In vitro prediction of methane production by lactating dairy cows

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Summary

As the world human population continues to increase, as well as affluency and urbanization, there is (and will be) a growing demand for animal derived food products. This is the case in developing countries, but also in developed countries. Ruminant animals are a major source of high quality protein (milk and meat) for humans and they have a competitive advantage compared to other animal species (e.g. pigs and poultry) that they can utilise human inedible forages for nutrition. However, ruminants are also a major contributor to the production of enteric methane (CH_4) in the world, contributing considerably to greenhouse gasses. Anaerobic microbial fermentation of feed in the rumen is accompanied by the production of CH_4, resulting in a loss of energy to the animal.

In the literature, several studies have reported CH_4 emissions from dairy cattle feeds and feed ingredients as well as additive using *in vitro* techniques. Many of these studies also investigated the possibility to reduce CH_4 emissions from rumen fermentation. However, literature reporting the relationship between *in vitro* and *in vivo* CH_4 emission are very scarce. There are no reports investigating simultaneously the CH_4 synthesis *in vitro* and *in vivo*, using the same animals and same feed ingredients and rations.

In experiments, performed at Wageningen University, the *in vivo* CH_4 emission by lactating cows on different feeding regimes was determined using climate controlled respiration chambers. Experiments were performed with grass silages differing in maturity and fertilization level, maize silages differing in stage of maturity, as well with sainfoin, a tannin-containing forage. These *in vivo* experiments are very expensive, laborious and time consuming. If *in vivo* CH_4 emission could be estimated using equations based on *in vitro* data, more rapid gains in our knowledge and understanding of factors influencing CH_4 emission from dairy cows could be achieved. As such, performing such costly *in vivo* experiments offers a unique opportunity to determine the relationship, if any, of *in vivo* CH_4 emissions by dairy cows to *in vitro* CH_4 production using an adapted version of the gas production technique. By performing *in vivo* and *in vitro* experiments simultaneously, the rumen fluid from the cows used in the *in vivo* experiments can be used for the *in vitro* experiments. In this manner, the rumen fluid used in the *in vitro* experiments is fully adapted to the experimental rations, as is used in the *in vivo* experiments.

The aim of this thesis was to determine the relationship (if any) between *in vivo* CH_4 production obtained using climate controlled respiration chambers and *in vitro* CH_4 production using the gas production technique. In **Chapter 2**, an *in vitro* experiment is described to quantify the total rumen fermentation (gas production) and CH_4 production of grass silages, differing in maturity at harvest (early maturity, mid maturity and late maturity) and differing in nitrogen fertilisation level (65 kg of N/ha , LF; and 150 kg of N/ha HF, respectively). The *in vitro* CH_4

production was compared to the *in vivo* enteric CH_4 production. The grass silages were fed as part of a total mixed ration (TMR) to lactating cows in the *in vivo* experiment. From the *in vivo* experiment, 12 rumen fistulated cows were used as donor cows of rumen inoculum for the *in vitro* incubations. *In vitro* gas and CH_4 production was determined using rumen fluid from cows consuming each of the six different grass silages. The results indicate that the *in vitro* gas production decreased with increasing maturity of the grass at harvest. However, the stage of maturity of the grass at harvest did not affect the *in vitro* CH_4 synthesis. The *in vitro* CH_4 data correlated poorly with the *in vivo* CH_4 data. Including chemical composition and *in vitro* gas production parameters in a stepwise regression resulted also not in a correlation between the observed *in vivo* CH_4 synthesis, expressed in g/kg fat and protein corrected milk, and the predicted CH_4 production ($R^2 = 0.40$; $P = 0.36$). The results of this experiment show that *in vitro* gas and CH_4 parameters do not accurately predict *in vivo* CH_4 emissions in grass silages.

In **Chapter 3** an *in vitro* experiment is described to determine the total gas and CH_4 production of grass silages differing in maturity at harvest. The *in vitro* experiment was performed, using rumen fluid from dairy cows fed those grass silages, in early and late lactation. The *in vitro* CH_4 data, obtained with the gas production technique, were compared to the obtained *in vivo* CH_4 data using climate controlled respiration chambers. The adaptation of the rumen microbes to the grass silage based rations fed, was also investigated. Silages were made from grass harvested in 2013 at four different stages of maturity, on May 6th, May 25th, July 1st and July 8th. The grass silages were used to formulated different rations, which were fed to 24 lactating cows in early and late lactation, which resulted in a different intake (16.5 vs 15.4 kg/d DMI) by the cows. The results show that the *in vitro* gas and CH_4 production (expressed in ml/g OM incubated), decreased with increasing maturity of the grass at harvest. The grass samples showed a higher *in vitro* gas and CH_4 production using rumen fluid from cows at a late stage of lactation than at an early stage of lactation. No correlation was observed between the *in vitro* CH_4 production (expressed in ml/g OM) and the *in vivo* obtained CH_4 production (expressed in g/kg OM intake or g/kg DM intake). Stepwise multiple regression, including the chemical composition and gas production parameters, when predicting the *in vivo* CH_4 production expressed in (g/kg OM intake) resulted in a relationship with $R^2 = 0.48$ and $P = 0.057$.

In **Chapter 4** an experiment is described to determine the *in vitro* total gas production and CH_4 production of maize silages differing in maturity, using rumen fluid from cows adapted to those maize silages. For the *in vivo* experiment, cows were fed with rations with the different maize silages. The maize silages were harvested at dry matter percentages of 25, 28, 32 and 40. Eight cows, fully adapted to their respective experimental rations served as donor cows for rumen

inoculum for the *in vitro* incubations. The *in vitro* gas production was not affected by the maturity of the whole-plant maize silage, irrespective whether the maize silage itself or as part of the TMR was incubated in the adapted rumen fluid. There was no relationship between *in vitro* and *in vivo* CH_4 production from the maize silages based rations.

In **Chapter 5** *in vitro* experiments were performed to determine the total gas production and CH_4 production of samples of sainfoin silage-based and grass silage-based TMRs, using rumen fluid from cows adapted to the respective TMRs. The results indicate that *in vitro* gas production, CH_4 production and total volatile fatty acid production were not affected by the dietary treatment (sainfoin vs grass). There was no relationship between *in vivo* CH_4 production and *in vitro* CH_4 production ($R^2 = 0.02$; $P = 0.481$).

In conclusion, the lack of a relationship between *in vivo* and *in vitro* CH_4 production was observed in all experiments. Stepwise multiple regression, including the chemical composition and gas production parameters determined across feeds investigated in the Chapter 3 to 5, improved the relationship but the resulting equation did not include gas production parameters. This lack of a significant correlation with *in vitro* CH_4 production parameters could result from several factors. In *in vitro* systems, rumen fluid is commonly diluted with a buffer. Furthermore, the used *in vitro* gas production system is a closed system with no removal of fermentation end products, no supply of substrate and no simulation of rumen passage. In experiments in respiration chambers, CH_4 originates from fermentation in the rumen, but also from fermentation in the large intestine and minor amounts from the manure. The latter two are not simulated in the *in vitro* technique. Moreover, the rumen is a continuous fermentation vessel with input (feed) and removal across the rumen wall and passage to the intestine. Nevertheless, although not found in the present work, one would expect, at least, a weak to moderately positive relationship between *in vitro* CH_4 production and *in vivo* CH_4 production. However, this could not be proven in the present work.

Samenvatting

Heden ten dage neemt de wereldbevolking nog steeds toe en in combinatie met een toenemende welvaart, ook de vraag naar dierlijk eiwit. Als leveranciers van melk en vlees zijn herkauwers hierbij van groot belang. Inherent aan het specifieke verteringsproces in de pens (i.e. fermentatie) van herkauwers wordt echter het broeikasgas methaan door deze dieren geproduceerd. Vanuit dit perspectief is het wenselijk om te beschikken over prospectieve schattingen van de methaanproductie om zo rantsoenen voor herkauwers te formuleren waarbij er zo min mogelijk methaan ontstaat per eenheid eetbaar product. In de literatuur zijn modellen beschreven om de methaanproductie te voorspellen op basis van voerkarakteristieken maar deze modellen zijn onvoldoende accuraat.

De hoeveelheid methaan die ontstaat bij de fermentatie van voer kan worden gemeten onder laboratorium omstandigheden (i.e. *in-vitro*) en tijdens dit promotieonderzoek is onderzocht in hoeverre de methaanproductie gemeten in het laboratorium een voorspellende waarde heeft voor de methaanproductie bij melkkoeien. De *in-vitro* methode omhelsde het automatische meten van methaanproductie van voer (substraat) geïncubeerd met pensvloeistof van de melkkoeien. De *in-vitro* experimenten die in dit proefschrift zijn beschreven zijn uniek in die zin dat ze parallel uitgevoerd zijn aan experimenten met melkkoeien die in respiratiekamers gehuisvest waren. De werkelijke methaanproductie kon daardoor nauwkeurig worden gemeten en tevens kon er voor de *in-vitro* experimenten gebruik worden gemaakt van pensvloeistof van koeien waarvan de pensmicroben volledig aangepast waren aan de rantsoenen. De experimenten zijn uitgevoerd met verschillende ruwvoeders; grassilage (hoofdstukken 2 en 3), snijmaissilage (hoofdstuk 4) en sainfoin (*Onobrychis viciifolia*, hoofdstuk 5). Zowel binnen als tussen de genoemde ruwvoeders liep de chemische samenstelling van de voeders sterk uiteen waardoor er theoretisch een grote variatie in methaanproductie verwacht mocht worden.

In het tweede en derde hoofdstuk van dit proefschrift zijn de twee experimenten met grassilage beschreven. De chemische samenstelling van de grassilages werd beïnvloed door een verschil aan te leggen in bemestingsniveau met stikstof (65 kg N/ha of 150 kg N/ha, hoofdstuk 2) en te maaien in verschillende stadia van fysiologische ouderdom (maaistadium) van het gras (hoofdstuk 2 en 3). De *in-vitro* productie van methaan bleek afhankelijk te zijn van zowel N-bemesting (hoofdstuk 2)

als maaistadium (hoofdstuk 3). Echter in beide experimenten kon geen verband worden aangetoond tussen de *in-vitro* methaanproductie en de methaanproductie zoals die gemeten werd door de koeien zelf in de respiratiekamers. In het derde experiment (hoofdstuk 4) is snijmais gebruikt waarvan de chemische samenstelling werd beïnvloed door de mais op verschillende tijdstippen te oogsten. Op deze manier kon mais worden geoogst met een grote variatie in droge stof gehalte (25-40 % droge stof) en zetmeel gehalte (275-385 g zetmeel/kg droge stof). Ondanks de grote variatie in chemische samenstelling, bleek de methaanproductie, uitgedrukt als % van de totale gasproductie, niet te verschillen tussen de verschillende snijmaissilages. Ook in deze studie werd geen relatie gevonden tussen de *in-vitro* methaanproductie en de methaanproductie door de koeien in de respiratiekamers (i.e. *in-vivo* methaanproductie). In het vierde experiment (hoofdstuk 5) is de *in-vitro* methaanproductie vergeleken tussen rantsoenen gebaseerd op grassilage en rantsoenen waarbij een deel van de grassilage vervangen is door sainfoin (*Onobrychis viciifolia)*. Sainfoin is een vlinderbloemige plant die tanninen bevat en het is vanuit de literatuur bekend dat tanninen de potentie hebben de methaanproductie te kunnen remmen. De vervanging van een deel van de grassilage door sainfoin bleek echter de totale gasproductie, en de productie van vluchtige vetzuren en methaan niet te beïnvloeden. Ook in deze studie kon geen relatie worden vastgesteld tussen de *in-vivo* en *in-vitro* methaanproductie.

Resumerend kan dus worden opgemerkt dat in alle 4 experimenten geen relatie tussen de *in-vitro* en *in-vivo* methaanproductie kon worden aangetoond. Daarnaast bleek ook over de 4 studies heen geen relatie te bestaan tussen de *in-vitro* en *in-vivo* methaanproductie (hoofdstuk 6). Gebruik van multiple regressiemodellen, waarbij naast de *in-vitro* methaanproductie ook de chemische samenstelling van de rantsoenen en verschillende parameters van de gasproductiecurven werden geïncludeerd in het model, resulteerde slechts in een geringe verhoging van de verklaarde variantie van de *in-vivo* methaanproductie. Concluderend kan dus worden opgemerkt dat op basis van de gebruikte *in-vitro* techniek, het niet mogelijk is om de methaanproductie door melkkoeien te voorspellen. De verklaring hiervoor is niet eenduidig maar de volgende factoren zijn hoogst waarschijnlijk van belang. In het huidige *in-vitro* systeem worden de eindproducten van de fermentatie (o.a. vluchtige vetzuren) niet afgevoerd en treedt er geen passage op van voerdeeltjes. Daarnaast is de hoeveelheid methaan die gemeten wordt in respiratiekamers niet alleen afkomstig van de fermentatie in de pens maar ook van de fermentatie in de dikke darm en in de faeces, waarschijnlijk is faeces kwantitatief van ondergeschikt belang. De laatste twee bronnen van methaan werden niet gemeten met de gebruikte *in-vitro* techniek. Verder nemen koeien in meerdere porties hun voer op zodat er steeds vers voer in de pens wordt gefermenteerd, ook dit aspect wordt niet gesimuleerd met de gebruikte *in-vitro* techniek. Ondanks de genoemde tekortkomingen van het

in-vitro meten van methaanproductie werd *a priori* wel een verbetering van de voorspelling van de *in-vivo* methaanproductie verwacht maar dit kon niet worden bewezen.

Chapter 1

General introduction

INTRODUCTION

The growing global demand for meat and dairy products in developed countries is expected to increase (FAO, 2006), which drives animal production towards more intensive systems. Unfortunately, intensive animal production is associated with environmental issues such as nitrogen and phosphorus pollution. Moreover, ruminants also emit methane (CH_4), which is a well-known greenhouse gas and thus relevant in the light of global warming (Moss et al., 2000). Methane production by ruminants has been estimated to be 18.9 Tg/year (McMichael et al., 2007). The FAO (2008) predicted that the worldwide demand for milk will be doubled by 2050. Therefore, it has become important to reduce CH_4 emissions associated with the production of milk and meat, to reduce the ecological footprint of it.

Ruminants are able to convert fibre-rich plant material into high quality animal products, such as milk and meat, due to the process of fermentation in their rumen. Fermentation is an oxidative process, which takes place under anaerobic conditions and is carried out by microorganisms residing in the rumen (Hungate, 1966). Glucose equivalents released from plant polymers, such as fibres, starch and sugars, are converted into volatile fatty acids (VFAs) and serve as an energy source for the animal. The stoichiometry of the reactions of the synthesis of the VFAs, acetic acid, propionic acid and butyric acid, is shown below (adapted from Boadi et al., 2004):

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 8[H]$$

$$C_6H_{12}O_6 + 4[H] \longrightarrow 2C_2H_5COOH + 2H_2O$$

$$C_6H_{12}O_6 \longrightarrow C_3H_7COOH + 4[H] + 2CO_2$$

The first stage of the conversion of glucose equivalents into VFAs induces an accumulation of reduced cofactors such as NADH, $NADH_2$ and NADPH. Clearly, re-oxidation of the reduced cofactors is needed to sustain VFA production (Moss et al., 2000). Generally, the relative proportion of both acetate (CH_3COOH) and butyrate (C_3H_7COOH) is higher than that of propionate (C_2H_5COOH), thereby leading to an excess of [H]. The formation of CH_4 is the principal pathway to maintain the hydrogen balance (Moss et al., 2000; Chaban et al., 2006).

$$CO_2 + 8[H] \longrightarrow CH_4 + 2H_2O$$

Due to the fact that the balance between production and consumption of hydrogen is specific for acetate, propionate and butyrate formation, the ratio of the individual VFAs reflects the amount of hydrogen available for methanogenesis and thus can be considered as a measure for

CH_4 production (Moss et al., 2000). Both propionate and CH_4 can be considered as a hydrogen sink and formation of these compounds neutralizes the inhibiting effect of accumulated reduced cofactors. However, this seems to be just one side of the equation because there is also evidence that the hydrogen pressure in the rumen thermodynamically affects the VFA formation pathways (Janssen, 2010). Thus, hydrogen pressure and the formation of individual VFAs might mutually influence each other. The characteristics of fermentation can be used to develop a strategy to change CH_4 production in the rumen, as a high propionate to acetate ratio leads to a reduced CH_4 production (Ellis et al., 2008). However, due to the net production of hydrogen during rumen fermentation, propionate to acetate and butyrate ratio always is lower than 1. Volatile fatty acids are not commonly used as a substrate for methanogenesis because their conversion into hydrogen and CO_2 is a prolonged process, which is inhibited by rumen turnover (Hobson and Steward, 1997). Methane emission from ruminants depends on the quality of the diet, diet composition and quantity of feed consumed (Johnson and Johnson, 1995).

Methanogenesis is the major pathway of eliminating hydrogen produced in the rumen. The reaction that involves the reduction of CO_2 to CH_4 through utilization of hydrogen is thermodynamically favourable to methanogens to generate metabolic energy in the form of ATP, which is consequently utilized for their maintenance and growth (Ellis et al., 2008). Using hydrogen produced as a last step of carbohydrate fermentation, methanogenic bacteria play a key role in rumen fermentation by maintaining a low concentration of hydrogen in the rumen and allowing the microorganisms involved to function optimally and support the complete oxidation of substrates.

Although much research has been conducted in order to understand the process of enteric CH_4 formation, the underlying mechanisms remain a topic of interest. The total production of CH_4 by the dairy herd is currently based on equations, taking account of the feed, lactation stage and genetics of the cows. As this approach is not very precise, research was initiated by the Dutch government to precisely measure the CH_4 production, using climate controlled respiration chambers. In the research program "Low Emission Animal Feed" which was funded by the Dutch Ministry of Economic Affairs (The Hague, The Netherlands), the Product Board Animal Feed (Zoetermeer, the Netherlands) and the Dutch Dairy Board (Zoetermeer, the Netherlands) different feeding strategies were tested in this way. This offered a unique possibility to relate the CH_4 emissions determined *in vivo* with *in vitro* measurements. Also the adaptation of the rumen fluid to a feed, and so too CH_4 synthesis in the rumen, could be investigated. Due to costs, only a very limited number of *in vivo* experiments can be performed using respiration chambers. *In vitro*, offers the opportunity to screen and measure many more feeds, feed ingredients, additive and total mixed

rations (TMR), obtaining much more background information about potentially viable feeding strategies to reduce CH_4 emissions by dairy cattle and other ruminant species.

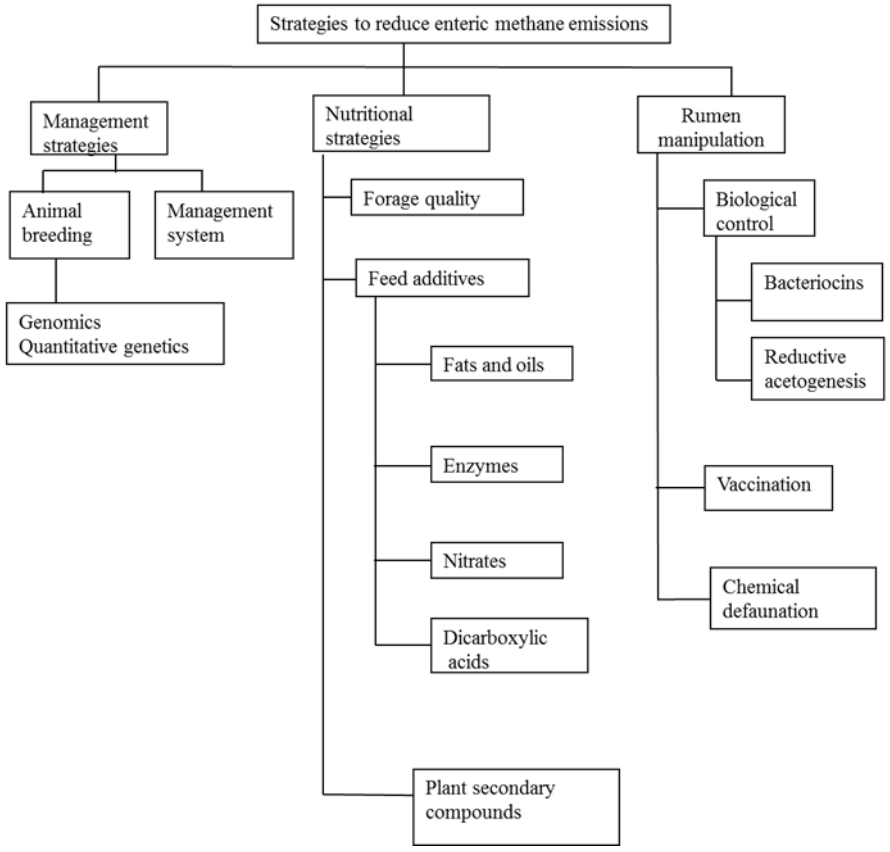

Figure 1.1. Overview of conceptual strategies to mitigate enteric CH_4 emission from ruminants (adapted from Eckard et al., 2010; Cottle et al., 2011; Knapp et al., 2014).

