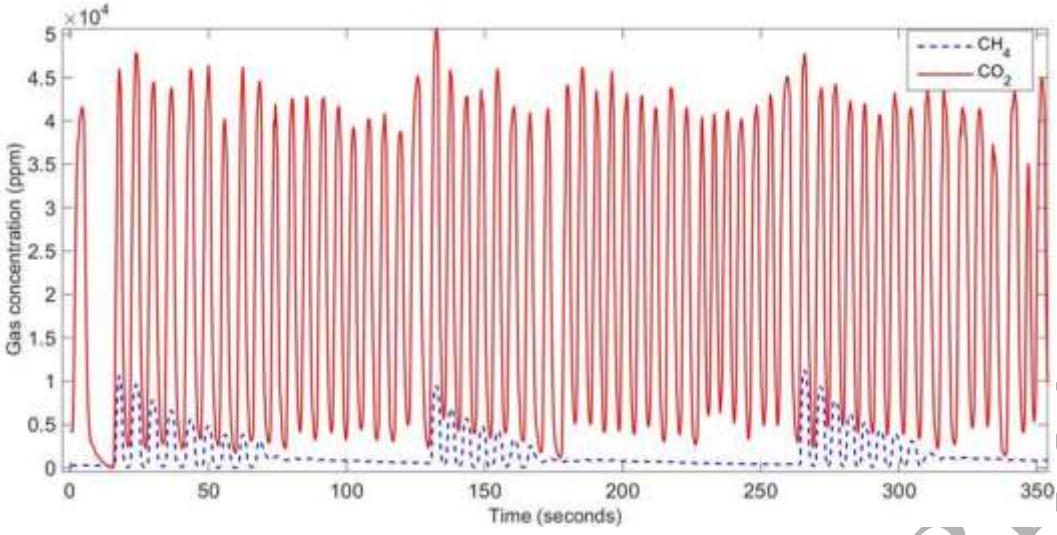

Figure 2. Example of the recorded signals for the concentration of CO_2 (solid line) and CH_4 (dashed line) in the air sampled from the cow's nostril. The signal for 350 seconds is derived from cow one eating HAY100, 3 hours after a meal.