STRATEGIES TO MITIGATE ENTERIC METHANE PRODUCTION BY RUMINANTS

Over the past years, several studies on enteric CH_4 emission mitigation have been published (Moss et al., 2000; Boadi et al., 2004; Beauchemin et al., 2008; Grainger and Beauchemin, 2011). Although more research has to be done in this field, strategies to mitigate CH_4 emission can be grouped into three main categories: 1) management strategies, 2) nutritional strategies and 3) rumen manipulation (Figure 1.1). However, these options still need to be investigated over an extended period of time in order to deliver some biological control (Eckard et al., 2010). Some of these strategies have different degrees of practicability, efficacy, and applicability at a farm level, although some have been demonstrated *in vitro* but still need to be confirmed *in vivo*. Strategic options that

can reduce CH_4 emissions from dairy production without comprising animal productivity and feed fermentation are most interesting in animal nutrition. Enteric CH_4 emission correlates positively to the amount of dry matter (DM) fermented in the rumen, which itself depends on feed composition and rumen fermentation characteristics (Beauchemin et al., 2008; Cottle et al., 2011). Improving forage quality through feeding forage with low fibre and high soluble carbohydrates can reduce CH_4 production (Beauchemin et al., 2008). Improving diet quality can improve animal performance and as well reduce CH_4 production (Eckard et al., 2010). The type of feed can have a major impact on CH_4 production, due to the impact on rumen fermentation, depending on the ratio of forage to concentrate (Moss et al., 2000). Also the effect of feed on enteric CH_4 reduction could be due to a direct inhibition on the microbial activity, also indirectly through the inhibition of the protozoa. Roughages are principal components of ruminant rations and both grass and maize silages are used widely.

In the context of ruminal CH_4 production, fermentation characteristics and rate of fermentation of the roughages are of particular interest, because roughages are implicated in relation to increased CH_4 emissions (Johnson and Johnson, 1995). Holtshausen et al. (2012) reported that the maturity of grass at harvest affects CH_4 production per unit of digested grass. Mature versus immature grass has a higher content of cell walls (i.e. crude fibre, NDF, ADF) which causes a shift in the VFA profile towards acetic acid, thus increasing the formation of CH_4 (Rinne et al., 1997; Moss et al., 2000). The rapidly growing forages have higher concentrations of sugars and soluble proteins, and an increased digestibility. As cell walls thicken during maturation, this causes a decrease in ruminal fermentation (Russell, 1998). Maturity of maize at harvest affects the formation of CH_4 as well.

The starch content of fully matured maize is much higher, compared to immature maize (Phipps et al., 1995) and it is well known that the fermentation of starch is associated with relative high proportions of propionic acid and lower amounts of CH_4 and a decreased ratio of acetate to propionate (Leng, 1993; McAllister et al., 1996). Alternatively, the fermentation of starch may lower the rumen pH, thereby inhibiting the growth of rumen protozoa and consequently limiting the transfer of hydrogen from protozoa to methanogens (Williams and Coleman, 1988). On the other hand, mature maize has a lower NDF content and a high starch content compared to immature maize (Filya, 2004), which is associated with a relatively high rate of fermentation causing relatively higher proportions of propionic acid and CH_4 reduction (Moss et al., 2000).

Utilization of slowly degradable starch, like maize rather than rapidly degradable starch has a potential to reduce CH_4 production, as more of the starch passes through the rumen and does not contribute to CH_4 synthesis. In qualitative terms the relationship between the maturity of both

maize and grass silages, and associated factors, such as growth rate and CP content, are already described in literature. However, the quantitative relationship between the fore mentioned factors and CH_4 production is not yet fully known.

IN VITRO AND IN VIVO METHANE PRODUCTION

The *in vitro* gas production (GP) technique is well standardized, cheap and a widely used method to estimate the nutritive value of ruminant feed by incubating the substrate in buffered rumen fluid (Cone et al., 1996; Getachew et al., 1998). This technique can also be used to estimate CH_4 production from different alternatives used in the light of CH_4 mitigation (Pellikaan et al., 2011a; Hatew et al., 2015). The amount of gas released from the fermentation process and the buffering of VFAs is related to the kinetics of a known amount of feedstuff (Dijkstra et al., 2005). The principle of the *in vitro* technique relies on the incubation of rumen inoculum with a substrate (e.g. feed, TMR, feed ingredients, forage or additive) under anaerobic conditions. Gas is produced throughout the fermentation process and its volume is recorded. Therefore, gas volume curves can be generated over time, which makes it possible to determine kinetic parameters of the GP, using nonlinear models (Groot et al., 1996). Headspace gas samples can be taken to analyse the gas compositions and to determine the actual CH_4 concentration in the headspace using gas chromatography. However, in the *in vitro* incubations, the fermentation process is only related to fermentation in the rumen, while for *in vivo* measurements the CH_4 production includes the combined effects of fermentation in the rumen, the large intestine and to some extent, manure.

Respiration chambers are commonly used to measure CH_4 of individual animals (Figure 1.2). Their use is technically demanding, expensive, and only few animals can be monitored at one time (McGinn et al., 2008). Although the design of the chambers varies, the principles remain the same. The respiration chambers technique (Verstegen et al., 1987) confines 1 or more animals in airtight chambers from which the inlet and outlet of air is monitored for its gases (oxygen, CO_2, CH_4 and hydrogen). The advantage of this technique is its high accuracy and also manure and urine can be collected from the chambers allowing the determination of feed digestibility and deriving an energy balance. Estimation of CH_4 emissions from manure management is based on the assumption that manure has a specific maximum CH_4 producing capacity (Steed and Hashimoto, 1995). In respiration chambers the amount of CH_4 emitted per cow is approximately 1000 times the amount of CH_4 produced by its manure (Külling et al., 2002). Needless to say that CH_4 measurement in respiration chambers is not feasible under practice farming conditions.

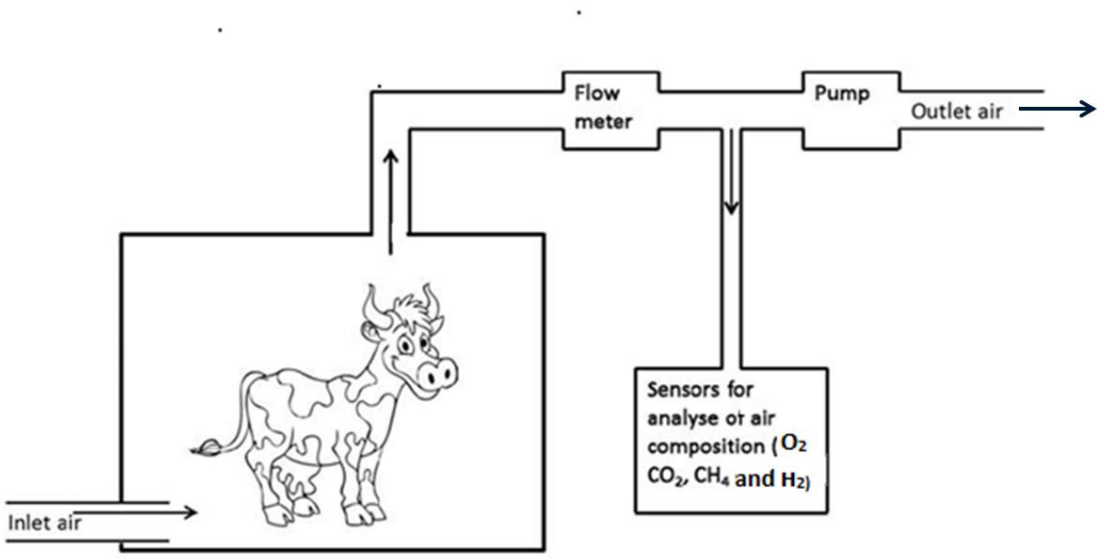

Figure 1.2. Overview of open circuit respiration chamber for methane measurements (adapted from Storm et al., 2012).

In this thesis, *in vitro* experiments are described which were run parallel to *in vivo* experiments using respiration chambers where CH_4 emission of dairy cows were accurately measured, in an attempt to develop a precise and accurate *in vitro* prediction formula for CH_4 production. This approach to relate *in vitro* to *in vivo* CH_4 production is limited in the literature. This thesis opportunistically utilised the data of four studies which: 1) examined the effects of improving grass silage quality through grassland management (Macome et al., 2017), 2) assessed the effect of maize silage quality on CH_4 production (Macome et al., unpublished data) and 3) investigated the potential of plants, containing condensed tannins by using sainfoin as a tool to inhibit methanogenesis and CH_4 production (Macome et al., unpublished data). The use of plants containing secondary compounds such as condensed tannins in dairy cow nutrition has been reported to have anti-methanogenic effects (Beauchemin et al., 2007; Martin et al., 2010). It has been suggested by Hristov et al. (2013) that tannins have a CH_4 mitigating potential up to 20%, but the anti-methanogenic effect depends on the tannin concentration in the diet (Jayanegara et al., 2009). However, next to the dietary dose of tannins, also the origin of tannins needs to be considered in relation to the efficacy of tannins to reduce CH_4 production (Pellikaan et al., 2011b).

AIM AND OBJECTIVES

The overall aim of this thesis was to determine if *in vivo* CH_4 emission from dairy cows, as measured in climate controlled respiration chambers, can be accurately predicted by the *in vitro* GP technique. The guiding hypothesis of the research was that the *in vitro* GP technique has a moderate to good predictability (R^2 = 0.40-0.70) with *in vivo* CH_4 emissions from dairy cows fed practical diets. Specific objectives were, to:

1. Determine whether *in vitro* gas and CH_4 production measurements, using an automated GP technique can mimic the *in vivo* CH_4 production as measured in climate-controlled respiration chambers (Warner et al., 2016), for different qualities of grass silage.
2. Establish an accurate and precise relationship between *in vivo* and *in vitro* CH_4 production from dairy rations fed to cows at two stages of lactation.
3. Investigate the effect of maize silage made from whole- plant maize, harvested at different maturities, on *in vitro* CH_4 synthesis.
4. Evaluate the effect of partial replacement of grass silage by sainfoin silage on *in vitro* gas and methane production and investigate the correlation between *in vivo* and *in vitro* CH_4 production.

OUTLINE OF THE THESIS

The *in vivo* data referred to in this thesis, were obtained from the research projects entitled: "Low Emission Animal Feed" and "LegumePlus". Within the former, research was conducted to relate the quality of grass and maize to *in vivo* CH_4 emissions while in the latter, replacement of grass silage by sainfoin silage was investigated. Both projects were conducted at Wageningen University using the same respiration chambers to measure *in vivo* CH_4 emissions.

This thesis contains six chapters of which four chapters describe *in vitro* experiments conducted to evaluate the effects of different feeding strategies on enteric CH_4 production and also to predict the *in vivo* CH_4 production using the *in vitro* GP technique. Methane production *in vitro* of samples of grass, differing in plant maturity and nitrogen fertilisation, was determined and compared to *in vivo* enteric CH_4 production in **Chapter 2**. In a second experiment (**Chapter 3**), CH_4 emission of cows fed grass silages differing in maturity, during two stages of lactation was compared to *in vitro* measurement of CH_4. In the subsequent chapter (**Chapter 4**), the *in vitro* rumen fermentation and CH_4 production of whole-plant maize silage harvested at four different maturities was investigated, and compared with obtained *in vivo* CH_4 production data.

In **Chapter 5**, the effect of partial replacement of grass silage by sainfoin silage on *in vitro* fermentation and CH_4 production was evaluated. Based on the results from all experiments, use of the *in vitro* measurements to estimate the *in vivo* CH_4 production is discussed in **Chapter 6** as well as the implication of feeding strategies as an opportunity to mitigate enteric CH_4

REFERENCES

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister. T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85, 1990-1996.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement. Aust. J. Exp. Agric. 48, 21-27.

Boadi, D., Benchaar, C., Chiquette, J., Massé, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows update review. Can. J. Anim. Sci. 84, 319-335.

Chaban, B., Ng, S.Y.M., Jarrell, K.F., 2006. Archaeal habitats - from the extreme to the ordinary. Can. J. Microbiol. 52, 73-115.

Cone, J.W, van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. 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.

Cottle, D.J., Nolan J.V., Wiedmann, S.G., 2011. Ruminant enteric methane mitigation: a review. Anim. Prod. Sci. 25, 491-514.

Dijkstra, J., Kebreab, E., Bannink, A., France, J., Lopez, S., 2005. Application of the gas production technique to feed evaluation systems for ruminants. Anim. Feed Sci. Technol. 123-124, 561-578.

Eckard, R.J., Grainger, C., de Klein, C.A.M., 2010. Options for abatement of methane and nitrous oxide from ruminant production: A review. Livest. Sci. 130, 47-56.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J., 2008. Aspects of rumen microbiology central mechanistic modelling of methane production in cattle. J. Agric. Sci. 146, 213-233.

Filya, I., 2004. Nutritive value and anaerobic stability of whole crop maize silage harvested at four stages of maturity. Anim. Feed Sci. Technol. 116, 141-150.

Food and Agricultural Organization of the United Nations (FAO), 2006. Livestock's Long Shadow: Environmental Issues and Options, Rome, Italy.

Food and Agriculture Organization of the United Nations (FAO), 2008. The State of Food Insecurity in the World, Rome, Italy.

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. 72, 261-281.

Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Technol. 166-167, 308-320.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77-89.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Hobson, P.N., Stewart, C.S., 1997. The Rumen Microbial Ecosystem, Chapman and Hall, London, UK.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci. 62, 40-45.

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B., Tricarico, J.M., 2013. Mitigation of methane and nitrous oxide emissions from animal. A review of enteric methane mitigation options. J. Anim. Sci. 91, 5045-5069.

Hungate, R.E., 1966. The Rumen and its Microbes. Academic Press, New York.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1-22.

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system. Anim. Feed Sci. Technol. 50, 230-237.

Johnson, K.H., Johnson, D.E., 1995. Methane emission from cattle. J. Anim. Sci. 73, 2483-2492.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., Tricarico, J.M., 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97, 3231-3261.

Külling, D.R., Dohme, F., Menzi, H., Sultter, F., Lischer, P., Kreuzer, M., 2002. Methane emissions of differently fed dairy cows and corresponding methane and nitrogen emissions from their manure storage. Environ. Monit. Assess. 79, 129-150.

Leng, R.A., 1993. Quantitative ruminant nutrition a green house science. Aust. J. Agric. Res. 44, 363-380.

Macome, F.M., Pellikaan, W.F., Schonewille, J.Th., Bannink, A., van Laar, H., Hendriks, W.H., Warner, W., Cone, J.W., 2017. *In vitro* rumen gas and methane production of grass silages differing in plant maturity and nitrogen fertilisation, compared to *in vivo* enteric methane production. Anim. Feed Sci. Technol. 230, 96-102.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351-365.

McAllister, T.A., Cheng, K.J., Okine, E.K., Mathison, G.W., 1996. Dietary, environment and microbiological aspects of methane in ruminants. Can. J. Anim. Sci. 76, 231-246.

McGinn, S.M., Chen, D., Loh, Z., Hill, J., Beauchemin, K.A., Denmead, O.T., 2008. Methane emissions from feedlot cattle in Australia and Canada. Aust. J. Exp. Agric. 48, 183-185.

McMichael, A.J., Powles, J.W., Butler, C.D., Uauy, R., 2007. Food, livestock production, energy, climate change, and health. The Lancet 370, 1253–1263.

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

Pellikaan, W.F., Hendriks, W.H., Uwimana, C., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011a. A novel method to determine simultaneously methane production during *in vitro* gas production using fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Pellikaan, W.F., Stringano, E., Leenaars, J., Bongers, D.J.G.M., van Laar-van Schuppen, S., Plant, J., Mueller-Harvey, I., 2011b. Evaluating effects of tannins on extent and rate *in vitro* gas and methane production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166-167, 377-390.

Phipps, R.H., Sutton, J.D., Jones, B.A., 1995. Forage mixtures for dairy cows: the effect on dry-matter intake and milk production of incorporating either fermented or urea-treated whole-crop wheat, brewer's grains, fodder beet or maize silage into diets based on grass silage. Anim. Sci. 61, 491-496.

Rinne, M., Jaakkola, S., Huhtanen, P., 1997. Grass maturity effects on cattle fed silage- based diets. Anim. Feed Sci. Technol. 67, 1-17.

Russel, J.B., 1998. Strategies that ruminal bacteria use to handle excess carbohydrate. J. Anim. Sci. 76, 1955-1963.

Steed, J., Hashimoto, A., 1995. Methane emissions from typical manure management systems. Bioresource Technol. 50, 123-130.

Storm, I.M.L.D., Hellwing, A.L.F., Nielsen, N.I., Madsen, J., 2012. Review: Methods for measuring and estimating methane emissions from ruminants. Animal 2, 160-183.

Verstegen, M.W.A., van der Hel, W., Brandsma, H.A., Henken, A.M., Bransen, A.M., 1987. The Wageningen respiration unit for animal production research: A description of the equipment and its possibilities. In: Energy Metabolism in Farms Animals: Effects of Housing, Stress and Disease. Pp 21-48. Martinus Nijhoff Publishers, Dordrecht, the Netherlands.

Warner, D., Hatew, B., Podesta, S.C., Klop, G., van Gastelen, S., van Laar, H., Dijkstra, J., Bannink, A., 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10, 34-43.

Williams, A.G., Coleman, G.S., 1988. The rumen protozoa. In: The Rumen Microbial Ecosystem. Hobson, P.N. (Ed), Elsevier Science Publishers Ltd, Essex, UK, Pp 77-129.

Chapter 2

In vitro rumen gas and methane production of grass silages differing in plant maturity and nitrogen fertilisation, compared to *in vivo* enteric methane production

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ABSTRACT

The potential of an *in vitro* gas production (GP) system to predict the *in vivo* enteric methane (CH_4) production for various ryegrass-based silages was evaluated, using adapted rumen fluid from cows. Rumen fluid from 12 lactating rumen-cannulated Holstein-Friesian cows were used for *in vitro* incubations and compared with *in vivo* CH_4 production data derived from the same cows fed the same grass silages. The cows consumed a total mixed ration consisting of six different grass silages and concentrate at an 80:20 ratio on a dry matter (DM) basis. The grass silages differed in plant maturity at harvest (28, 41 and 62 days of regrowth) and N fertilisation (65 and 150 kg of N/ha). Rumen fluid from cows consuming each of the six grass silages was used to determine the *in vitro* organic matter (OM) fermentation and *in vitro* CH_4 synthesis, using an automated GP technique. *In vitro* GP decreased with increasing maturity of the grass. *In vitro* CH_4 production, expressed either in ml/g of OM, in ml/g of degraded OM (DOM) or as a % of the total GP, increased with increased N fertilisation ($P < 0.05$). Maturity of grass at harvest did not affect the CH_4 synthesis expressed in ml/g of DOM and CH_4 expressed as % of the total gas, whereas N fertilisation increased the *in vitro* CH_4 synthesis, expressed in any unit. The *in vitro* data correlated poorly with the *in vivo* data. Across the six grass silages tested, the *in vitro* CH_4 production, expressed in ml/g of OM after 8, 12, 24, and 72 h of incubation did not correlate with the *in vivo* enteric CH_4 production, expressed in g/kg of DM intake ($R^2 = 0.01–0.08$). Stepwise multiple regression showed a weak, but positive correlation between the observed *in vivo* CH_4 synthesis, expressed in g/kg FPCM and the predicted CH_4 per kg FPCM, using the amount of *in vitro* organic matter degraded ($R^2 = 0.40$; $P = 0.036$). *In vitro* gas and CH_4 parameters did not improve the accuracy of the prediction of the *in vivo* CH_4 data.

INTRODUCTION

Methane (CH_4) is a potent greenhouse gas and, along with carbon dioxide and nitrous oxide, CH_4 emission from livestock production is a major contributor to global warming (Moss et al., 2000). Enteric CH_4 formed by fermentation of feed in the gastrointestinal tract of ruminants constitutes a loss of dietary energy to the animal (Johnson and Johnson, 1995). It is generally accepted that the ingredient composition of ruminant diets has a major impact on rumen fermentation and, thus, on enteric CH_4 production (Moss et al., 2000). Therefore, the quantitative impact of the various dietary ingredients on CH_4 production is important to derive prospective estimates on CH_4 production to formulate diets that generate the smallest possible amount of CH_4 per unit of edible product.

Grass silage is a principal component of rations in intensive and extensive ruminant production systems. It is well established that factors such as chemical composition and degradability of ruminant diets greatly influence CH_4 production (Hristov et al., 2013). Mature grass has more neutral detergent fiber (NDF) and less crude protein (CP) compared to immature grass, causing a shift in the profile of volatile fatty acids towards acetic acid and, thereby, increasing the formation of CH_4 per unit of digested grass (Rinne et al., 1997; Moss et al., 2000).

Assessment of *in vitro* gas production (GP) is largely used to evaluate the nutritive value of ruminant feeds by incubating substrate in buffered rumen fluid (Cone et al., 1996; Getachew et al., 1998; Dijkstra et al., 2005). This *in vitro* approach however, can also be used to evaluate different feeding strategies for their potential to mitigate CH_4 production (Pellikaan et al., 2011; Holtshausen et al., 2012; Hatew et al., 2015). There is a lack of studies comparing *in vivo* and *in vitro* CH_4 production, using rumen fluid from cows adapted to a specific dietary treatment and using those diet components as substrate for *in vitro* incubations. Hatew et al. (2015) reported the potential of using the *in vitro* GP and CH_4 production technique to predict the enteric CH_4 emission measured in controlled respiration chambers. However, in the study conducted by Hatew et al. (2015) the main focus was directed to starch sources in concentrates.

Recently, Warner et al. (2016) quantified the *in vivo* CH_4 production of dairy cows fed grass silages, differing in maturity and produced from grasslands with differences in nitrogen fertilisation. The objective of the present study was to determine whether *in vitro* gas and CH_4 production measurements, using an automated GP technique, can mimic the *in vivo* CH_4 production as measured in climate-controlled respiration chambers (Warner et al., 2016), for different qualities of grass silage.

MATERIALS AND METHODS

Donor animals and experimental diets

The present *in vitro* experiments were conducted simultaneously with an *in vivo* trial previously reported by Warner et al. (2016) where 54 lactating Holstein-Friesian cows were used for *in vivo* CH_4 measurements, using climate-controlled respiration chambers. In brief, the *in vivo* experiment consisted of a randomised block design with nine blocks of six cows each, and within each block, cows were randomly assigned to one of the six experimental treatments. Each experimental period consisted of a 12-d adaptation period in a tie stall, followed by 5 d of CH_4 measurements in a climate-controlled respiration chamber. Cows were fed a total mixed ration (TMR), consisting of compound feed (20% on DM basis) and silage (80% on DM basis) from either low or high fertilised ryegrass based swards (65 kg of N/ha, LF; and 150 kg of N/ha, HF, respectively). Grass was harvested at either early maturity (EM; 28 days of regrowth), mid maturity (MM; 41 days of regrowth) or late maturity (LM; 62 days of regrowth). Dry matter yields and the chemical composition of the experimental grass silages and the compound feed are shown in Table 2.1.

Table 2.1. Dry matter yield and chemical composition of silages from low or high fertilised ryegrass (65 kg of N/ha, LF or 150 kg of N/ha, HF) and harvested at either early maturity (EM; 28 days of regrowth), mid maturity (MM; 41 days of regrowth) or late maturity (LM; 62 days of regrowth). Data derived from Warner et al. (2016).

Item	LF[a]			HF		
	EM	MM	LM	EM	MM	LM
Dry matter yield, kg/ha	2023	3214	3535	2055	3609	5793
Chemical composition						
Dry matter, g/kg	436	654	762	430	575	540
Organic matter	903	924	934	895	902	914
Crude protein	149	106	78	197	173	120
Ether extracts	33	27	22	35	33	25
Sugar	98	190	179	54	79	69
Neutral detergent fibre	476	501	561	459	507	603
Acid detergent fibre	282	288	315	280	298	353
Acid detergent lignin	20	24	26	21	22	32

[a] LF, HF = silage from low or high fertilised ryegrass, respectively; EM, MM, LM = ryegrass harvested at early, mid or late maturity, respectively.
[b] Unless indicated otherwise, units are expressed as g/kg of DM.

Twelve out of the 54 cows were fitted with a permanent rumen cannula (10 cm i.d., Type 1C; Bar Diamond Inc., Parma, ID, USA) and only these 12 cows served as a donor of rumen fluid

for the *in vitro* experiments. In two separate runs rumen fluid was collected directly after completion of the 5 d *in vivo* CH_4 measurements, ensuring that the cows were fully adapted to their respective experimental rations. The six grass silages served as substrate in two *in vitro* runs where rumen fluid was collected from six cows fed the TMR containing the same grass silages. As the *in vivo* study was performed in a series of experiments using respiration chambers, the time between the two runs was two months. A grass silage substrate was incubated only with rumen fluid from a cow that was fed the corresponding grass silage based TMR.

In vitro gas and methane production

Gas production (GP) profiles were determined by using a fully automated GP equipment (Cone et al., 1996). Grass silage samples were freeze-dried and ground over a 1-mm sieve using a cross beater mill (Peppink 100 AN, Olst, The Netherlands). An amount (~0.5 g DM) of each grass silage was accurately weighed into 250-ml fermentation bottles (Schott, Mainz, Germany). Each substrate was weighed in triplicate in bottles. Blanks (rumen fluid without sample) were run in duplicate in each series. Rumen fluid was obtained before feeding the cows (between 0600 h and 0630 h), and subsequently transferred into a pre-warmed insulated flask, previously filled with carbon dioxide, to the laboratory. All other handlings were as described by Cone et al. (1996).

Prior to the inoculation, the fermentation bottles were placed in a shaking water bath, maintained at 39 °C and pre flushed with CO_2. The bottles were then inoculated with 60 ml of buffered rumen fluid with a rumen fluid to buffer ratio of 1:2 (v/v) and connected to the fully automated equipment (Cone et al., 1996). Ten microliter of the headspace gas was collected from the bottles at distinct incubation times (0, 2, 4, 8, 12, 24, 30, 36, 48, 60 and 72 h of incubation) and directly injected into a gas chromatography to determine the CH_4 concentration in the gas sample as described by Pellikaan et al. (2011). After 72 h of incubation, the amount of degraded OM (DOM) was determined gravimetrically after filtration and drying over a glass crucible. Methane profiles were fitted with a monophasic model and gas production data were fitted using a triphasic model, as described by Cone et al. (1996) and Groot et al. (1996) to determine the asymptotic gas production (A) of the soluble (A1) and insoluble fraction (A2), and the time needed to reach half of A and A2 (B and B2, respectively) as a measure for the rate of fermentation. The parameters C and C2 determine the sharpness of the profile (Groot et al., 1996; Cone et al., 1997). Phase 1 is related to the fermentation of the soluble fraction, and phase 2 to the fermentation of the non-soluble fraction (Cone et al., 1997).

Chemical analysis

The dry matter (DM) content was determined after drying at 103 °C overnight (ISO 6496; ISO, 1999) and ash content after incineration for 3 h at 550 °C (ISO 5984; ISO 2002). Nitrogen (N) was determined, using the Kjeldahl method (AOAC, 1990), and a factor of 6.25 was used to convert N into crude protein (CP). Organic matter content (OM) was determined following the AOAC (1990) protocol. Neutral detergent fibre (NDF) was analysed according to Van Soest et al. (1991), after a pre-treatment with a heatstable amylase, and was expressed exclusive of residual ash (aNDFom). Contents of acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest (1973) and ADF was expressed exclusive of residual ash (ADFom). Sugar contents were determined as described by Van Vuuren et al. (1993) using 40% ethanol.

Statistical analysis

Prior to statistical analysis, triplicate *in vitro* data from the different bottles were averaged. All data were subjected to ANOVA using the general linear model procedure in SAS (version 9.3; SAS Institute Inc. Cary, NC, USA), based on the model:

$$Y_{ij} = \mu + M_i + F_j + (M \times F)_{ij} + e_{ij}$$

where Y_{ij} = response variable (e.g. CH_4, GP, fermentation kinetics parameters); μ = overall mean; M_i = maturity stage of the grass (i = early, mid or late maturity); F_j = rate of N fertilisation (j = low or high); $(M \times F)_{ij}$ = interaction term between maturity and rate of N fertilisation; and e_{ij} = residual error. Differences between treatment means were compared using the least square means procedure and Tukey's statement for multiple comparisons. Results are reported as least square means and their associated standard errors.

In order to determine the relationship between *in vitro* parameters, gas and CH_4 production and *in vivo* CH_4 production, multiple regression analysis was performed using *in vivo* CH_4 production as dependent variable and the various *in vitro* curve fit parameters of gas and CH_4 production and chemical composition, as predictor variables. Stepwise regression was performed by incorporating parameters into the model showing the highest significant partial-correlation coefficient for its relation with the residual variance in *in vivo* CH_4 production. The level of statistical significance was declared at $P \leq 0.05$.

RESULTS

Relationship between *in vitro* and *in vivo* methane production

For the 12 donor cows used in the present study, Warner et al. (2016) observed *in vivo* a positive effect of the maturity of the grass at harvest on the enteric CH_4 production. With CH_4 expressed in g CH_4/kg DMI, it ranged from 22.5 to 25.0 for LF grass and from 20.6 to 26.3 for HF grass. With CH_4 expressed as a % of GEI it showed an increase with advancing maturity of grass at harvest, and ranged from 6.6 to 7.6 for LF, and from 6.0 to 7.8 for HF grass. Organic matter digestibility decreased with increasing maturity of the grass. Similar results were observed for the amount of *in vitro* degraded organic matter, and a positive relationship with *in vivo* OM digestibility was observed (R^2 = 0.51; P = 0.008; Figure. 2.1). The *in vitro* obtained GP data, being a measure for total fermentation of the different substrates are shown in Table 2.2. This Table also shows the *in vitro* obtained CH_4 production, as well as the *in vivo* CH_4 production (Warner et al., 2016). However, the *in vivo* CH_4 production expressed in g/kg DOMI did not correlate with the *in vitro* CH_4 production, expressed in g/kg DOM.

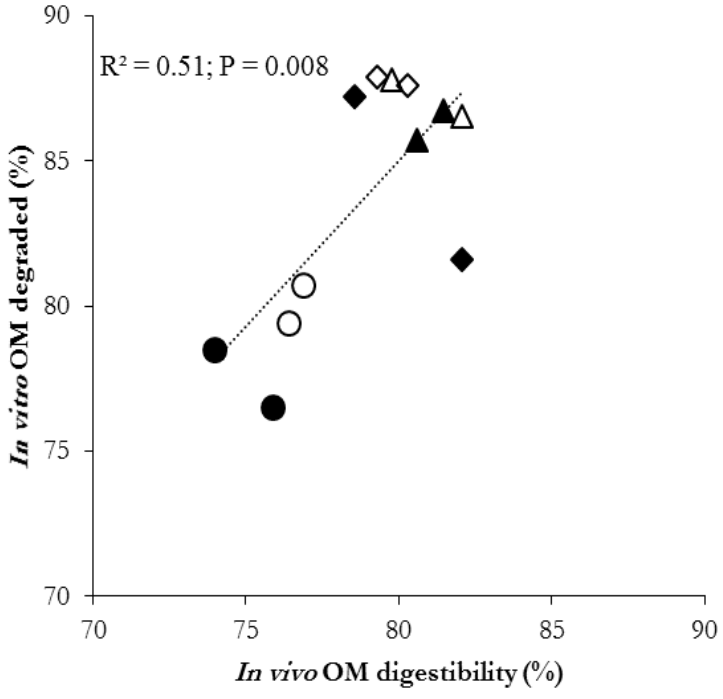

Figure 2.1. Relationship *in vivo* OM digestibility and *in vitro* OM degraded (of silages of grass harvested at three stages of maturity (28, 41 and 62 days of regrowth; EM, MM and LM, respectively) and with two N fertilisation rates (65 kg of N/ha, LF; 150 kg of N/ha, HF). Δ = EM-LF; ◊ = MM-LF; O = LM-LF; ▲ = EM-HF; ♦ = MM-HF; ● = LM-HF.

Table 2.2. *In vitro* organic matter digestibility, gas and methane production and curve fit parameters after *in vitro* fermentation of silages from low or high fertilised ryegrass (65 kg of N/ha, LF or 150 kg of N/ha, HF) and harvested at either early maturity (EM; 28 days of regrowth), mid maturity (MM; 41 days of regrowth) or late maturity (LM; 62 days of regrowth), and *in vivo* CH_4 data as partly published by Warner et al. (2016).

Item	LF[a]			HF			SEM[c]	P-value		
	EM	MM	LM	EM	MM	LM		F	M	F × M
DOM	0.872	0.878	0.801	0.863	0.845	0.776	0.0128	0.079	0.003	0.649
Gas production (GP)										
72-h GP (ml/g OM)	306.6	288.4	279.9	316.5	297.6	276.2	1.20	0.002	<0.001	0.002
A1 (ml/g OM)	61.6	65.8	60.1	96.4	71.5	42.7	0.97	<0.001	<0.001	<0.001
A2 (ml/g OM)	191.0	171.4	150.6	168.7	177.6	165.9	0.56	0.584	<0.001	<0.001
B2 (h)	7.4	7.1	8.6	6.0	7.0	8.0	0.01	<0.001	<0.001	<0.001
C2	3.0	2.7	2.7	2.5	2.7	2.6	0.01	<0.001	<0.001	<0.001
R_{max} (ml/h)	21.6	18.9	13.4	20.6	19.4	15.7	0.06	<0.001	<0.001	<0.001
CH_4 production										
72-h CH_4 (ml/g OM)	55.7	52.6	46.3	61.8	68.7	57.4	2.82	0.003	0.045	0.278
72-h CH_4 (ml/g DOM)	64.0	61.2	59.8	71.6	81.3	74.0	3.26	0.003	0.540	0.280
CH_4 (% of total gas)	18.2	18.2	16.5	19.5	23.1	20.8	0.98	0.004	0.179	0.329
A (ml/g OM)	62.9	61.6	59.2	68.0	78.3	69.5	4.62	0.030	0.490	0.498
B (h)	12.3	16.6	21.5	9.6	13.2	17.9	1.37	0.028	0.002	0.948
C	1.2	1.1	1.1	1.1	1.2	1.1	0.09	0.946	0.665	0.619
R_{max} (ml/h)	3.7	3.2	1.6	5.2	4.4	3.2	0.42	0.013	0.018	0.907
In vivo CH_4 production										
OM digestibility	0.810	0.798	0.767	0.811	0.804	0.750	0.0098	0.681	0.007	0.526
CH_4 (g/day per cow)	384.1	385.2	343.7	316.4	351.3	326.8	22.80	0.076	0.385	0.544
CH_4 (g/kg of OMI)	26.4	26.6	25.0	25.2	27.0	27.5	1.56	0.668	0.806	0.531
CH_4 (g/kg of DOMI)	32.6	34.0	34.9	29.8	34.7	33.4	2.96	0.896	0.373	0.582

[a] LF, HF = silage from low or high fertilised ryegrass, respectively; EM, MM, LM = ryegrass harvested at early, mid or late maturity, respectively. DOM is *in vitro* degraded organic matter; A, A1 and A2 are the asymptotic methane or gas production in phase 1 and phase2, respectively; CH_4 is methane; B and B2 are the time at which half of asymptotic CH_4 or gas production is reached; C and C2 are the sharpness of the switching characteristics for the profile of CH_4 or gas production; R_{max} is maximum rate of gas or CH_4 production.

[b] *In vivo* CH_4 production of donor cows fed the same diet as the substrate incubated, was measured simultaneously in climate-controlled respiration chambers (Warner et al., 2016).

[c] SEM is pooled standard error of the means.

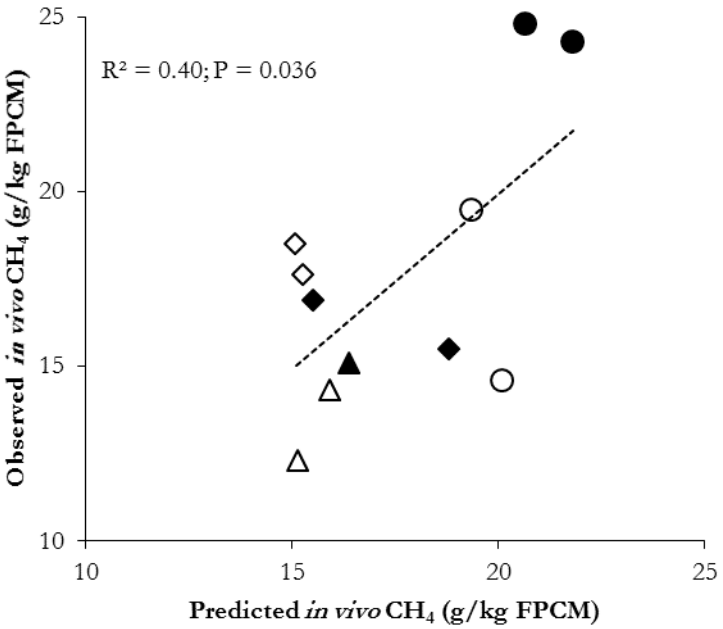

Figure 2.2. Relationship between observed *in vivo* (CH_4 g/kg of fat and protein corrected milk (FPCM)) and predicted *in vivo* CH_4 (g/kg of FPCM) by the regression equation CH_4 g/kg FPCM = 66.87 – 0.589 × OMD of silages of grass harvested at three stages of maturity (28, 41 and 62 days of regrowth; EM, MM and LM, respectively) and with two N fertilisation rates (65 kg of N/ha, LF; 150 kg of N/ha, HF). Δ = EM-LF; ◊ = MM-LF; O = LM-LF; ▲ = EM-HF; ♦ = MM-HF; ● = LM-HF. Results are shown for 11 donor cows (1 cow was discarded from *in vivo* analysis, as described by Warner et al., 2016).

There was no linear relationship between CH_4 production measured *in vivo* and *in vitro*. *In vitro* CH_4 production after 8, 12, 24 and 72 h of incubation, expressed in ml/g of OM did not correlate with the *in vivo* CH_4 production, expressed in g/kg DMI (R^2 = 0.01–0.08). There was also no correlation between 72-h *in vitro* CH_4 production, expressed in ml/g of OM and *in vivo* CH_4 production, expressed in g/kg of OMI (R^2 = 0.00). To predict the *in vivo* CH_4 production from the *in vitro* gas and CH_4 parameters and chemical composition, stepwise multiple regression was performed. The predicted *in vivo* CH_4 (g/kg FPCM) and observed *in vivo* CH_4, expressed in g/kg FPCM, showed a weak, but positive correlation (R^2 = 0.40; P = 0.036; Figure. 2.2).

The inclusion of *in vitro* gas and CH_4 parameters did not improve the prediction of the *in vivo* CH_4 data. *In vivo* CH_4 expressed in g/kg OMI, g/kg DMI, and CH_4 as %GEI was not predicted well, because no variable met the pre-set 0.05 significance level to enter the regression model.

***In vitro* gas and methane production**

The DOM was significantly influenced by the grass silage maturity but only a trend was obtained for the effect of N fertilisation (Table 2.2). The interaction between maturity and N fertilisation was not significant for DOM. Total 72-h GP decreased with increasing silage maturity and was larger with HF grass (Table 2.2). However, the decrease in GP from MM to LM was more pronounced for HF grass than LF grass. The estimated values for the asymptotic GP of the soluble fraction (A1) followed the same trend as 72-h GP, with the largest value for EM grass at HF. However, for LF the decrease in the A1 asymptote did not follow the same pattern observed for GP. In contrast, the estimated values for the asymptotic GP of the insoluble fraction (A2) did not increase for the LF and HF grasses.

The time needed to reach half of the asymptotic GP of the insoluble fraction (B2) increased between EM and LM, indicating a negative relationship between the rate of fermentation and the maturity of the grass silages. This notion is confirmed by a decrease in the maximum rate of GP production (R_{max}) with increasing maturity, and decrease being more pronounced at LF than HF (Table 2.2).

Methane production after 72-h, expressed as either ml/g of OM, ml/g of DOM or as % of the total gas, was greater at HF than LF (Table 2.2). Maturity of the grass silages only affected the 72-h CH_4 production when expressed as ml/g of OM (Table 2.2) with decreased CH_4 production with advancing maturity. The interaction between maturity and rate of N fertilisation was not significant for CH_4 production. The estimated values for the asymptotic CH_4 production (A) generally followed the same trend as 72-h CH_4 production expressed as ml/DOM or as a % of total gas (Table 2.2). Asymptotic CH_4 production was found to be larger ($P = 0.030$) at HF than LF, as well as CH_4 as % of total gas, but values did not differ with stage of maturity. The time needed to reach half of the asymptotic CH_4 production (B) increased with advancing maturity (Table 2.2) and was less at HF than LF. The maximum rate of gas and CH_4 production (R_{max}) decreased with advancing grass maturity and was affected by the rate of N fertilisation. It was observed from the results in (Table 2.2), that R_{max} for GP was 3.3% larger in the HF samples, compared to LF, and for CH_4 the variation was 50.5% larger in HF compared to LF.

DISCUSSION

Relationship between *in vitro* and *in vivo* methane production

In the current *in vitro* experiment we used rumen fluid from donor cows adapted to the dietary treatments, which is generally not the case for the *in vitro* studies reported in literature. Despite the *in vitro* measurements being performed simultaneously with the *in vivo* experiments and with adapted rumen fluid from the donor cows used as a part of the *in vivo* experiment (Warner et al., 2016), there was no relationship between *in vivo* CH_4 expressed in ml/g of DM intake or in ml/g DOM intake and *in vitro* ml/g OM incubated or ml/g DOM ($R^2 = 0.04$, $P = 0.847$; $R^2 = 0.01$, $P = 0.781$). The main reason for the lack of a relationship might be due to the fact that the *in vivo* variation between the animals was too large within the treatments, compared to differences between treatments. Furthermore, the absence of a relationship between *in vitro* and *in vivo* CH_4 production could also be influenced by the fact that passage, rumen acidity and the profile of volatile fatty acids, which influence the *in vivo* measurements and hindgut fermentation are not exactly simulated *in vitro* (Hatew et al., 2015).

In general, increased grass maturity resulted in a decreased ruminal degradability, due to a lower CP content and higher NDF and lignin contents. A positive correlation between the *in vitro* maximum fractional rate of substrate degradation and the *in vivo* fractional degradation rate of OM for the tested grass silages (based on the *in situ* nylon bag technique; see Heeren et al., 2014) was observed ($R^2 = 0.70$, $P = 0.037$; data not shown).

Using the estimated curve fit parameters (A, B, C), the time point at which the *in vitro* CH_4 production is equal to the *in vivo* CH_4 production, expressed in ml/g OM incubated can be estimated. There was a large variation in the estimated time points, which essentially augmented with advancing grass maturity: 16.45 h (LF-EM); 22.22 h (LF-MM); 30.42 h (LF-LM); 9.38 h (HF-EM); 12.58 h (HF-MM); and 18.50 h (HF-LM) after the start of the incubation. It shows that the *in vitro* CH_4 production correlated poorly with the *in vivo* CH_4 production for a fixed time point. However, these results suggest again that grass requires more time for degradation with increasing maturity, resulting in less fermentation and, consequently, less CH_4 formation compared with younger grass over the same time period.

It was anticipated that the variation in *in vitro* CH_4 is similar when grass silage is incubated instead of the TMR. Furthermore, the concentrate used was the same in all rations and the differences in CH_4 production, also in *in vivo* experiment were caused by the differences in grass quality.