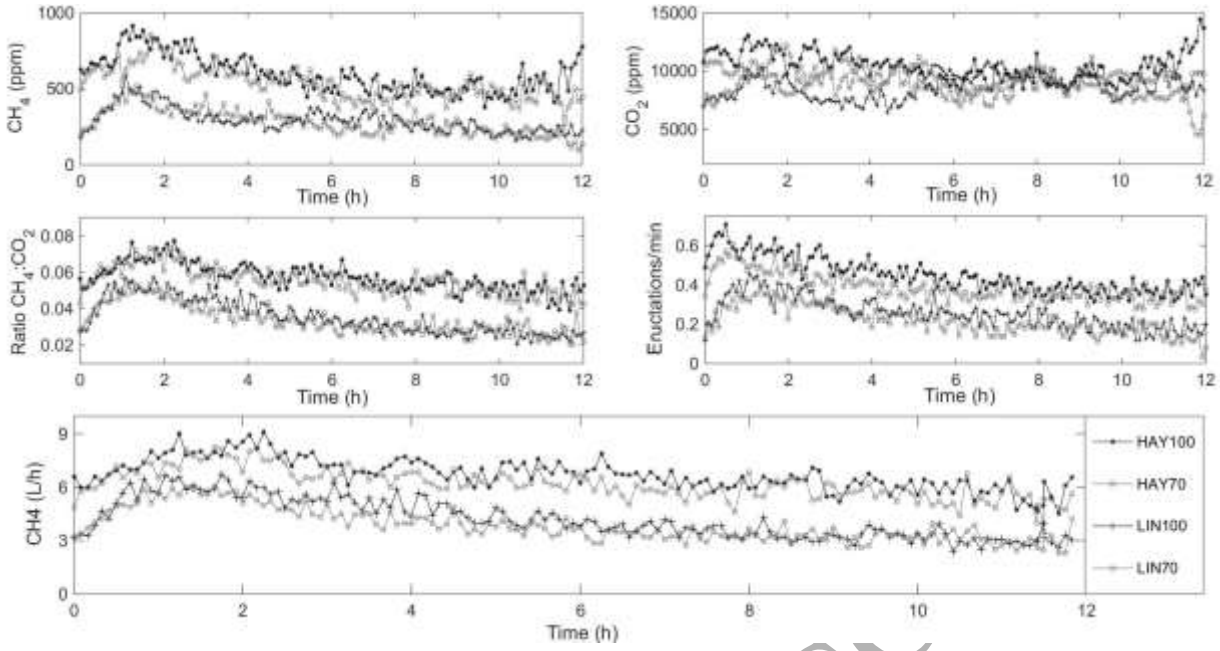

Figure 3. Time after feeding dynamics of CH_4, CO_2, CH_4:CO_2 ratio and eructation frequency, CH_4 (L/h) according to the diet treatment: haylage diet with 100% daily feed allowance (HAY100); haylage diet with 70% daily feed allowance (HAY70); linseed-based diet with 100% daily feed allowance (LIN100); linseed-based diet with 70% daily feed allowance (LIN70). Each point is a 5-min mean of the 4 cows.

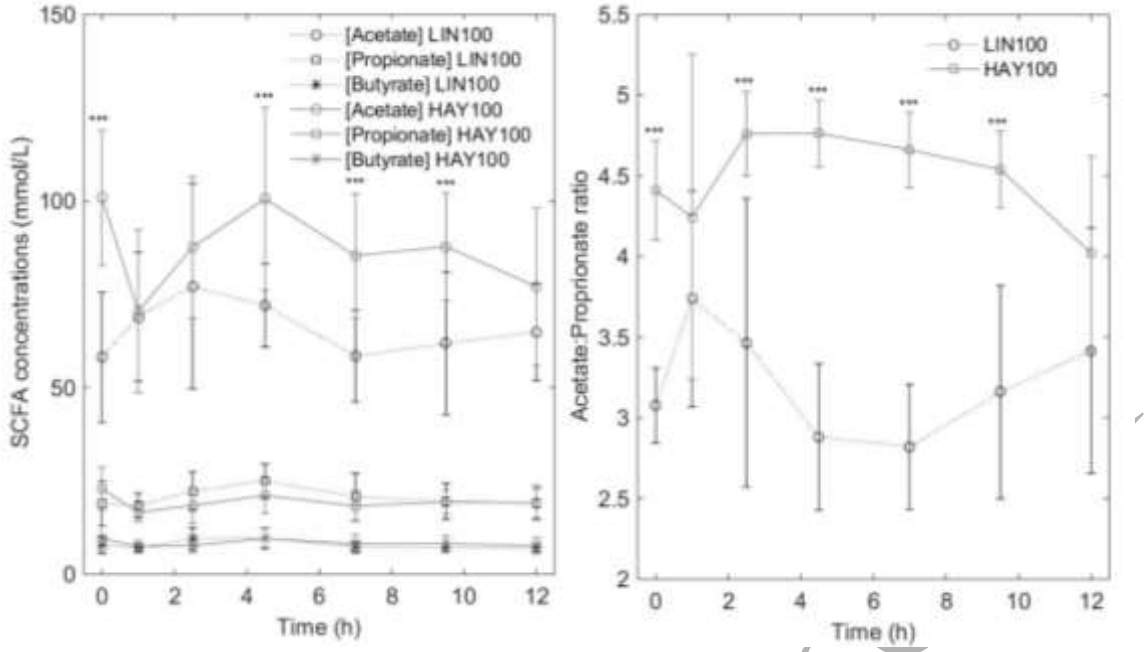

Figure 4. Average VFA concentrations (mmol/L) in the rumen of cows eating the two diets (LIN100 and HAY100) and evolution of the acetate:propionate ratio in the rumen for both diets.