Effect of grass silage quality on *in vitro* rumen fermentation characteristics

The six grass silages tested in this *in vitro* experiment varied in nutrient composition as the result of different maturity stages and N fertilisation levels (Table 2.1). The HF grass silages contained more CP and less sugar than the LF grass silages. Advancing maturity resulted in a decreased CP content and increased sugar and fibre contents. The sugar content was highest for MM, which could have been caused by weather conditions at harvest and wilting. The decline in GP with increasing maturity observed (Table 2.2) is in accordance with findings from Cone et al. (1999). The decline in GP in phase 1 of the HF and LF samples, which is the GP caused by fermentation of the water soluble fraction (Cone et al., 1997), is associated with a decreasing CP content with advanced maturity. The decline in 72-h GP for the LF grass in phase 2, which is the GP caused by fermentation of the non-soluble fraction, is associated with a decrease in degradable non-soluble components (cell walls) with advancing maturity. The increased time to reach half of the asymptotic gas production (B2) indicates a slower fermentation with increased maturity (Table 2.2).

In vitro CH_4 production, expressed in ml/g of OM, in ml/g of DOM or as a % of total gas was affected by the N fertilisation, whereas grass maturity showed only a minor effect and only on *in vitro* CH_4 expressed in mg/g of OM. Warner et al. (2016), using 12 animals as donor for the rumen inoculum for the *in vitro* incubations, reported an increase in enteric CH_4, expressed in g/kg FPCM at increasing maturity. Opposite results were observed in this *in vitro* experiment, with decreasing CH_4 production in ml/g of OM with advancing maturity, accompanied by a lower total GP. Methane expressed as a % of total gas varied from 16.5% to 18.2% for LF, and from 19.5% to 23.1% for HF. Therefore, for the purposes of this study, the best way to express CH_4 production as a % of total GP. Advancing maturity did not influence the% CH_4 in the total gas for LF grass, but the CH_4 percentage was higher for HF grass. In particular, fermentation of the soluble fraction (A1) increased by, on average, 12% with increasing N fertilisation, whereas fermentation of the non-soluble fraction (A2) did not change with N fertilisation. This effect likely explains the overall positive effect of N fertilisation on *in vitro* CH_4 production, whereas this effect was not observed *in vivo* (Table 2.2). The R_{max} of GP decreased with increasing maturity in line with larger B2 values. A similar pattern was observed for CH_4 production with larger B values, indicating that the rate of CH_4 production decreased with increasing grass maturity.

CONCLUSIONS

In vitro CH_4 production of grass silages used in this research did not correlate with the *in vivo* CH_4 production, expressed in g/kg of OMI, g/kg DMI or g/kg of DOMI. The *in vivo* variation between the donor cows was too large, compared to the differences between the different treatments. It is clear that *in vitro* degraded OM rather matches with *in vivo* digestibility, but this is not valid for CH_4 production. The results indicate that *in vitro* CH_4 measurements for screening purposes need to be handled with care as effects of grass silage quality observed in an *in vitro* batch culture may not be observed *in vivo* and vice versa. However, the conclusions are only based on a limited dataset (n =12 cannulated cows and six grass silages).

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REFERENCES

AOAC, 1990. Official methods of analysis, 15th edn. Assoc. Off. Anal. Chem., Arlington, VA, USA.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. 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.

Cone, J.W., van Gelder, A.H., Driehuis, F., 1997. Description of gas production profiles with a three phasic model. Anim. Feed Sci. Technol. 66, 31-45.

Cone, J.W., van Gelder, A.H., Soliman, I.A., de Visser, H., van Vuuren, A.M., 1999. Different techniques to study rumen fermentation characteristics of maturing grassland grass silage. J. Dairy Sci. 82, 957-966.

Dijkstra, J., Kebreab, E., Bannink, A., France, J., Lopez, S., 2005. Application of the gas production technique to feed evaluation systems for ruminant. Anim. Feed Sci. Technol. 123, 561-578.

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds. Anim. Feed Sci. Technol. 72, 261-281.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77-89.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Heeren, J.A.H., Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Bannink, A., Warner, D., de Jonge, L.H., Dijkstra, J., 2014. Rumen degradation characteristics of ryegrass herbage and ryegrass silage are affected by interactions between stage of maturity and nitrogen fertilisation rate. Anim. Prod. Sci. 54, 1263-1267.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci. 60, 40-45.

Hristov, A.N., Oh, J., Frikins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B., Tricarico, J.M., 2013. Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91, 5045-5069.

ISO 6496, 1999. Animal Feeding Stuffs. Determination of Moisture and other Volatile Matter Content. Geneva, Switzerland.

ISO 5984, 2002. Animal Feeding Stuffs. Determination of Crude Ash. Geneva, Switzerland.

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

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

Pellikaan, W.F., Hendriks, W.H., Uwimana, C., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011. A novel method to determine simultaneously methane production during the *in vitro* gas production using a fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Rinne, M., Huhtanen, P., Jaakkola, S., 1997. Grass maturity effects on cattle fed silage-based diets. 2. Cell wall digestibility, digestion and passage kinetics. Anim. Feed Sci. Technol. 67, 19-35.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Van Soest, P.J., 1973. Collaborative study of acid detergent fibre and lignin. J. Assoc. Off. Anal. Chem. 56, 781-784.

Van Vuuren, A.M., van der Koelen, C.J., Valk, H., de Visser, H., 1993. Effects of partial replacement of ryegrass by low protein feed on rumen fermentation and nitrogen loss by dairy cows. J. Dairy Sci. 76, 2982-2993.

Warner, D., Hatew, B., Podesta, S.C., Klop, G., van Gastelen, S., van Laar, H., Dijkstra, J., Bannink, A., 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10, 33-43.

Chapter 3

In vitro gas and methane production in rumen fluid from dairy cows fed grass silages differing in plant maturity, compared to obtained *in vivo* data

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ABSTRACT

The relationship between *in vitro* rumen CH_4 production of grass silages, using the gas production technique, and *in vivo* data obtained with the same cows and rations in respiration chambers was investigated. Silages were made from grass harvested in 2013 on May 6th, May 25th , July 1st and July 8th. The grass silages were used to formulate 4 different rations which were fed to 24 cows in early and late lactation, resulting in a slightly different dry matter intake (DMI; 16.5 kg/d vs 15.4 kg/d). The experimental rations consisted of 70% grass silage, 10% maize silage and 20% concentrates on a dry matter basis. Cows were adapted to the rations for 17 days before rumen fluid was collected via esophageal tubing and *in vitro* gas and CH_4 production were analysed. *In vitro* total gas and CH_4 production of the (ensiled) grass expressed as ml/g OM decreased with advancing maturity of the grass. *In vitro* gas and CH_4 production using grass based rations as substrate were greater using rumen fluid from cows fed at late lactation compared to early lactation. The *in vitro* CH_4 production after 48 hours of incubation expressed in ml/g OM did not correlate with the *in vivo* CH_4 production expressed in g/kg organic matter intake or g/kg DMI (R^2 = 0.00-0.18, $P \geq 0.287$). The differences in CH_4 emission per unit of intake observed *in vivo* were rather small between the different rations, which also contributed to the observed poor relationship. Utilizing stepwise multiple regression improved the correlation only slightly. *In vitro* gas and CH_4 production varied based on whether donor cows were previously adapted to the respective ration or not, suggesting that careful adaption to the experimental diet should be envisaged in *in vitro* gas and CH_4 production experiments.

INTRODUCTION

Grass silage is a principal component of ruminant rations in many countries. The maturity of grass at harvest affects the methane (CH_4) production by the cows per unit of digested grass (Warner et al., 2016). Enteric CH_4 is formed by fermentation of feed in the gastrointestinal tract and constitutes a considerable loss of dietary energy to the animal (Johnson and Johnson, 1995), and contributes to greenhouse gas emissions (Moss et al., 2000).

Assessment of *in vitro* gas production (GP) is largely used to evaluate the nutritive value of ruminant feeds by incubating substrate in buffered rumen fluid (Cone et al., 1996; Getachew et al., 1998; Dijkstra et al., 2005). This *in vitro* approach can also be used to evaluate different feeding strategies for their potential to mitigate CH_4 production (Pellikaan et al., 2011; Holtshausen et al., 2012; Hatew et al., 2015). There is a lack of studies reporting *in vivo* CH_4 production by cattle upon changes in the maturity of a grass silage at harvest (Randby et al., 2012; Warner et al., 2017), and there is a dearth of direct *in vitro-in vivo* comparisons with respect to CH_4 production. Results from a previous experiment (Macome et al., 2017) showed a weak, but positive relationship between *in vitro* and *in vivo* CH_4 production, expressed as g CH_4/kg of fat and protein corrected milk (FCPM). Performing *in vitro* and *in vivo* experiments simultaneously gives the unique opportunity to use rumen fluid in the *in vitro* experiments from the same cows as used in the *in vivo* experiments and using the same feed ingredients and rations, allowing a direct comparison between *in vitro* and *in vivo* CH_4 measurements. It is highly valuable to have fast and reliable *in vitro* techniques to estimate the CH_4 emission from ruminants, as *in vivo* studies, in particular using respiration chambers, are very laborious and expensive.

The objective of the present study was to measure the *in vitro* gas and CH_4 production of grass silages, differing in maturity stages at harvest, and of rations consisting of the respective grass silages. The obtained *in vitro* CH_4 production was related to simultaneously obtained *in vivo* CH_4 production data (Warner et al., 2017). Also, the effect of adaptation of the microbial population to the respective grass silage based rations was investigated.

MATERIALS AND METHODS

Experimental design, donor animals and rations

The *in vitro* experiments were conducted simultaneously with the *in vivo* experiments (Warner et al., 2017). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University and carried out under the Dutch Law on Animal Experimentation. *In vivo* CH_4 measurements and animal handlings were described in more detail by Warner et al. (2017). The present *in vitro* study relates to a total of 24 lactating Holstein-Friesian dairy cows housed at the experimental farm of Wageningen University & Research (Wageningen, the Netherlands) and grouped in a randomized block design. Within each block of 4 cows, cows were assigned to an early lactation or a late lactation group, and within groups, cows were randomly assigned to 1 of 4 dietary treatments consisting of grass silage of varying maturity at harvest. Cows were, on average, 96 days in milk (DIM) for the early lactation group, and 218 DIM for the late lactation group. Early and late lactation cows showed relatively different levels of DM intake (DMI) (16.5 vs 15.4 kg/d, respectively).

Grass swards were harvested in 2013 at 4 different dates as grass maturity advanced: May 6th (G1), May 25th (G2), July 1st (G3) and July 8th (G4). Cut grass was wilted for two or three days and ensiled in bales of ca. 500 kg using 12 layers of stretch plastic without addition of inoculants. Cows were offered a ration with 70% grass silage, 10% maize silage and 20% concentrate on a dry matter (DM) basis. The least mature grass silage (GS1) was highly digestible and, therefore, 5% wheat straw was included in the respective ration (replacing an equal amount of GS1 on as-fed basis) to obtain a sufficient structural value to meet the requirements of the cows (Warner et al., 2017).

For the *in vitro* measurements, core samples were collected from the grass silage bales fed to the cows housed in the climate-controlled respiration chambers for *in vivo* CH_4 measurements. This procedure was repeated before each *in vitro* run to obtain fresh grass silage sample at each run and a sample comparable to that offered to cows in the respiration chambers. Maize silage, wheat straw and concentrate were sampled once before each experimental period.

Feeding and rumen inoculum sampling

Cows were housed in tie stalls for the first 12 days of each 17-day period to facilitate adaptation to their respective rations. A 5-day measurement period followed in which cows were housed individually in climate respiration chambers (Warner et al., 2017). During the last 4 days of adaptation, cows received 95% of the average daily intake of the cow with the smallest daily intake

within the group of 4 cows, provided that cows with the largest feed intake were never restricted to less than 80% of the *ad libitum* feed intake. Feed intake was restricted to ensure a uniform DMI within each block. Samples of 750 ml rumen fluid were collected from 4 donor cows within each period for the *in vitro* measurements, resulting in 4 different types of rumen fluid per period.

Each grass silage was incubated with all 4 types of rumen fluid; thus, with the rumen fluid of the cow adapted to the respective ration and with the rumen fluid of the cows adapted to the remaining 3 rations. Total ration substrates, whose compositions were the same as those of the ration fed in the *in vivo* experiment, were only incubated with the corresponding (adapted) rumen fluid. As donor cows were not previously fitted with a rumen cannula, rumen fluid was collected via esophageal tubing. A recent paper suggested that the rumen bacterial community composition does not differ between esophageal tubing and rumen cannula sampling (Paz et al., 2016).

Gas and methane production

In vitro rumen fermentation was determined using fully automated GP equipment, as described by Cone et al. (1996). Approximately 0.50 g of oven-dried and ground (1 mm) substrate (grass silage or total ration) was weighed into 250-ml fermentation bottles (Schott, Mainz, Germany) and 60 ml of buffered rumen fluid was added. Each grass silage or total ration was analyzed in triplicate. *In vitro* incubation measurements lasted for 48 hours. Rumen fluid was obtained from 4 donor cows within the respective group per period prior to the *in vitro* measurements early in the morning before feeding the animals, and was transported in a pre-warmed insulated CO_2 filled flask. All other handlings were as described by Cone et al. (1996).

Each grass silage was incubated with rumen fluid from a donor cow adapted to the respective grass silage ration and from 3 cows adapted to one of the 3 other grass silage rations (TRGS1 through TRGS4). Gas production curves were fitted with a multiphasic model as described by Cone et al. (1996) and Groot et al. (1996). This model determines the asymptotic GP (A) caused by fermentation of the soluble fraction (A1), the insoluble fraction (A2) and the microbial turnover (A3), the time needed to reach half of A1, A2 and A3 (B1, B2 and B3) as a measure of the rate of fermentation, and the sharpness of the curve in phase 1, phase 2 and phase 3 (C1, C2 and C3). Data on phase 3, B1 and C1 are not reported, as these parameters do not contribute to the feeding value of the substrate. As fitting the curves with the three-phasic model with 9 unknown parameters often gives non-physiological results, the values for A1 and A2 were fixed. The GP after 3 h is assumed to be A1 and the GP between 3 and 20 h is assumed to be A2, as described by Van Gelder et al. (2005). During the GP incubations, 10 µl of headspace gas was collected from the bottles at 9 time points (0, 2, 4, 8, 12, 24, 28, 36, and 48 h), and directly injected

into a gas chromatograph to determine the CH_4 concentration in the headspace gas as described by Pellikaan et al. (2011).

Each incubation was done in triplicate over three *in vitro* runs. The *in vitro* CH_4 production data were fitted with a mono-phasic model (Groot et al., 1996). The maximum rate of the GP of the non-soluble fraction (R_{max2}, ml/h) and of CH_4 production (R_{max}, ml/h) was calculated as described by Bauer et al. (2001).

Chemical analyses

Grass silage samples were dried at 70 °C for 48 hours in a forced-air ventilation oven and ground in a Wiley mill (Peppink 100 AN, Olst, The Netherlands), fitted with a 1-mm sieve. The DM content of the ground samples was determined after drying at 103 ºC overnight (ISO 6496; ISO, 1999), and ash content was determined by incineration for 3 h at 550 °C (ISO 5984; ISO, 2002). Nitrogen was measured by the Kjeldahl method (AOAC, 1990), and crude protein (CP) was calculated as N × 6.25. Neutral detergent fibre (NDF) was analyzed according to Van Soest et al. (1991) after a pre-treatment with a heat stable amylase and expressed exclusive of residual ash (aNDFom) (ISO 16472; ISO 2006). Contents of acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest (1973) (ISO 13906; ISO 2008), and ADF was expressed exclusive of residual ash (ADFom). Sugar analysis was determined as described by Van Vuuren et al. (1993) with modifications by Abrahamse et al. (2008), using 40% ethanol.

Statistical analyses

Triplicate *in vitro* data were pooled per cow and substrate. All triplicate results for each substrate (i.e. grass silage and total ration) per run were averaged prior to statistical analysis. The experimental unit for the *in vitro* measurements was the value of the averaged data (fermentation bottles). Data were subjected to ANOVA in a randomized block design with a 4 × 4 factorial arrangement of treatments (4 stages of grass maturity × 4 rumen inocula) by the mixed model procedures of SAS (2010). Substrate (n = 4; either grass silage or total rations) and rumen fluid (n = 4) were considered fixed effects, block (n = 3) was considered a random effect, and stage of lactation (n = 2) was considered a blocking factor. As grass silages and total rations were used as substrate for the *in vitro* measurements, two statistical models were used:

$$Y_{ijk} = \mu + SUB_i + RF_j + Block_k + (SUB \times RF)_{ij} + e_{ijk}$$

where Y_{ijk} = response variable (e.g. CH_4, GP, fermentation kinetics parameters), μ = overall mean, SUB_i = the fixed effect of grass silage substrate (i = 4; maturity stages GS1 through GS4), RF_j = the fixed effect of rumen fluid (j = 4; samples from 4 cows receiving GS1 through GS4), $Block_k$ = the random effect of block (k = 3; *in vitro* runs), (SUB × RF) = interaction term and e_{ijk} = residual error.

$$Y_{ij} = \mu + SUB_{i+} e_i$$

where Y_i = response variable (e.g. CH_4, GP, fermentation kinetics parameters), μ = overall mean, SUB_i = total ration substrate (i = 4; maturity stages TRGS1 through TRGS4), and e_i = residual error.

Differences between treatment means were compared by the least squares means procedure and a Tukey's statement was used for multiple comparisons. Results are reported as least squares means and their associated standard error of means. Results on rumen fluid adaptation were reported as an average GP or CH_4 parameter across grass silages. Effect of dietary treatments and their interactions were declared significant at $P \leq 0.05$. The PROC REG statement was used in SAS to predict the *in vivo* CH_4 production. In order to determine which *in vitro* measurements were not related, stepwise multiple regression was performed. Forward stepwise multiple regression was performed by incorporating *in vitro* CH_4 production and chemical composition variables showing the largest significance into the model. Significance was declared at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

Table 3.1. Chemical composition of grass silages of increasing maturity stage at harvest (GS1 to GS4), maize silage, wheat straw and compound feed.

Component (g/kg DM)	Grass silages*				Maize silage	Wheat straw	Compound feed†
	GS1	GS2	GS3	GS4			
DM (g/kg as is)	456	510	407	431	333	887	918
OM	894	898	909	921	964	902	922
CP	286	209	145	124	71	33	247
Crude fat	46	31	28	26	29	9	62
Sugars	78	82	65	87	—	—	94
Starch	—	—	—	—	352	—	219
NDF	365	469	518	546	353	753	162
ADF	199	259	305	319	192	438	87
ADL	3	7	16	21	7	51	3

* GS1 is grass silage harvested on May 6th, GS2 on May 25th, GS3 July 1st and GS4 on July 8th.
† Ingredients (g/kg DM): citrus pulp (311.0), rumen-protected soybean meal (269.3: SoyPass, Trident, Peterborough, FL), wheat (199.8), maize (120.6), rumen-inert palm fat (39.6; Hidropalm, Norel, Madrid, Spain), urea (18.4), calcium biphosphate (13.4), salt (9.4) calcined magnesia (8.9), mineral premix (2.5; Research Diet Services, Wijk bij Duurstede, the Netherlands), chromium oxide (1.5).

RESULTS

Chemical composition

Upon maturation, the CP content of the grass silages decreased from 286 to 124 g/kg DM, whereas the NDF content increased from 365 to 546 g/kg and the ADF content from 199 to 319 g/kg DM (Table 3.1). The youngest grass silage (GS1) contained only 3 g ADL/kg of DM, while the oldest grass silage (GS4) contained 21 g ADL/kg of DM. The aforementioned changes in chemical composition of the different grass silages were also obtained in the corresponding total rations (data not shown).

In vitro gas and CH_4 production of the grass silages

The *in vitro* GP characteristics by fermentation of the grass silages are shown in Table 3.2. The GP decreased with progressing maturity of the grass at harvest. A similar pattern was observed for the asymptotic GP of the soluble (A1) and non-soluble fraction (A2) (Table 3.2). The half time of the asymptotic GP of the non-soluble fraction (B2) showed a slower fermentation with progressing maturity of the grass. The maximum rate of GP in phase 2 decreased with progressing maturity, showing the same trend as B2. Furthermore, an effect of the origin of the rumen fluid on A1 and C2 was observed (Table 3.2).

The *in vitro* CH_4 production characteristics by fermentation of the grass silages as well as the effect of the origin of the rumen fluid are shown in Table 3.3. The results indicated that the *in vitro* CH_4 production (expressed as ml/g OM incubated), and the fitted parameters B, C and R_{max} were affected by the maturity of the grass at harvest. *In vitro* methane production, expressed in ml/g OM, decreased with progressing maturity of the grass at harvest. The half time of the asymptotic CH_4 production (B) increased with progressing maturity of the grass, indicating a slower CH_4 synthesis with progressing grass maturity. Moreover, the origin of the rumen fluid had a strong effect on the total *in vitro* CH_4 production and fitted parameters.

In vitro gas and CH_4 production of the total rations

The *in vitro* GP, CH_4 production and fermentation characteristics of the incubated total ration samples are shown in Tables 3.4 and 3.5. Contrary to the grass silages, the total ration samples were all incubated in rumen fluid obtained from donor cows adapted to their corresponding diet. The *in vitro* GP decreased upon progressing grass maturity (Table 3.4). Parameter A1 decreased upon progressing grass maturity, indicating that less soluble sugars and

Table 3.2. *In vitro* gas production (GP) and curve fit parameters of grass silages made from grass differing in maturity at harvest (GS1 to GS4) and incubated with 4 different types of rumen fluid (RFGS1 to RGS2).

Item*	Variables†					
	GP (ml/g OM)	A1 (ml/g OM)	A2 (ml/g OM)	B2 (h)	C2	R_{max2} (ml/h)
Substrate (SUB)						
GS1	286.4^{a}	63.5^{a}	193.0^{a}	6.9^{b}	2.9^{a}	23.0^{a}
GS2	292.9^{a}	56.9^{ab}	195.1^{a}	7.9^{a}	2.8^{ab}	20.0^{a}
GS3	272.8^{b}	48.7^{b}	177.1^{b}	8.0^{a}	2.7^{b}	17.3^{b}
GS4	265.0^{b}	50.1^{b}	166.3^{c}	8.0^{a}	2.6^{b}	16.0^{b}
SEM	4.52	3.81	2.90	0.15	0.05	0.42
Rumen fluid (RF)						
RFGS1	291.4^{a}	71.0^{a}	177.6^{b}	6.9^{b}	2.4^{b}	19.0
RFGS2	285.2^{a}	59.7^{b}	184.1^{b}	7.3^{a}	2.6^{b}	19.5
RFGS3	268.7^{b}	48.0^{b}	180.2^{b}	7.7^{a}	2.9^{a}	19.2
RFGS4	271.2^{b}	40.6^{b}	188.3^{a}	8.3^{a}	3.0^{a}	18.5
SEM	4.53	3.81	2.91	0.15	0.05	0.42
P-value						
SUB	<0.0001	0.001	<0.0001	<0.0001	<0.0001	<0.0001
RF	<0.0001	<0.0001	0.001	<0.0001	<0.0001	0.168
SUB × RF	0.193	0.972	0.311	0.962	0.918	0.031

^{a,b,c} Values within a column and item with different superscript differ significantly ($P < 0.05$).

* GS1-GS4 are grass silages differing in maturity from young to old with increasing number (harvested on May 6th, May 25th, July 1st and July 8th, respectively); RFGS1-RFGS4 are the type of rumen fluid used for *in vitro* incubations from donor cows fed a total ration containing grass silage GS1–GS4, respectively; SEM is the standard error of the mean.

† A1 and A2 are the asymptotic GP caused by fermentation of the soluble fraction (A1) and non-soluble fraction (A2); B2 is time at which half of A2 is reached; C2 is the sharpness of the curve in phase 2 (non-soluble fraction); R_{max2} is the maximum rate of GP in phase 2.

Table 3.3. *In vitro* methane (CH_4) production and curve fit parameters of grass silages differing in maturity at harvest (GS1 to GS4) and incubated with 4 different types of rumen fluid (RFGS1 to RGS2).

Item*	Variables†					
	CH_4 (ml/g OM)	A (ml/g OM)	B (h)	C	R_{max} (ml/h)	CH_4 (% of total gas)
Substrate (SUB)						
GS1	48.0[a]	50.7	10.4[b]	1.9[a]	3.2[a]	16.7
GS2	49.3[a]	53.6	11.8[a]	1.8[a]	2.9[ab]	16.8
GS3	46.8[b]	53.2	11.9[a]	1.5[b]	2.8[b]	17.2
GS4	45.0[c]	52.5	12.5[a]	1.4[b]	2.8[b]	17.0
SEM	0.87	1.56	0.45	0.05	0.13	0.30
Rumen fluid (RF)						
RFGS1	51.7[a]	57.8[a]	10.4[b]	1.5[b]	3.4[a]	17.8[a]
RFGS2	49.8[a]	55.6[a]	11.0[b]	1.5[b]	3.3[a]	17.5[a]
RFGS3	44.9[b]	50.1[b]	12.0[a]	1.6[b]	2.7[b]	16.7[a]
RFGS4	42.7[b]	46.4[b]	13.3[a]	1.9[a]	2.3[b]	15.7[b]
SEM	0.87	1.56	0.45	0.05	0.13	0.30
P-value						
SUB	0.004	0.186	<0.0001	<0.0001	0.030	0.071
RF	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
RF × SUB	0.854	0.894	0.920	0.990	0.759	0.363

[a,b,c] Values within a column and item with different superscript differ significantly ($P < 0.05$).

* GS1-GS4 are grass silages differing in maturity from young to old with increasing number (harvested on May 6th, May 25th, July 1st and July 8th, respectively); RFGS1-RFGS4 are the type of rumen fluid used for *in vitro* incubations from donor cows fed a total ration containing grass silages GS1–GS4, respectively; SEM is standard error of the mean.

† A is the asymptotic CH_4 production; B is the time at which half of A is reached; C2 is the sharpness of the curve; R_{max} is the maximum rate of CH_4 production.

Table 3.4. *In vitro* gas production (GP) and curve fit parameters of total rations (TRGS1-TRGS4), containing grass silage made from grass differing in maturity at harvest.

Item*	Variables†					
	GP (ml/g OM)	A1 (ml/g OM)	A2 (ml/g OM)	B2 (h)	C2	R_{max2} (ml/h)
TRGS1	303.4[a]	86.4[a]	179.5	5.9[b]	2.5	22.3[a]
TRGS2	302.4[a]	65.4[ab]	194.9	7.1[a]	2.7	21.1[a]
TRGS3	268.8[b]	50.8[b]	177.8	7.4[a]	2.6	18.1[b]
TRGS4	264.7[b]	45.2[b]	182.8	8.4[a]	2.7	16.9[b]
SEM	6.06	5.81	5.03	0.24	0.05	0.42
P-value	<0.0001	0.009	0.105	<0.0001	0.024	<0.0001

[a,b,c] Values within a column with different superscript differ significantly ($P < 0.05$).

* TRGS1-TRGS4 are the total rations containing the respective grass silage GS1-GS4 (harvested on May 6th, May 25th, July 1st and July 8th, respectively); SEM is the standard error of the mean.

† A1 and A2 are the asymptotic GP caused by fermentation of the soluble fraction (A1) and non-soluble fraction (A2); B2 is time at which half of A2 is reached; C2 is the sharpness of the curve in phase 2 (non-soluble fraction); R_{max2} is the maximum rate of GP in phase 2.

Table 3.5. *In vitro* methane (CH_4) production and curve fit parameters of total rations (TRGS1-TRGS4), containing grass silage differing in maturity.

Item*	Variables†					
	CH_4 (ml/g OM)	A (ml/g OM)	B (h)	C	R_{max} (ml/h)	CH_4 (% of total gas)
TRGS1	49.3[b]	52.4[b]	8.2[c]	1.6	4.1[a]	16.2[b]
TRGS2	55.0[a]	60.8[a]	10.3[b]	1.5	3.7[a]	18.2[a]
TRGS3	45.5[bc]	51.1[b]	10.8[b]	1.4	3.2[b]	16.9[b]
TRGS4	42.8[c]	48.6[b]	12.8[a]	1.5	2.3[b]	16.2[b]
SEM	1.40	2.02	0.51	0.07	0.31	0.38
P-value	<0.0001	0.002	<0.0001	0.221	0.002	0.001

[a,b,c] Values within a column with different superscript differ significantly ($P < 0.05$).

* TRGS1-TRGS4 are the total rations containing the respective grass silage GS1-GS4 (harvested on May 6th, May 25th, July 1st and July 8th, respectively); SEM is the standard error of the mean.

† A is the asymptotic CH_4 production; B is the time at which half of A is reached; C is the sharpness of the curve; R_{max} is the maximum rate of CH_4 production.

Table 3.6. *In vivo* methane (CH_4) production of the 24 lactating dairy cows in early and late lactation fed rations containing grass silage at increasing maturity stage at harvest (GS1-GS4) and that served as donors of rumen fluid for *in vitro* incubations.

Item*	Variables†			
	CH_4 g/kg OMI	CH_4 g/kg DMI	CH_4 g/kg DOMI	CH_4 g/kg FPCM
Substrate (SUB)				
GS1	22.0[b]	20.2[b]	28.8[b]	10.6[b]
GS2	24.6[a]	22.2[b]	31.4[b]	13.0[a]
GS3	24.4[a]	22.3[b]	33.3[b]	14.4[a]
GS4	24.7[a]	24.1[a]	38.0[a]	13.4[a]
SEM	0.65	0.51	1.21	0.67
Stage of lactation (SL)				
Early	24.2	22.2	33.0	12.4
Late	23.6	22.1	32.8	13.3
SEM	0.52	0.36	0.91	0.59
P-value				
SUB	0.001	0.001	0.004	0.002
SL	0.389	0.809	0.837	0.083

[a,b] Values within column with different superscript differ significantly ($P < 0.05$).

* GS1-GS4 are grass silages differing in maturity from young to old with increasing number (harvested on May 6th, May 25th, July 1st and July 8th, respectively); SEM is the standard error of the mean; early lactation is 96 days in milk; late lactation is 218 days in milk.

† CH_4 g/kg OMI is the CH_4 production expressed in grams per kilogram of organic matter intake; CH_4 g/kg DMI is the CH_4 production expressed in grams per kilogram of dry matter intake; CH_4 g/kg DOMI is the CH_4 production expressed in grams per kilogram of digested organic matter intake; CH_4 g/kg FPCM is the CH_4 production expressed in grams per kilogram of fat and protein corrected milk.

proteins were available upon maturation of the grass silages. The half time of the asymptotic GP of the non-soluble fraction (B2) increased as grass matured, indicating a slower fermentation. The maximum rate of GP of the non-soluble fraction (R_{max2}) was strongly affected by the maturity of the grass at harvest ($P < 0.0001$), showing a decreased R_{max2} as grass matured.

The *in vitro* CH_4 production (expressed as ml/g OM incubated or as a % of total gas), and the fitted parameters A, B, and R_{max} were affected by the maturity of the grass (Table 3.5). In particular, parameter B increased, indicating a slower CH_4 synthesis with progressing grass maturity at harvest.

In vivo CH_4 production

The *in vivo* data presented in Table 3.6 were extracted for the 24 cows used in the present *in vitro* study from a larger experiment, performed simultaneously in respiration chambers (Warner et al., 2017). The *in vivo* CH_4 production increased with progressing maturity of the grass at harvest, irrespective of the unit of expression. No effect of the stage of lactation on the CH_4 production was observed *in vivo* for the 24 cows used in the present *in vitro* experiment, except for a trend of increased CH_4 per unit of FPCM at late lactation ($P = 0.083$).

DISCUSSION

Effect of grass silage maturity on gas and CH_4 production

It is generally accepted that grass harvested at an early maturity stage is a valuable forage for dairy cows, due to its large nutritive value (Randby et al., 2012). Overall, the decrease in CP content and the increase in cell wall content (NDF and ADF) of the ensiled grass with progressing maturity at harvest was expected and is in agreement with other studies (Rinne et al., 1997; Cone et al., 1999). A decline in GP with progressing maturity using both grass silage and total ration samples was observed, which is in accordance with Cone et al. (1999). The decrease in GP of the soluble fraction (A1) of the grass silage and total ration samples, is associated with the decline in the CP content with advanced maturity. The decline in *in vitro* GP caused by fermentation of the non-soluble fraction (A2), is associated with a decrease in degradable cell walls with progressing maturity, accompanied by a decrease in the rate of fermentation (increased B2).

The *in vitro* CH_4 production, expressed in ml/g OM incubated or as a % of total gas, for both the grass silages and the total ration samples was affected by the grass maturity. It is likely that an increase in fibre content of the more mature grass silages increases the acetic acid production during fermentation in rumen fluid, thus making more hydrogen available for methanogens, resulting in a larger *in vitro* CH_4 production. However, in the current study a decrease in CH_4 production of grass silage (mainly perennial ryegrass) with progressing maturity at harvest was observed. In line with our results, Holtshausen et al. (2012) reported a decrease in *in vitro* gas and CH_4 production of grass silage (mixture of timothy and meadow fescue) with progressing maturity at harvest, resulting from a shift in the volatile fatty acid pattern observed. In contrast, Purcell et al. (2011) in their study on perennial ryegrass pastures reported an increased *in vitro* rumen gas and CH_4 production for a high herbage mass (i.e. smaller OM digestibility and CP content in the grass compared to a low herbage mass), although the difference between treatments was small, likely owing to the similar fibre content and the unchanged volatile fatty acid pattern.

The rate of maximum GP in phase 2 (R_{max2}) decreased with progressing maturity, which is in line with the greater B2 values. A similar pattern was observed for CH_4 production with greater B values, indicating that the rate of maximum CH_4 production decreased with progressing maturity of the grass at harvest, as reported by Macome et al. (2017). Our hypothesis was that the *in vitro* CH_4 production of older grass, due to the increased NDF content, would increase with increasing maturity of the grass. Instead, we observed a smal decrease of CH_4 production (ml/g OM) with progressing maturity, but a tendency for an increase in % CH_4 in the total gas. Analysis of volatile

fatty acid pattern of *in vitro* residues at the end of the incubation may reveal as to why our hypothesis was not met in the current study.

In the current study, the origin of the rumen fluid showed a strong effect on the *in vitro* CH_4 production of the different grass samples. This effect was visible even for the less extreme combinations; for instance, when the grass silage was incubated with rumen fluid from a donor cow not previously adapted to the respective diet, yet adapted to a diet with the chemical composition of the grass silage closest to the one of the incubated grass silage. These results suggest that adaptation to the respective diet is essential for *in vitro* GP and CH_4 fermentation studies.

Relationship between *in vitro* and *in vivo* methane production

No relationship between the *in vivo* CH_4 production, expressed in g/kg organic matter intake (OMI), and *in vitro* CH_4 production expressed in g/kg OM incubated, was observed for any different incubation time point. The strongest correlation between *in vitro* and *in vivo* CH_4 production (g/kg OMI) was observed at 12 hours of *in vitro* incubation ($R^2 = 0.23$, $P = 0.226$).

A relationship between 48 hours *in vitro* and *in vivo* CH_4 production (g/kg of OMI or of DMI), using a single linear correlation, was lacking ($R^2 = 0.00–0.18$, $P \geq 0.287$). Also, when the *in vivo* CH_4 production was expressed in g/kg FPCM, there was no correlation ($R^2 = 0.06$, $P = 0.529$) with the *in vitro* CH_4 production (g/kg OM incubated). The reason for this lack of relationships could be due to the relative small differences in CH_4 production observed in the *in vivo* experiment, varying from 22.0 to 24.7 g/kg OMI (Warner et al., 2017). Using the calculated curve fit parameters (A, B, C), the time point can be calculated at which the *in vitro* CH_4 synthesis is equal to that observed *in vivo*. We observed a considerable variation in the calculated time points (GS1 = 6.91 h, GS2 = 8.01 h, GS3 = 10.23 h and GS4 = 10.46 h), suggesting an effect of grass maturity on the time point at which the CH_4 production *in vitro* was equal to that observed *in vivo*. It cannot be ruled out that within this observed variation the *in vivo* and the *in vitro* CH_4 production correlated poorly. Nonetheless, these results indicate that silage from mature grass requires more time for degradation in an *in vitro* batch culture, resulting in less fermentation compared with silage from young grass over the same time period.

The *in vitro* CH_4 production parameters and the chemical composition of the grass silages were further used to predict the *in vivo* CH_4 production using a stepwise multiple regression. A significant correlation between observed and predicted *in vivo* CH_4 production (both expressed in g/kg OMI or in g/kg FPCM) was observed (Figure 3.1 and 3.2). The equations used to predict *in vivo* CH_4 production based on *in vitro* parameters and the chemical composition of the grass silages were:

In vivo CH_4 (g/kg OMI)

$$= -9.68 - 0.233 \times \textit{in vitro}\ CH_4 + 1.88 \times CH_4\% + 8.55 \times CH_4C\ (R^2 = 0.48)$$

In vivo CH_4 (g/kg FPCM)

$$= 1.29 + 1.98 \times \textit{in vitro}\ CH_4\% - 0.261 \times CH_4A - 0018 \times DM\ (R^2 = 0.60)$$

where, *in vitro* CH_4 is total methane in ml/g OM incubated, CH_4% is the percentage CH_4 in the total gas production, CH_4C is the sharpness of the monophasic curve fitted to the *in vitro* CH_4 production, CH_4A is the asymptote of the monophasic model fitted to the *in vitro* methane production, DM is the dry matter content of the grass silage, and CH_4% is the percentage of CH_4 in the total gas production.

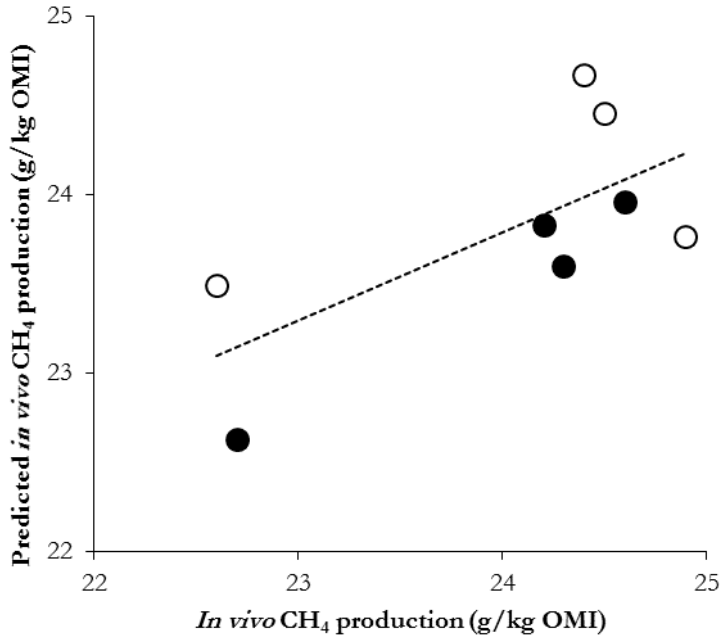

Figure 3.1 Relationship between observed *in vivo* CH_4 production and predicted CH_4 production, expressed in g/kg organic matter intake (OMI). Open symbols are for cows in early lactation (96 days in milk) and closed symbols for cows in late lactation (218 days in lactation). *In vivo* CH_4 (g/kg OMI) = – 9.68 – 0.233 × *in vitro* CH_4 + 1.88 × CH_4% + 8.55 × CH_4C (R^2 = 0.48), where *in vitro* CH_4 is total methane in ml/g OM incubated, CH_4% is the percentage CH_4 in the total gas production, and CH_4C is the sharpness of the monophasic curve fitted to the *in vitro* CH_4 production.

The prediction quality may be improved by increasing the sample size and contrast among treatments. Nonetheless, our results suggest that, although *in vitro* CH_4 measurements may be used for screening purpose, effects of grass silage quality observed *in vivo* are not easily reproduced in an *in vitro* batch culture system.

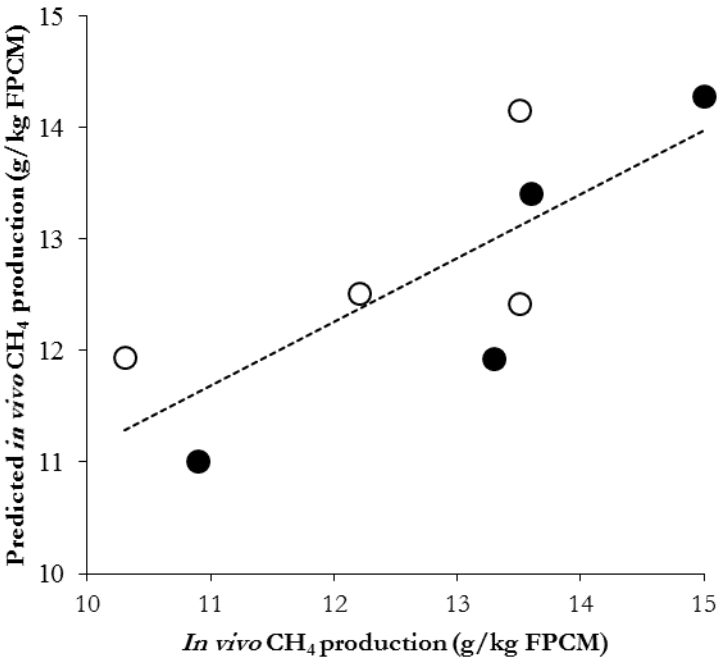

Figure 3.2. Relationship between observed *in vivo* CH_4 production and predicted CH_4 production, expressed as g/kg fat and protein corrected milk production (FPCM). Open symbols are for cows in early lactation (96 days in milk) and closed symbols for cows in late lactation (218 days in milk). *In vivo* CH_4 (g/kg FPCM) = 1.29 + 1.98 × *in vitro* CH_4% – 0.261 × CH_4A – 0018 × dry matter content (DM) (R^2 = 0.60), where CH_4A is the asymptote of the monophasic model fitted to the *in vitro* methane production and CH_4% is the percentage CH_4 in the total gas production.

CONCLUSIONS

The nutritional quality of grass silages decreased with progressing maturity of grass at harvest. *In vitro* gas and CH_4 production (ml/g of OM) of incubated grass silages decreased with advancing grass maturity at harvest. Based on our results on 24 cows, *in vitro* CH_4 production did not correlate with *in vivo* CH_4 production measured simultaneously on the same cows in respiration chambers. The lack of relationship might be partly explained by the relatively small numerical difference in *in vivo* CH_4 production per unit of intake. The prediction of *in vivo* CH_4 production with *in vitro* parameters only slightly improved when using stepwise multiple regression. The *in vitro* gas and CH_4 production and curve fit parameters varied based on whether donor cows were adapted to the respective ration or not. These results suggest that careful adaption to the experimental diet should be envisaged in *in vitro* gas and CH_4 production experiments.

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REFERENCES

Abrahamse, P.A., Dijkstra, J., Vlaeminck, B., Tamminga, S., 2008. Frequent allocation of rotationally grazed dairy cows changes grazing behavior and improves productivity. J. Dairy Sci. 91, 2033-2045.

AOAC, 1990. Official Methods of Analysis, 15th edn. Association of Official Analytical Chemists, Arlington, VA, USA.

Bauer, E., Williams, B.A., Voigt, C., Mosenthin, R., Verstegen, M.W.A., 2001. *In vitro* fermentation of various carbohydrate-rich feed ingredients combined with chyme from pigs. Arch. Anim. Nutr. 64, 394-411.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. 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.

Cone, J.W., van Gelder, A.H., Soliman, I.A., de Visser, H., van Vuuren, A.M.,1999. Different techniques to study rumen fermentation characteristics of maturing grass and grass silage. J. Dairy Sci. 82, 957-966.

Getachew, G., Blümmel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds. Anim. Feed Sci. Technol. 72, 261-281.

Dijkstra, J., Kebreab, E.; Bannink, A., France, J., Lopez, S., 2005. Application of the gas production technique to feed evaluation systems for ruminant. Anim. Feed Sci. Technol. 123, 561-578.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 7-89.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between *in vitro* an *in vivo* methane production measured simultaneously with different dietary starch sources levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci. 62, 40-45.

ISO 6496, 1999. Animal Feeding Stuffs. Determination of Moisture and Other Volatile Matter Content. International Organization for Standardization, Geneva, Switzerland.

ISO 5984, 2002. Animal Feeding Stuffs. Determination of Crude Ash. International Organization for Standardization, Geneva, Switzerland.

ISO 16472, 2006. Animal Feeding Stuffs. Determination of Amylase-treated Neutral Detergent Fibre Content (aNDF). International Organization for Standardization, Geneva, Switzerland.

ISO 13906, 2008. Animal Feeding Stuffs. Determination of Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) Contents. International Organization for Standardization, Geneva, Switzerland.

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

Macome, F.M., Pellikaan, W.F., Schonewille, J.Th., Bannink, A., van Laar, H., Hendriks, W.H., Warner, D., Cone, J.W., 2017. *In vitro* rumen gas and methane of grass silages differing in plant maturity and nitrogen fertilisation, compared to *in vivo* enteric methane. Anim. Feed Sci. Technol. 230, 96-102.

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

Paz, H.A., Anderson, C.L., Muller, M.J., Kononoff, P.J., Fernando, S.C., 2016. Rumen bacterial community composition in Holstein and Jersey cows is different under same dietary condition and is not affected by sampling method. Front. Microbiol. 7, doi 10.3389/fmicb.2016.01206.

Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, D.J.G.M., Becker, P.M., Cone, J.W., 2011. A novel method to determine simultaneously methane production during the *in vitro* gas production using a fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Purcell, P.J., O'Brien, M., Boland, T.M., O'Donovan, M., O'Kiely, P., 2011. Impacts of herbage mass and sward allowance of perennial ryegrass sampled throughout the growing season on *in vitro* rumen methane production. Anim. Feed Sci. Technol. 166-167, 405-411.

Randby, A.T., Weisbjerg, M.R., Nørgaard, P., Heringstad, B., 2012. Early lactation feed intake and milk yield responses of dairy cows offered grass silages harvested at early maturity stages. J. Dairy Sci. 95, 304-317.

Rinne, M., Huhtanen, P., Jaakkalo, S., 1997. Grass maturity effects on cattle fed silage-based diets. 2. Cell wall digestibility, digestion and passage kinetics. Anim. Feed Sci. Technol. 67, 19-35.

SAS, 2010: Statistical Analysis Software. SAS/STAT 9.3 User's Guide. SAS Inst., Cary, NC. USA.

Van Gelder, A.H., Hetta, M., Rodrigues, M.A.M., de Boever, J.L., Den Hartigh, H., Rymer, C., van Oostrum, M., van Kaathoven, R., Cone, J.W., 2005. Ranking of *in vitro* fermentability of twenty feedstuffs with an automated gas production technique. Results of a ring test. Anim. Feed Sci. Technol. 123-124, 243-253.

Van Soest, P.J., 1973. Collaborative study of acid detergent fibre and lignin. J. Assoc. Off. Anal. Chem. 56, 781-784.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Van Vuuren, A.M., van der Koelen, C.J., Valk, H., de Visser, H., 1993. Effects of partial replacement of ryegrass by low protein feed on rumen fermentation and nitrogen loss by dairy cows. J. Dairy Sci. 76, 2982-2993.

Warner, D., Hatew, B., Podesta, S.C., Klop, G., van Gastelen, S., van Laar, H., Dijkstra, J., Bannink. A., 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10, 34-43.

Warner, D., Bannink, A., Hatew, B., van Laar, H., Dijkstra, J., 2017. Effects of grass silage quality and level of feed intake on enteric methane production in lactating dairy cows. J. Anim. Sci. 95, 3687-3700.

Chapter 4

In vitro gas and methane production of silages from whole-plant maize harvested at four different stages of maturity and a comparison with *in vivo* methane production

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ABSTRACT

The current study investigated the relationship among *in vitro* and *in vivo* methane (CH_4) production by cows fed maize silage (MS) based rations. *In vivo* CH_4 production was measured in climate respiration chambers using 8, rumen canulated, Holstein-Friesian cows. *In vitro* CH_4 production was measured using rumen fluid from the 8 cows that were fully adapted to their respective experimental rations. The animals were grouped in two blocks, and randomly assigned to one of the four total mixed rations (TMRs) that consisted of 75% experimental MS, 20% concentrate and 5% wheat straw (dry matter (DM) basis). The experimental MS were prepared from whole-plant maize that was harvested at either a very early (25% DM; MS25), early (28% DM; MS28), medium (32% DM; MS32) or late (40% DM; MS40) stage of maturity. The four experimental TMRs and the corresponding MSs, served as substrate in two separate *in vitro* runs (each run representing one block of four animals) using rumen fluid from cows fed the TMR in question. No relationship was found between *in vivo* CH_4 production and *in vitro* CH_4 production measured at various time points between 2 and 48 h. None of the *in vitro* GP and CH_4 production parameters was influenced by an interaction among substrate and origin of rumen fluid. *In vitro* measured 48-h gas production (GP) was not affected by the maturity of whole-plant maize, irrespective whether MS alone or as part of TMR was incubated in adapted rumen inoculum. Incubation of the experimental TMRs did not affect the kinetics parameters associated with gas or CH_4 production, but in case MS alone was incubated, the asymptote of GP of the soluble fraction was slightly decreased with increasing maturity of MS at harvest. *In vitro* CH_4 production expressed in a % of total gas was not affected by the maturity of whole- plant maize at harvest. Several *in vitro* parameters were significantly affected (GP) or tended to be affected (CH_4) by diet fed to donor cows. It was concluded that the current *in vitro* technique is not suitable to predict *in vivo* CH_4 production from maize silage based rations.

INTRODUCTION

Whole-plant maize silage is commonly used in rations of dairy cows in many parts of the world. It has a high content of starch and generally good ensiling characteristics (Khan et al., 2015). The nutritional value of such maize silage (MS) largely depends on the content and degradability of the starch. The starch content as well as the vitreousness of maize kernels increases with maturity, and the fractional rate of ruminal starch degradation of maize decreases with maturity (Philippeau and Michalet-Doreau, 1997). The stage of maturity of the maize plant at harvest has, therefore, a significant impact on the nutritive value of MS, feed intake, milk yield (Johnson et al., 1999; Cammell et al., 2000; Warner et al., 2013) and methane (CH_4) production (Hatew et al., 2016). Enteric CH_4 is a potent greenhouse gas (Moss et al., 2000) and constitutes a loss of dietary energy to the animal (Johnson and Johnson, 1995).

Assessment of *in vitro* gas production (GP) is largely used to evaluate the nutritive value of ruminant feeds by incubating substrate in buffered rumen fluid (Cone et al., 1996; Getachew et al., 1998; Dijkstra et al., 2005). This *in vitro* approach can also be used to evaluate different feeding strategies for their potential to mitigate CH_4 production (Holtshausen et al., 2012; Hatew et al., 2015; Pellikaan et al., 2011). Currently, only a limited number of studies are available reporting *in vivo* CH_4 production of cattle upon changes in maturity of whole-plant maize at harvest (Cammell et al., 2000; Mc Geough et al., 2010; Hatew et al., 2016), and there is a dearth of direct *in vitro-in vivo* comparisons with respect to CH_4 production (Yáñez-Ruiz et al., 2016). Such comparisons are essential to evaluate the robustness of *in vitro* techniques to represent and simulate rumen fermentation including *in vivo* CH_4 production. Furthermore, it offers the possibility to predict *in vivo* CH_4 production which is of practical interest to mitigate enteric CH_4 production by dairy cows.

The use of rumen fluid from donor cows is inherent to the *in vitro* GP technique. It is generally accepted that rumen fluid of donor cows has to be adapted to the substrate that is subjected to *in vitro* GP and CH_4 measurements. To the authors' knowlegde however, there is a lack of studies addressing the issue on adaptation of rumen inoculum on MS based rations. It was, therefore, considered opportune to address the issue of adaptation of rumen inoculum to substrate in the current study as well.

The objective of this research was to investigate the relationship between *in vitro* and *in vivo* CH_4 production of silages from whole-plant maize harvested at four different stages of maturity. We hypothesized that *in vitro* CH_4 production is related to the *in vivo* CH_4 production in case identical dietary material is used both *in vivo* and *in vitro*, and those dietary materials are inoculated with rumen fluid obtained from donor animals adapted to those dietary materials.

MATERIALS AND METHODS

Donor animals and substrates

The *in vitro* experiment was conducted in parallel with the *in vivo* trial of Hatew et al. (2016) where 28 lactating Holstein-Friesian cows were used for *in vivo* CH_4 measurements, using climate-controlled respiration chambers. Eight out of the 28 cows in the latter study had a permanent rumen cannula and these 8 animals served as donors of rumen fluid for the current *in vitro* incubations. Housing, animals, feeding regimes and all procedures related to the *in vivo* trial are described in detail by Hatew et al. (2016). Briefly, cows were allocated to 7 blocks of 4 cows each, based on parity, DIM, fat and protein corrected milk at the start of the trial, and presence of a rumen cannula. Within blocks, cows were randomly assigned to one of the four total mixed rations (TMR) that consisted of 75% experimental MS, 20% concentrate and 5% wheat straw (DM basis). The experimental MS were harvested in 2013 at four different stages of maturity, viz. very early (September 20th, 25% DM; MS25), early (September 28th, 28% DM; MS28), medium (October 9th, 32% DM; MS32) or late (October 31st, 40% DM; MS40). Five days before the start of the adaptation period, cows received a high MS diet containing a non-experimental MS. The four experimental TMRs and the corresponding MSs (Table 4.1) served as substrate in two separate *in vitro* runs (each run with one block of four animals) using rumen fluid from cows fed the TMR in question. The *in vitro* incubations were run simultaneously with the *in vivo* CH_4 measurements and the timespan among the two *in vitro* runs was one week.

Rumen fluid was collected on the last day of each 12-d experimental period, thereby assuming that the cows were adapted to their respective experimental rations. The experimental MSs (n = 4) were used as sole substrates and incubated separately with each of the rumen fluid inocula types (n = 4). Furthermore, the MS based TMRs (n = 4) were used as substrate and incubated with rumen fluid from cows adapted to the corresponding TMR. The MS either used for *in vitro* incubation or to formulate the corresponding TMR that was fed to the cows, originated from the same squared plastic wrapped bag.

Table 4.1. Chemical composition of maize silages differing in maturity at harvest and of total mixed rations. Data are adopted from Hatew et al. (2016).

Parameter	Maize silages (MS)[1]				TMR[2]			
	MS25	MS28	MS32	MS40	TMRMS25	TMRMS28	TMRMS32	TMRMS40
Growing days[3]	128	136	147	169	NA[4]	NA	NA	NA
DM content (g/kg)	283	292	318	396	437	444	463	522
Chemical composition (g/kg DM)								
Ash	39	37	37	35	56	55	55	53
CP	83	83	80	79	145	145	142	142
NDF	407	394	359	349	369	359	332	325
ADF	242	233	207	195	219	212	193	183
ADL	11	11	9	10	13	12	11	12
Crude fat	26	27	25	24	26	26	25	24
Starch	275	305	356	385	243	266	304	326

[1] Whole-plant maize was harvested at targeted DM contents of either 25, 28, 32 or 40% for MS25, MS28, MS32 and MS40, respectively.
[2] Total mixed rations had maize silage : wheat straw : concentrate ratio of 75 : 5 : 20 (DM basis). TMR25, TMR28, TMR32, and TMR40 contained either MS25, MS28, MS32 or MS40, respectively.
[3] Number of days from planting until harvesting of the whole plant for ensiling.
[4] NA = Not applicable.

Gas and methane production

Gas production profiles of the experimental TMRs and MS were determined using fully automated GP equipment (Cone et al., 1996) with GP being measured over 48 h. Samples of each substrate were freeze dried and ground over a 1-mm sieve using a Wiley mill (Peppink 100AN, Olst, The Netherlands). Approximately 0.5 g (DM basis) of each sample was weighed into 250 ml fermentation bottles (Schott, Mainz, Germany). Each substrate was weighed in triplicate bottles. Bottles of blanks (rumen fluid without sample) were run in duplicate in each series. Equal amounts of rumen fluid (~250 ml total) were collected from the front ventral, middle ventral and caudodorsal region of the rumen of individual donor cows before the morning feeding in pre-warmed insulated flasks flushed with CO_2. Then, rumen fluid was filtered through cheese cloth and subsequently mixed (1:2 v/v) with an anaerobic buffer/mineral solution (Cone et al., 1996) under continuous flushing with CO_2. Prior to inoculation, the fermentation bottles were placed in a shaking water bath kept at 39 °C and pre-flushed with CO_2. The bottles were then inoculated with 60 ml of buffered rumen fluid and connected to fully automated GP equipment (Cone et al., 1996). Ten µl of the headspace gas was collected from the bottles at distinct incubation times (0, 2, 4, 8, 12, 24, 30, 36 and 48 h) and directly injected into a gas chromatography to determine the CH_4 concentration as described by Pellikaan et al. (2011).

Gas and methane curve fitting

Cumulative gas and CH_4 production data were fitted using the model described by Cone et al. (1996) and Groot et al. (1996). The non-linear least squares regression procedure was used (SAS Institute Inc., 2010) and the data were fitted according to following equation:

$$Y = \sum_{i=1}^{n} \frac{Ai}{1+\left(Bi/t\right)^{Ci}},$$

where Y = cumulative gas or CH_4 production (ml/g OM incubated), n = total number of phases, i = number of phases, Ai = estimated asymptotic gas production in phase i (ml/g of incubated OM), Bi = incubation time (h) where half of phase i gas or CH_4 production has been reached, Ci = sharpness of the switching characteristic for phase i, and t = time of incubation (h). Gas production was fitted using a tri-phasic model following the procedure as described by Cone et al. (1997), where phase 1 and 2 are assumed to relate to the fermentation of the soluble and non-soluble fraction, respectively, while phase 3 is assumed to be related with microbial turnover (Cone et al., 1997). The time points related to the asymptotes of GP in phase 1, 2 and 3 (A1, A2 and A3, respectively) were set at 3 h for A1, 17 h later for A2 and 28 h later (relative to A2) for A3 after the

incubation of the substrate so as to enable the estimation of the various parameters (*Ai*, *Bi* and *Ci*, respectively). The aforementioned time points were empirically determined by Cone et al. (1996) and Groot et al. (1996).

Data on CH_4 production were fitted according to the model already described with $n = 1$. The cumulative amount of CH_4 produced was obtained as described in detail by Pellikaan et al. (2011). Briefly, measured CH_4 concentrations in individual bottles were expressed relative to the maximum concentration in each bottle, and were fitted iteratively with the monophasic model. CH_4 concentrations at each individual valve opening were then calculated, and cumulative CH_4 calculated as the sum of the increase in headspace CH_4 concentration among two successive valve openings and the amount of CH_4 vented from the bottle.

The maximum rate of gas or CH_4 production (R_{max}, ml/g OM/h) and the time at which this maximum rate of gas or CH_4 production is reached (TR_{max}, h) were calculated using the equations modified by Yang et al. (2005):

$$R_{max} = \frac{Ai \times Bi^{Ci} \times Ci \times (TR_{max})^{(-Ci-1)}}{[1+Bi^{Ci} \times (TR_{max})^{-Ci}]^2}$$

$$TR_{max} = Bi \times \left[\frac{Ci-1}{Ci+1}\right]^{1/Ci}$$

where *i, Ai* , *Bi* , and *Ci* are defined as previously described. If $Bi \leq 1$ then R_{max} occurs at t = 0 h.

Calculations and statistical analyses

Prior to statistical analysis, triplicate *in vitro* data were pooled per cow and substrate. *In vitro* CH_4 production on a mass basis (g/kg OM) was calculated as follows: CH_4 (l/kg OM) / 25.621 × 16. The values 16 and 25.621 represent the molar mass (g/mol) of CH_4 and the volume of gas at 39 °C, respectively. The volume of gas (l/mol) was calculated using the ideal gas law, i.e. V = nRT/P, where n = amount of gas (moles), R = the gas constant (J/(K·mol)), P = atmospheric pressure (i.e. 101.3 kPa), and T = the temperature expressed on the Kelvin scale (i.e. 312.15 K). In case MS was the sole substrate available for *in vitro* fermentation, data were analysed by ANOVA using the PROC MIXED procedure (SAS Institute Inc., 2010) using the model:

$$Y_{ijk} = \mu + RUN_i + SUB_j + RF_k + (SUB \times RF)_{jk} + e_{ijk}$$

where Y_{ijk} = response variable (CH_4 or GP), μ = overall mean, RUN_i = Run (i = 1 to 2), SUB_j = substrate (j = 1 to 4; MSs with maturity stage of whole-plant maize being very early, early, medium and late maturity at the moment of harvest), RF_k = rumen fluid (k = 1 to 4; from cows fed TMR containing one of the 4 MSs), $(SUB \times RF)_{jk}$ = interaction term, and e_{ijk} = residual error term. When TMR instead of MS was used as a substrate, the factors RF_k and $(SUB \times RF)_{jk}$ were excluded from the afore mentioned model and SUB represented the TMR. Run was used as a random variable. Differences among treatment means were compared using Tukey-Kramer's multiple comparison procedure using the LSMEANS statement (SAS Institute Inc., 2010). Linear regression analysis between *in vitro* CH_4 production (g/kg OM incubated) and *in vivo* CH_4 production (g/kg OMI) of the 8 donor cows was performed for each time point (0, 2, 4, 8, 12, 24, 30, 36 and 48 h) for which *in vitro* CH_4 production was measured. The regression analyses were conducted under the assumption that the 8 data points could be considered independent using the PROGREG statement (SAS Institute Inc., 2010). Throughout, the level of statistical significance was pre-set at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

RESULTS

Chemical composition of the maize silages

Upon maturation of whole-plant maize, the DM contents of the respective silages ranged from 283 to 396 g/kg which is in line with values observed in practice; i.e. 280 to 425 g/kg (Khan et al., 2011). The starch contents ranged from 275 to 385 g/kg DM and were found to be negatively related with the NDF and ADF contents (Table 4.1). The contents of CP and ADL were similar across the different MS. The aforementioned changes in chemical composition of the different MS are mirrored by the corresponding TMRs.

Table 4.2. Summary statistics of *in vivo* methane (CH_4) production of the 8 lactating dairy cows that served as donors of rumen fluid for *in vitro* incubations.

Methane emission variable	Mean	Maximum	Minimum	SD[1]	CV[2]
CH_4 (g/day)	371	403	323	26.4	7.1
CH_4 (g/kg OMI)	21.7	24.0	19.0	1.77	8.2
CH_4 (g/kg DMI)	20.5	22.8	18.0	1.67	8.1
CH_4 (g/kg OMD)[3]	30.1	34.2	27.2	2.34	7.8

[1] SD, standard deviation.
[2] CV, coefficient of variation.
[3] OMD, organic matter degraded.
The data were extracted from Hatew et al. (2016).

In vivo methane production

The coefficient of variation related to the various indices of *in vivo* CH_4 production ranged from 7.1 to 8.2% (Table 4.2). The greatest coefficients of variation of CH_4 production were calculated when *in vivo* CH_4 production was expressed either as g/kg DMI or g/kg OMI; i.e. 8.1 and 8.2%, respectively. In these cases, the respective maximum values were similar and found to be 26.7% and 26.3% greater than the corresponding minimum values.

Maize silage and *in vitro* gas production

In vitro gas production (GP 48-h) and the related kinetics parameters (Table 4.3) were not affected ($P \geq 0.131$) by the interaction among maturity of maize plant at harvest and the dietary background of the rumen fluid (i.e. rumen fluid obtained from cows fed either TMRMS25, TMRMS28, TMRMS32 or TMRMS40). The maturity of maize plant at harvest did not affect the measured 48-h GP (Table 4.3). In contrast, the dietary background of the rumen fluid significantly influenced the measured 48-h GP and it was found to be 6.6% lower ($P = 0.021$) when rumen fluid was obtained from cows fed TMR containing MS28 instead of MS40. The asymptote of GP of the soluble fraction (A1) and its associated half time of maximum GP (B1) were not affected ($P \geq 0.108$) by the dietary background of rumen fluid. However, A1 decreased with increasing maturity of MS at harvest ($P = 0.009$) while the half time of the soluble fraction (B1) was found to be significantly shorter when MS25 and MS28 instead of MS32 and MS40 were incubated. The latter observations are in line with the greater ($P = 0.001$) rate of maximum GP of the soluble fraction (R_{max1}) after the incubation of two immature MSs. The time at which the maximum gas production of the soluble fraction (TR_{max1}) was reached was found to be similar among treatments. The switching characteristics of the GP curve related to the soluble fraction (C1), was neither affected by the maturity of MS nor the dietary background of the rumen fluid ($P \geq 0.310$).

The asymptote GP of the non-soluble fraction (A2) and its associated half time of maximum GP (B2) were not affected by the maturity of MS at harvest ($P = 0.227$ and $P = 0.111$, respectively) but the maximum rate of GP related to the non-soluble fraction (R_{max2}) increased with increasing maturity of MS ($P = 0.013$). The dietary background of rumen fluid had a significant impact on A2, B2 and R_{max2}. The time at which the maximum gas production of the non-soluble fraction (TR_{max2}) was reached was not affected by the maturity of MS ($P = 0.625$) but the dietary background of rumen fluid had a significant impact on TR_{max2} ($P = 0.024$). The switching characteristic of the GP curve related to the non-soluble fraction (C2) tended ($P = 0.062$) to be affected by the maturity of MS at harvest while the dietary background of the rumen fluid had a significant influence on C2 ($P = 0.038$). The relevance of the latter results however, can be disputed

because the use of either the lowest or the highest C2 value (2.2 and 2.4, respectively) has only a minor impact on the curve of total GP.

The asymptote of GP in phase 3 (A3) was not affected by maturity of whole plant maize at harvest (P = 0.645) but its related half time of maximum GP (B3) was significantly affected (P = 0.025) by maturity at harvest. The dietary background of rumen fluid had an influence (P = 0.046) on A3, indicating a slight decrease of the rumen microbial turnover with increasing maturity of whole-plant maize at harvest. The dietary background of rumen fluid did not influence B3 (P = 0.175). The dietary background of rumen fluid, but not maturity of MS, tended (P = 0.065) to affect the maximum rate of GP related to microbial turnover (R_{max3}). In contrast, TR_{max3} increased with increasing maturity of MS (P = 0.029) but the dietary background of rumen fluid did not influence TR_{max3} (P = 0.221). The switching characteristic of the GP curve related to microbial turnover (C3) was neither affected by the maturity of MS nor the dietary background of the rumen fluid (P ≥ 0.127).

Maize silage and *in vitro* methane production

Methane production (CH_4 48-h) and its associated kinetics parameters (A, B, C, R_{max} and TR_{max}) were not affected (P ≥ 0.381) by the interaction among maturity of maize plant at harvest and the dietary background of the rumen fluid (Table 4.4).The 48-h CH_4 production expressed as ml/g OM tended (P = 0.054) to be affected by the maturity of whole plant maize at harvest. In contrast, relative CH_4 production (% of total GP 48-h) was not affected (P = 0.139) by the maturity of whole plant maize at harvest. Moreover, the various kinetics parameters (A, B, C, R_{max} and TR_{max}) related to CH_4 production did not differ (P ≥ 0.328) among substrates. The dietary background of rumen fluid tended to influence CH_4 production, either expressed as ml/g OM or as % of total GP (P = 0.071 and P = 0.087, respectively), and the lowest values were found when MS was inoculated with rumen fluid from cows fed late maturity MS.

The asymptotic CH_4 production (A) and its associated half time (B) were not influenced (P ≥ 0.182) by the dietary background of the rumen fluid. Likewise, R_{max} and TR_{max} were not affected (P ≥ 0.204) by the dietary background of the rumen fluid. The switching characteristic of the curve (C) tended to be affected (P = 0.095) by the dietary background of the rumen fluid but these differences in C have minor impact on the CH_4 production curve and are therefore considered not relevant.

Table 4.3. *In vitro* gas production and associated kinetics parameter estimates for main effect of substrate (SUB; maize silages differing in maturity at harvest) and rumen fluid (RF; rumen fluid from cows fed TMR either adapted or not adapted to the specific maturity of the maize silage in question).

Variables[1]	Substrate (SUB)[2]				Dietary background of rumen fluid (RF)[3]				SEM	P-value		
	MS25	MS28	MS32	MS40	TMRMS25	TMRMS28	TMRMS32	TMRMS40		SUB	RF	SUB × RF
	ml/g OM											
GP 48-h	331.7	324.0	318.8	319.9	317.7^{ab}	335.3^{a}	329.0^{ab}	313.3^{b}	10.64	0.264	0.021	0.714
A1	63.4^{a}	55.2^{a}	46.9^{b}	45.6^{b}	54.2	59.9	48.1	49.1	3.49	0.009	0.108	0.985
A2	203.4	208.7	208.9	209.8	197.7^{b}	212.3^{a}	212.6^{a}	208.2^{a}	4.27	0.227	0.001	0.703
A3	64.9	60.1	63.0	64.5	65.8^{a}	63.1^{a}	68.3^{a}	56.0^{b}	5.39	0.645	0.046	0.805
	h											
B1	1.7^{b}	2.1^{b}	3.4^{a}	3.2^{a}	2.7	2.4	2.0	3.3	0.66	0.016	0.176	0.962
B2	7.6	7.2	6.8	6.8	7.0^{ab}	6.7^{b}	7.8^{a}	6.8^{b}	0.25	0.111	0.016	0.999
B3	22.4^{b}	22.3^{b}	23.7^{a}	24.2^{a}	23.1	24.1	22.7	22.8	0.69	0.025	0.175	0.990
	dimensionless											
C1	1.2	1.1	1.7	1.2	1.4	1.1	1.1	1.6	0.34	0.310	0.327	0.551
C2	2.3	2.3	2.4	2.5	2.2^{b}	2.4^{a}	2.4^{ab}	2.4^{ab}	0.06	0.062	0.038	0.953
C3	5.0	5.3	5.3	5.4	5.4	5.2	5.0	5.4	0.43	0.283	0.127	0.807
	ml/g OM/h											
R_{max1}	28.2^{a}	20.5^{a}	12.5^{b}	9.0^{c}	20.5	19.2	15.2	15.4	3.40	0.001	0.145	0.131
R_{max2}	18.7^{b}	20.6^{b}	21.9^{a}	22.9^{a}	19.4^{b}	23.3^{a}	19.4^{b}	22.0^{ab}	1.02	0.013	0.007	0.999
R_{max3}	3.8	3.6	3.6	3.7	4.0	3.5	3.8	3.4	0.15	0.878	0.065	0.804
	h											
TR_{max1}	0.2	0.2	1.3	0.6	0.6	0.3	0.2	1.2	0.64	0.304	0.431	0.699
TR_{max2}	5.0	4.8	4.6	4.8	4.5^{b}	4.6^{b}	5.3^{a}	4.7^{ab}	0.22	0.625	0.024	0.999
TR_{max3}	20.6^{b}	20.7^{b}	22.0^{a}	22.6^{a}	21.4	22.3	20.8	21.3	0.51	0.029	0.221	0.993

[1] GP 48-h, cumulative gas production measured after 48 h of incubation; A1, A2 and A3, asymptote of gas production in phase 1, phase 2 and phase 3, respectively; B1, B2, B3, incubations time at which half of maximum gas production has been formed in phase 1, phase 2 and phase 3, respectively; C1, C2, and C3, the sharpness of the switching characteristic for the profile in phase 1, phase 2 and phase 3, respectively; $R_{max1,}$ $R_{max2,}$ $R_{max3,}$ maximum gas production rate in phase 1, phase 2 and phase 3, respectively ; $TR_{max1,}$ TR_{max2} and $TR_{max3,}$ time occurrence of R_{max} in phase 1, phase 2 and phase 3, respectively.

[2] Main effect of substrate, MS25, MS28, MS32 and MS40, maze silage made of whole-plant maize harvested at a targeted DM content of 25, 28, 32 and 40%, respectively.

[3] Main effect of rumen fluid, TMRMS25, TMRMS28, TMRMS32 and TMRMS40, rumen fluid from donor cows fed TMR containing 75% (DM basis) of MS25, MS28, MS32 and MS40, respectively.

TMR and *in vitro* gas and methane production

Incubation of the experimental TMRs (Table 4 5), did neither affect ($P \geq 0.406$) the measured 48-h GP nor the asymptotes of GP related to soluble fraction (A1) and microbial turnover (A3). A tendency however, was observed for the asymptote of GP in phase 2 (A2) ($P = 0.070$). The half time of maximum GP of the three phases (B1, B2, B3) were similar ($P \geq 0.210$) among the treatments and only the switching characteristic of the profile in phase 2 (C2) tended ($P = 0.079$) to increase when the TMR contained more mature MS. In all three phases, R_{max} and TR_{max} related to GP were similar ($P \geq 0.128$) among the different TMRs. The measured 48-h CH_4 production either expressed as ml/g OM or as % of total GP, and the associated kinetics parameters were found to be similar ($P \geq 0.143$) among the experimental TMRs (Table 4 5).

Relationship among *in vitro* and *in vivo* CH_4 production

In vitro 48-h CH_4 production was found to be greater than *in vivo* CH_4 production (Table 4.6, Figure 4.1A), and *in vitro* 48-h CH_4 production was not significantly related to *in vivo* CH_4 production. The explained variance in *in vivo* CH_4 production was greatest ($R^2 = 0.16$) when 12-h *in vitro* incubation results were regressed against *in vivo* CH_4 production, but the relationship (Figure 4.1B) was found to be non-significant ($P = 0.321$).

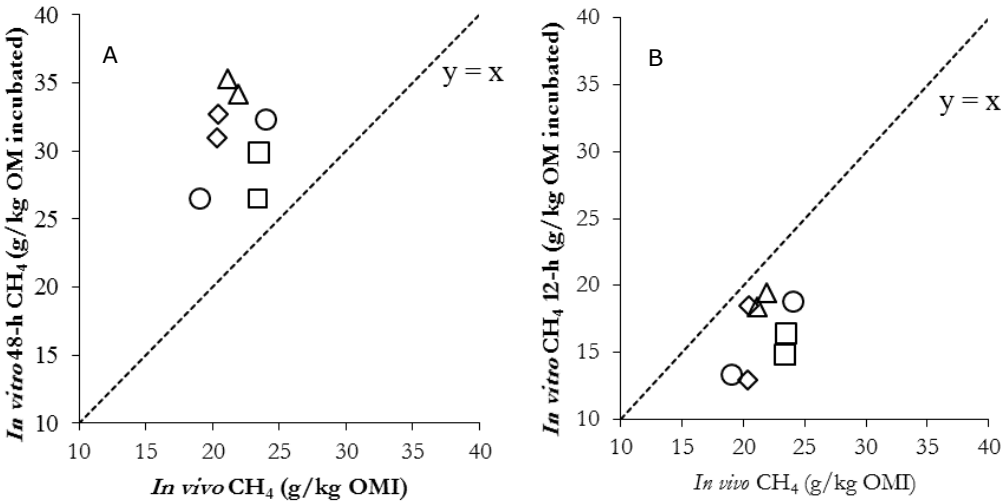

Figure 4.1. Relationships between *in vivo* (g/kg OMI) and *in vitro* (g/kg OM incubated) CH_4 production after 48 h (Panel A, $R^2 < 0.01$; $P = 0.900$) and 12 h (Panel B, $R^2 = 0.16$; $P = 0.321$) of incubation, using adapted rumen fluid as *in vitro* inoculum and substrates identical to the rations fed during the *in vivo* experiment (Hatew et al., 2016) (Δ, TMRMS25; □, TMRMS28; ◊, TMRMS32; ○, TMRMS40). The abbreviations TMRMS25, TMRMS28, TMRMS32 and TMRMS40 indicate total mixed rations containing maize silage (75% DM basis) made from whole-plant maize harvested at targeted DM contents of 25, 28, 32 and 40%, respectively.

Table 4.4. *In vitro* methane production and associated kinetics parameter estimates for main effect of substrate (SUB; maize silages differing in maturity at harvest) and rumen fluid (RF rumen fluid from cows fed TMR either adapted or not adapted to the specific maturity of the maize silage in question).

Variable[1]	Substrate (SUB)[2]				Dietary background of rumen fluid (RF)[3]				SEM	P-value		
	MS25	MS28	MS32	MS40	TMRMS25	TMRMS28	TMRMS32	TMRMS40		SUB	RF	SUB × RF
CH_4 48-h												
ml/g OM	50.3	49.4	44.0	45.9	48.3	47.6	50.2	43.5	2.62	0.054	0.071	0.889
% of GP 48-h	15.2	15.2	13.8	14.3	15.2	14.2	15.3	13.9	0.43	0.139	0.087	0.875
A (ml/g OM)	61.5	55.2	54.0	52.0	60.2	56.5	57.4	48.6	3.67	0.328	0.182	0.422
B (h)	19.1	12.1	18.1	12.2	18.8	17.2	13.2	12.3	5.16	0.606	0.721	0.381
C	1.5	1.6	1.6	1.6	1.4	1.7	1.6	1.7	0.08	0.665	0.095	0.991
R_{max} (ml/g OM/h)	2.9	2.9	2.9	2.9	3.0	3.0	2.9	2.6	0.32	0.953	0.299	0.867
TR_{max} (h)	4.5	4.7	4.3	4.3	3.7	4.6	4.5	4.9	0.53	0.850	0.204	0.999

[1] CH_4 48-h, absolute cumulative methane production measured after 48 hours of incubation (ml/g OM) and relative methane production expressed as a % of GP 48-h, the cumulative gas production measured after 48 hours of incubation; A, asymptote of methane production; B, incubation time at which half of maximum methane production has been formed; C, the sharpness of the switching characteristic for the profile; R_{max}, maximum of methane production rate; TR_{max}, time occurrence of R_{max}.

[2] Main effect of substrate, MS25, MS28, MS32 and MS40, maize silage made of whole-plant maize harvested at a targeted DM content of 25, 28, 32 and 40%, respectively.

[3] Main effect of rumen fluid, TMRMS25, TMRMS28, TMRMS32 and TMRMS40, rumen fluid from donor cows fed TMR containing 75% (DM basis) of MS25, MS28, MS32 and MS40, respectively.

Table 4.5. *In vitro* gas production and fermentation parameters incubation of a total mixed ration (TMR), incubated with inoculum obtained from cows fed a TMR with an identical ingredient composition. Rations were based on maize silages differing in maturity at harvest.

Variable[1]	Treatment[2]				SEM	P-value
	TMRMS25	TMRMS28	TMRMS32	TMRMS40		
GP 48-h (ml/g OM)	311.3	300.8	309.0	289.8	8.31	0.406
A1 (ml/g OM)	63.1	55.7	48.2	44.344 44.3	9.18	0.570
A2 (ml/g OM)	188.3	189.6	199.9	193.3	1.95	0.070
A3 (ml/g OM)	59.9	55.5	60.8	52.2	6.70	0.609
B1 (h)	1.7	2.3	1.8	2.4	0.47	0.501
B2 (h)	7.8	6.7	7.7	7.0	0.30	0.210
B3 (h)	22.2	24.2	23.1	23.9	1.25	0.494
C1	1.3	1.3	1.1	1.2	0.10	0.107
C2	2.2	2.4	2.4	2.5	0.05	0.079
C3	4.6	4.8	4.7	5.3	0.21	0.283
R_{max1} (ml/g OM/h)	26.1	17.9	25.2	14.9	8.06	0.695
R_{max2} (ml/g OM/h)	16.6	20.4	18.9	20.5	0.87	0.128
R_{max3} (ml/g OM/h)	3.2	2.9	3.2	3.0	0.24	0.672
TR_{max1} (h)	0.3	0.4	0.1	0.5	0.21	0.336
TR_{max2} (h)	5.0	4.7	5.3	5.0	0.21	0.357
TR_{max3} (h)	20.1	22.2	21.0	22.3	1.18	0.489
CH_4 48-h						
ml/g OM	56.3	45.8	51.6	47.6	2.83	0.219
% of GP 48-h	18.1	15.2	16.3	15.2	0.69	0.143
A (ml/g OM)	65.5	53.7	66.9	57.4	4.29	0.251
B (h)	12.6	13.4	18.4	14.7	3.79	0.630
C	1.4	1.5	1.3	1.5	0.11	0.735
R_{max} (ml/g OM/h)	3.1	3.6	3.9	3.5	0.99	0.944
TR_{max} (h)	3.4	2.6	2.6	2.8	0.43	0.572

[1] GP 48-h, cumulative gas production measured after 48 hours of incubation; A1, A2 and A3, asymptote of gas production in phase 1, phase 2 and phase 3, respectively; B1, B2 and B3, incubation time at which half of maximum gas production has been formed in phase 1, phase 2 and phase 3, respectively; C1, C2 and C3, the sharpness of the switching characteristic for the profile in phase1, phase 2 and phase 3, respectively; R_{max1}, R_{max2}, R_{max3}, maximum gas production rate in phase 1, phase 2 and phase 3, respectively; TR_{max1}, TR_{max2}, TR_{max3}, time occurrence of R_{max} in phase 1, phase 2 and phase 3, respectively; CH_4 48-h, absolute cumulative methane production measured after 48 hours of incubation (ml/g OM) and relative methane production expressed as a % of GP 48-h: A, asymptote of methane production; B, incubation time at which half of maximum of methane production has been formed; C, the sharpness of the switching characteristics for the profile; R_{max}, maximum methane production rate; TR_{max}, time occurrence of R_{max}.

[2] TMRMS25, TMRMS28, TMRMS32 and TMRMS40%, total mixed ration containing maize silage (75% on DM basis) made from whole-plant maize harvested at targeted DM contents of either 25%, 28%, 32% or 40 % of DM of DM of maize silage, respectively.

Table 4.6. Slope, intercept and the coefficient of determination (R^2) of the linear relationships between *in vivo* CH_4 production (g/kg OMI) and *in vitro* CH_4 production measured at different time points (g/kg OM incubated).

Time point of incubation (h)	Slope	Intercept	R^2	P-value
2	0.108	0.38	0.07	0.540
4	0.083	4.3	0.02	0.738
8	0.076	9.4	< 0.01	0.899
12	0.582	3.9	0.16	0.321
24	-0.165	27.1	0.01	0.849
30	0.224	21.3	0.01	0.777
36	0.186	23.5	0.01	0.842
48	0.097	28.9	< 0.01	0.900

DISCUSSION

From the perspective of optimization of ration formulation and mitigating CH_4 emission, it is relevant to have fast and reliable methods to estimate the CH_4 production from the rumen. Thus, *in vitro* methods that accurately predict CH_4 production are of great interest because measurements of CH_4 production using respiration chambers are laborious and expensive. Currently there is a dearth of direct *in vitro-in vivo* comparisons with respect to CH_4 production (Yáñez-Ruiz et al., 2016) and the present study provides the first experimental data on an *in vitro-in vivo* comparison of CH_4 production in dairy cows fed MS based rations.

Relationship among *in vitro* and *in vivo* CH_4 production

In contrast to expectation, the current data clearly show a lack of relationship among *in vivo* and *in vitro* CH_4 productions despite the fact that identical dietary material is used and rumen fluid is obtained from fully adapted animals. Similarly, Hatew et al. (2015) showed that *in vitro* CH_4 production was not related with *in vivo* CH_4 production (both expressed in ml CH_4 per kg of OM) from different combinations of sources and levels of starch in the diet of adapted dairy cattle. A straight forward explanation for the observed discrepancy among *in vivo* and *in vitro* CH_4 production cannot be provided but the substrate subjected to *in vitro* fermentation is ground to fine particles which may not accurately represent the cow's chewing. Furthermore, *in vitro* the finely ground particles cannot escape from fermentation through passage. Thus, the degradability of the feed is at least potentially, overestimated under *in vitro* conditions at 48-h. The latter is of particular interest because Hatew et al. (2016) reported that the effective rumen degradability of starch decreases with advancing maturity of the maize plants at harvest, causing an increase in duodenal starch flow resulting in a reduced CH_4 emission. Moreover, a rumen retention time of 48 h seems not realistic under practical feeding conditions. Using ^{13}C labelled MS, Warner et al. (2013) reported rumen retention times (reciprocal of fractional passage rate) of 19 – 42 h for DM or 21 to 26 h for starch. Previously, Ellis et al. (2016) also found shorter *in vitro* incubation times to result in greater differences in CH_4 production among silage treatments than longer *in vitro* incubation times. From these perspectives it can be suggested that shorter *in vitro* incubation times may provide better fits with *in vivo* CH_4 production. However, also the relationship among *in vivo* and *in vitro* CH_4 production after 12 h of *in vitro* incubation of the substrates, was found to be non-significant and the model explained only 16% of the variation in *in vivo* CH_4 production. Apart from the reasons already mentioned, factors such as rumen acidity and profile of fatty acids are not exactly mimicked *in vitro* (Pinares-Patiño et al., 2007; Dijkstra et al., 2012; Hatew et al., 2015) and the density of the

microbial population is lower under *in vitro* conditions due to the use of diluted rumen fluid under these conditions (Yáñez-Ruiz et al., 2016). Thus, *in vitro* fermentation conditions differ substantially from *in vivo* conditions and this difference may explain the lack of relationship among *in vitro* and *in vivo* CH_4 production.

Maturity of whole plant maize at harvest and *in vitro* gas and methane production

In line with results reported by Cone et al. (2008), the maturity of whole plant maize at harvest did not affect cumulative GP-48h. It thus appears that the *in vitro* degradability of the organic matter was not affected by the maturity of whole-plant maize at harvest. The lack of effect of maturity on GP-48h probably relates to the shift from NDF to starch when whole-plant maize matures. During the process of maturation of whole-plant maize, the maize kernels are filled with starch, thereby, shifting carbohydrates from NDF to starch (Phipps et al., 2000; Bal et al., 2000). Furthermore, both the digestibility of NDF and the fractional degradation rate of maize starch in the rumen decreases with increasing maturity (Philippeau and Michalet-Doreau, 1997). Clearly, starch cannot escape from fermentation under *in vitro* conditions and all starch is available for microbial digestion. The aforementioned characteristics of whole-plant maize are in line with the observations that both the asymptotic GP of the soluble fraction (A1) and the associated maximum rate of gas production (R_{max1}) decreased with increasing maturity while the half time of the soluble fraction (B1) increased with increasing maturity. Furthermore, the maximum rate of GP of the non-soluble fraction (R_{max2}) increased with increasing maturity, thereby, reflecting the greater digestibility of starch versus NDF. It should be noted however, that *in vivo*, both the effective rumen degradability of OM as well as the total tract digestibility of OM decreased with advanced maturity of whole-plant maize at harvest (Hatew et al., 2016), and these *in vivo* results differ from the present *in vitro* findings.

In contrast to expectations, relative 48-h CH_4 production (% of GP-48h) was not affected by the maturity of whole-plant MS at harvest, irrespective MS or its corresponding TMR was incubated. This result is not easy to explain because it is well known that a shift from NDF to starch increases the fermentation rate of OM, thereby, shifting VFA production from acetate to propionate, which renders less hydrogen available for the synthesis of CH_4 (McAllister and Newbold, 2008). Unfortunately, the profile of VFA in the present *in vitro* study is unknown which hinders proper interpretation of the current data, but it might be suggested that the fermentation rate of OM *in vitro* was not effectively influenced by the maturity of whole-plant MS at harvest. This notion is in line with the fact that the maximum rate of GP (R_{max}) was found to be similar

among treatments, but it is in contrast with *in situ* results reported by Hatew et al. (2016) who used the same MS.

Dietary background of rumen fluid and *in vitro* gas and methane production

The lack of interaction among substrate (MS) and dietary background of rumen fluid on total GP and CH_4 in the current study is in contrast with the common idea that adapted rumen inoculum is needed to predict accurate yields of GP and CH_4 (Yáñez-Ruiz et al., 2016). Unfortunately, the current result is not easy to explain due to a lack of studies addressing the issue of adaptation of inoculum to MS based rations. However, Cone and van Gelder (2006) reported that the fermentation rate of native potato starch was enhanced by using rumen fluid adapted to the fermentation of native potato starch instead of rumen fluids not adapted to that substrate. Likewise, Hatew et al. (2015) reported significantly greater yields of GP and CH_4 when beet pulp was inoculated with rumen fluid adapted to beet pulp instead of rumen fluid obtained from cows fed rations without any beet pulp. Thus, in the latter experiments (Cone and van Gelder, 2006; Hatew et al., 2015), GP and CH_4 yield were only affected in case rumen fluid was adapted either or not to the substrate in question. It can, therefore, be speculated that a sharp contrast in chemical composition among the incubated substrate and the ration to which the rumen bacteria are adapted (i.e. inoculum) is needed to affect GP and CH_4 yields. The current speculation cannot be substantiated due to a dearth of relevant studies addressing the issue on adaptation of inoculum but it appears to be in line with the observation that the lowest GP and A2 values were found when MS was incubated with rumen fluid that was obtained from cows adapted to TMRMS25 and TMRMS40.

In the current study, rumen fluid was collected 12 days after the onset of feeding the experimental TMRs. This 12-d adaptation time can be considered somewhat short to attain full adaptation of the rumen microbiota (Yáñez-Ruiz et al., 2016). On the other hand, the animals were already receiving a high MS diet for 5 days prior to receiving the MS of interest (Hatew et al., 2016). Thus, the rumen microbiota already had a (short) adaptation to a ration of this particular type (high MS) prior to the start of the experimental periods. We therefore anticipated that the process of adaptation to the experimental TMRs was facilitated and that the results of the current *in vitro* measurements were not compromised by the relative short experimental periods.

CONCLUSION

In vitro 48-h gas and CH_4 production using adapted rumen inoculum was not affected by the maturity of whole plant maize. No statistically significant relationship was found among *in vivo* and *in vitro* CH_4 production. The current *in vitro* technique appears to be unsuitable to predict *in vivo* CH_4 production in dairy cows fed maize silage based rations.

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REFERENCES

Bal, M.A., Shaver, R.D., Jirovec, A.G., Shinners, K.J., Coors, J.G., 2000. Crop processing and chop length of corn silage: effects on intake, digestion, and milk production by dairy cows. J. Dairy Sci. 83, 1264-1273.

Cammell, S.B., Sutton, J.D., Beever, D.E., Humphries, D.J., Phipps, R. H., 2000. The effect of crop maturity on the nutritional value of maize silage for lactating dairy cows. 1. Energy and nitrogen utilization. J. Anim. Sci. 71, 381-390.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W. Oudshoorn. L., 1996. 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.

Cone, J.W., van Gelder, A.H., Driehuis, F., 1997. Description of gas production profiles with a three phasic model. Anim. Feed Sci. Technol. 66, 31-45.

Cone, J.W., van Gelder, A.H., 2006. Adaptation of the rumen microbial population to native potato starch degradation determined with gas production technique and the nylon bag technique. J. Anim. Physiol. Anim. Nutr. 90, 511-518.

Cone, J.W., van Gelder, A.H., van Schooten, H.A., Groten, J.A.M., 2008. Effects of forage maize type and maturity stage on *in vitro* rumen fermentation characteristics. Neth. J. Agric. Sci. 55, 139-154.

Dijkstra, J., Kebreab, E., Bannink, A., France, J., Lopez, S., 2005. Application of the gas production technique to feed evaluation systems for ruminants. Anim. Feed Sci. Technol. 123, 561-578.

Dijkstra, J., Ellis, J.L., Kebreab, E., Strathe, A.B., Lopez, S., France, J., Bannink, A., 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol. 172, 22-33.

Ellis, J.L., Bannink, A., Hindrichsen, I.K., Kinley, R.D., Pellikaan, W.F., Milora, N., Dijkstra, J., 2016. The effect of lactic acid bacteria included as a probiotic or silage inoculant on *in vitro* rumen digestibility, total gas and methane production. Anim. Feed Sci. Technol. 211, 61-74.

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds. Anim. Feed Sci. Technol. 72, 261-281.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77-89.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra. J., 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Hatew, B., Bannink, A., van Laar, H., de Jonge, L.H., Dijkstra, J., 2016. Increasing harvest maturity of whole-plant corn silage reduces methane emission of lactating dairy cows. J. Dairy Sci. 99, 354-368.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci., 60, 40-45.

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

Johnson, L., Harrison, J.H., Hunt, C., Shinners, K., Dogget, C.G., Sapienza, D., 1999. Nutritive value of corn silage as affected by maturity and mechanical processing: A contemporary review. J. Dairy Sci. 82, 2813-2825.

Khan, N.A., Cone, J.W., Pellikaan, W.F., Khan, M.A., Struik, P.C., Hendriks, W.H., 2011. Changes in fatty acid content and composition in silage maize during grain filling. J. Sci. Food Agric. 91, 1041-1049.

Khan, N.A., Ali, P.Q., Yu, M., Cone, J.W., Hendriks, W.H., 2015. Nutritive value of maize silage in relation to dairy cow performance and milk quality. J. Sci. Food Agric. 95, 238-252.

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to methanogens. Aust. J. Exp. Agric. 48, 7-13.

Mc Geough, E.J., O'Kiely, P., Foley, P.A., Hart, K.J., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, and performance of finishing beef cattle offered maize silages harvest at 4 different stages of maturity. J. Anim. Sci. 88, 1479-1491.

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

Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011. A novel method to determine simultaneously methane production during the *in vitro* gas production using fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Philippeau, C., Michalet-Doreau, B., 1997. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. Anim. Feed Sci. Technol. 68, 25-35.

Phipps, R.H., Sutton, J.D., Beever, D.E., Jones, A.K., 2000. The effect of crop maturity on the nutritional value of maize silage for lactating dairy cows. 3. Food intake and milk production. Anim. Sci. 71, 401-409.

Pinares-Patiño, C.S., Waghorn, G.S., Machmuller, A., Vlaming, B., Molano, G., Cavanagh, A., Clark, H., 2007. Methane emissions and digestive physiology of non-lactating dairy cows fed pasture forage. Can. J. Anim. Sci. 87, 601-613.

SAS. 2010. Statistical Analysis Software. 9.3 User's Guide. SAS 2002-2010, SAS Institute Inc, Cary, NC, USA.

Warner, D., Dijkstra, J., Hendriks, W.H., Pellikaan, W.F., 2013. Passage kinetics of ^{13}C-labeled corn silage components through the gastro intestinal tract of dairy cows. J. Dairy Sci. 96, 5844-5858.

Yáñez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., O'Kiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z., Hristov, A.N., 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants - a review. Anim. Feed Sci. Technol. 216, 1-18.

Yang, H.J., Tamminga, S., Williams, B.A., Dijkstra, J., Boer, H., 2005. *In vitro* gas and volatile fatty acids production profiles of barley and maize and their soluble and washout fractions after feed processing. Anim. Feed Sci. Technol. 120, 125-140.

Chapter 5

Methane production of sainfoin (*Onobrychis viciifolia*) silage-based and grass silage-based total mixed rations *in vitro* compared to that of lactating cows

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ABSTRACT

In vitro methane (CH_4) production was compared with *in vivo* CH_4 production data simultaneously measured in climate controlled respiration chambers. *In vitro* ruminal CH_4 synthesis and fermentation characteristics of sainfoin (SAIN) were determined using rumen fluid from dairy cows receiving grass silage or SAIN silage-based diets throughout a 25-day adaption period. Rumen fluid obtained at day 8, 18 and 33 of the experimental periods was used for *in vitro* incubations with the corresponding diets. *In vitro* gas, CH_4 and total volatile fatty acid (VFA) production were not affected by the dietary treatment, whereas the molar proportions of acetic and propionic acid, and the non-glucogenic to glucogenic VFA ratio were affected. A non-significant relationship was observed between the *in vitro* and *in vivo* measured CH_4 production. Rumen fluid from cows adapted for 25 days to SAIN silage-based diets did not show any *in vitro* CH_4-reducing properties and overall fermentation was not affected. However, a slight reduction in *in vitro* gas and CH_4 production was observed on the first day after animals were changed to their SAIN silage-based diet. *In vitro* measurement of CH_4 production using rumen fluid from adapted dairy cows did not predict their *in vivo* CH_4 production.

INTRODUCTION

Enteric methane (CH_4) is a potent greenhouse gas (Moss et al., 2000) and constitutes a loss of dietary energy to the animal (Johnson and Johnson, 1995). Methane losses using climate-controlled respiration chambers (CRCs) to accurately quantified *in vivo* CH_4 production by dairy cows are referred to as the 'golden standard' (Knapp et al., 2014). *In vivo* measurements either done in CRCs or by use of SF6 technique are, however, labour intensive and costly, they raise ethical concerns, but also have each their methodological limitations (Pinares-Patiño and Waghorn, 2014). It is, therefore, warranted to investigate more cost effective and rapid methods that allow evaluation of different feeding strategies for their potential to mitigate CH_4 production. *In vitro* gas production (GP) is largely used to evaluate the nutritive value of ruminant feeds (Menke and Steingass, 1988; Getachew et al., 1998). Over the past decade, this *in vitro* approach is also more and more used to evaluate the impact of specific feedstuffs/rations on CH_4 production (Holtshausen et al., 2012; Dal Pizzol et al., 2017). However, direct *in vitro-in vivo* comparisons on CH_4 production are required to derive accurate prospective estimates on CH_4 production by dairy cows.

Hitherto, the number of studies reporting direct *in vitro-in vivo* comparisons with respect to CH_4 production is scarce (Yáñez-Ruiz et al., 2016**).** Recently, Macome et al. (2017) attempted to predict *in vivo* CH_4 production, measured in CRCs, by *in vitro* CH_4 production using a fully automated GP technique (Pellikaan et al., 2011). In that study (Macome et al., 2017), no significant relationship was found between *in vitro* and *in vivo* CH_4 production, irrespective of the unit of CH_4 production. The lack of a relationship (Macome et al., 2017) was, at least partly, explained by the higher variation in CH_4 production (g/kg digestible organic matter, OM) among animals compared to the variation among treatments, i.e. grass silages from herbage differing in maturity stage and produced from grasslands with differences in nitrogen fertilisation. Hatew et al. (2015) on the other hand, observed a positive correlation between the *in vitro* and *in vivo* CH_4 production per unit effective rumen fermentable OM in dairy cows adapted to rations with different sources and levels of starch. It was, therefore, considered important to revisit the issue of *in vitro-in vivo* comparisons on CH_4 production.

Sainfoin (*Onobrychis viciifolia*) is a tanniniferous legume that is well adapted for growth in dry environments on calcareous soils (Hayot Carbonero et al., 2011). Sainfoin has almost completely ceased to be used over the past decades, but it is currently reappraised as forage because of its palatability (Parker and Moss, 1981), nutritional characteristics (Karnezos et al., 1994) and health promoting properties (McMahon et al., 1999). Moreover, sainfoin contains condensed tannins (CT) Hayot Carbonero et al. (2011) and has potential to mitigate CH_4 production (Hristov et al., 2013;

Huyen et al., 2016a). The latter property of sainfoin can be considered of interest to further explore the relationship, if any, between *in vitro* and *in vivo* CH_4 production.

The objective of the current study was to determine the relationship between *in vitro* and *in vivo* CH_4 production using sainfoin *versus* grass silage based substrates as well as rations, respectively. It was hypothesized that *in vitro* CH_4 production is related to *in vivo* CH_4 production in case the substrates for *in vitro* use are inoculated with rumen fluid obtained from donor animals adapted to rations with an identical ingredient composition as the substrate in question. *In vivo* CH_4 production was measured in CRCs.

MATERIAL AND METHODS

Donor animals and substrates

The current *in vitro* experiment was performed in parallel with the *in vivo* trial reported by Huyen et al. (2016a) where 6 rumen cannulated, lactating Holstein-Friesian cows were used for *in vivo* CH_4 measurements, using CRC[1]. Housing, animals, feeding regime and all procedures related to the *in vivo* trial are described in detail elsewhere (Huyen et al., 2016a).

The *in vivo* study had a crossover design where each of the two experimental periods lasted 33 days. Cows were paired based on parity and milk production and within pairs, the cows were randomly assigned to one of the experimental diets provided as total mixed rations (TMRs); a grass silage and maize silage based control TMR (CON) or a grass-sainfoin silage and maize silage based TMR (SAIN). The CON diet consisted of grass silage (600 g/kg dry matter, DM), maize silage (100 g/kg DM), concentrate (240 g/kg DM) and linseed (60 g/kg DM). In the SAIN diet, the grass silage component was partly replaced by sainfoin silage (grass silage : sainfoin silage, 50 : 50 on DM basis), with sainfoin silage being prepared as a mixture of two sainfoin cultivars ('Zeus' and 'Esparcette', 70 : 30 on DM basis) grown on a clay and sand type soil, respectively, at the experimental facilities of the Plant Sciences Group (Unifarm) of Wageningen University. Both sainfoin cultivars were harvested at the end of the flowering period in the second vegetative cycle and separately ensiled in round bales (Huyen et al., 2016a). The chemical composition of the experimental TMRs is shown in Table 5.1.

[1] All animal handlings were in accordance with European Union directive 2010/63/EU and approved of by the Institutional Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands).

Table 5.1. Chemical composition of the experimental grass silage based (CON) and sainfoin silage based (SAIN) total mixed rations.

	Experimental rations	
Component	CON	SAIN
Dry matter, $g \cdot kg^{-1}$ product	444.9	357.2
	----------g/kg dry matter----------	
Organic matter	918.9	891.3
Crude protein	162.7	171.9
Neutral detergent fibre	395.7	359.1
Acid detergent fibre	236.7	244.5
Crude fat	37.8	35.1
Starch	97.9	90.9

For a full description of the experimental rations, the reader is referred to Huyen et al. (2016a).

During the first 7 days of each experimental period, all cows received CON *ad libitum* and from day 8 up until 33 the cows received their allocated TMR (CON or SAIN) fed at 95% of *ad libitum*. On days 5, 15, 22 and 29 of each experimental period at 15:00 h, the animals were moved to individual CRCs to measure CH_4 production starting the next day at 09:00h for a consecutive 5-day, 2-day, 2-day and 3-day period, respectively. For the current *in vitro* study, rumen fluid samples were collected on days 8, 18 and 33 of each experimental period at 06:00h before the morning feeding, hence six successive *in vitro* incubation runs were conducted. The two experimental TMRs served as substrate for *in vitro* runs using rumen fluid from cows fed the TMR in question and originated from the TMR that was fed to the cows. Corresponding *in vivo* CH_4 production data for the collected rumen fluid times (day 8, 18 and 33) were obtained from day 8 to 10, day 16 to 17 and day 29 to day 32 with the animals housed in CRCs, respectively. Results obtained during the latter measurement periods are reported by Huyen et al. (2016a).

Gas and methane production

Gas production profiles were determined using fully automated GP equipment, as described previously (Cone et al., 1996) with GP being measured over 48 h. Samples of each substrate were freeze dried and ground over a 1-mm sieve using a cross beater mill (Peppink 100AN, Olst, The Netherlands). Approximately 0.50 g DM of each sample was weighed into 250 ml fermentation bottles (Schott, Mainz, Germany) with each substrate weighed in triplicate bottles. Bottles of blanks (rumen fluid without sample) were included in duplicate in each series. Equal volumes of rumen fluid (~ 250 ml) were collected from the front ventral, the middle ventral and caudodorsal region of the rumen of individual donor cows. Rumen fluid from each cow was collected in pre-warmed insulated flasks flushed with CO_2 with all other handlings as described by Cone et al. (1996). Methane concentration in the headspace of the fermentation bottles was determined as described by Pellikaan et al. (2011a).

Gas and methane curve fitting

Cumulative CH_4 and GP curves were fitted with a modified Michaelis-Menten equation Groot et al. (1996) by non-linear least squares regression procedure in SAS (SAS, 2010) using a monophasic and triphasic equation, respectively:

$$Y = \sum_{i=1}^{n} \frac{Ai}{1+\left(Bi/t\right)^{Ci}} \quad (1)$$

where Y = cumulative gas or CH_4 production (ml/g incubated OM), n = total number of phases, i = number of phases (i = 1 to n), Ai = estimated asymptotic GP in phase i (ml/g of incubated OM), Bi = incubation time (h) where half of phase i gas or CH_4 production has been reached, Ci = sharpness of the switching characteristic for phase i, and t = time of incubation (h). Gas production data was described using a tri-phasic model (n = 3) following the procedure of van Gelder et al. (2005), where phase 1 and 2 are assumed to be related to the fermentation of the soluble and non-soluble fraction, respectively, while phase 3 represents microbial turnover. The asymptote of GP for phase 1 (A1) was defined as the GP after 3h, for phase 2 (A2) as the difference in GP between 3 and 20 h, and for phase 3 (A3) as the difference in GP between 20 and 48 h (Van Gelder et al., 2005)

Data on CH_4 production were fitted according to the model already described with n = 1 and A1 being the asymptote of the CH_4 production at time infinite. The cumulative amount of CH_4 produced was obtained as described in detail by Pellikaan et al. (2011a). Briefly, measured CH_4 concentrations in individual bottles were expressed relative to the maximum concentration in each bottle, and were fitted iteratively with the monophasic model. Methane concentrations at each individual valve opening were then calculated, and cumulative CH_4 calculated as the sum of the increase in headspace CH_4 concentration between two successive valve openings and the amount of CH_4 vented from the bottle.

Chemical analysis and fermentations end products

The DM content was determined after drying at 103 °C overnight (ISO 6496; 1999) and ash content after incineration for 3 h at 550 °C (ISO 5984; ISO, 2002). Nitrogen (N) content was determined using the Kjeldahl method (ISO 5983-1; ISO, 2005) and a factor of 6.25 was used to convert N into crude protein. Neutral detergent fibre (NDF) was analysed according to Van Soest et al. (1991) and was expressed without residual ash. The starch content was determined enzymatically (ISO 15914; ISO, 2004).

After 48 h of incubation, fermentation was stopped by placing the bottle on ice. Thereafter, a 0.75 ml sample of the supernatant was taken and mixed with an equal volume (1:1, v/v) of a stock solution composed of 25 ml of 85% (v/v) ortho-phosphoric acid dissolved in 200 ml Millipore water and 300 ml of a 4 g/l 4-methylvaleric acid (internal standard) for VFA analysis, and stored at -20 °C pending analysis. Volatile fatty acids were analysed by means of gas chromatography (GC; Thermo Ultra CE Instruments, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1:9) and fitted to a flame ionisation detector (FID), using a capillary column (30 m, i.d. 0.53 mm, film thickness 1.0 μm; Agilent J&W HP-FFAP, Santa Clara, CA, USA) with hydrogen as the carrier gas (25 kPa pressure). The starting temperature of the column was set at 80 °C for 1 min followed by a 20 °C min^{-1} increase to 120 °C, followed by a 6.1 °C min^{-1} increase to 205 °C where temperature was maintained for 2 min.

Calculations and statistical analyses

Data from triplicate bottles for each substrate were averaged before statistical analysis. *In vitro* CH_4 production on a mass basis (g/kg OM) was calculated as CH_4 (l/kg OM) / 25.621 × 16, with the values 16 and 25.621 representing the molar mass (g/mol) of CH_4 and the volume of gas at 39 °C, respectively. The volume of gas (l/mol) was calculated using the ideal gas law, i.e. V = nRT/P, where n = amount of gas (moles), R = the gas constant J (K/mol), P = atmospheric pressure (i.e. 101.3 kPa), and T = the temperature expressed on the Kelvin scale (i.e. 312.15 K). Total VFA were calculated as the sum of acetic acid (HAc), propionic acid (HPr), butyric acid (HBu), isobutyric acid (iHBu), valeric acid (HVa) and isovaleric acid (iHVa). The proportions of branched chain volatile fatty acids (BCVFA) in fermentation fluids were calculated as the sum of iHBu and iHVa scaled to total VFA. The non-glucogenic to glucogenic ratio (NGR) was calculated as:

$$NGR = (HAc + 2HBu + HVa) / (HPr + HVa) \quad (2)$$

Effects of substrates on gas and CH_4 productions were analysed using a repeated statement in ANOVA, using the MIXED procedure in SAS according to the following model:

$$Y_{ij} = \mu + SUB_i + DAY_j + (SUB \times DAY)_{ij} + e_{ij} \quad (3)$$

where Y_{ij} = response variable (e.g. CH_4, GP or fermentation kinetic parameter), μ = overall mean, SUB_i = TMR substrate (i = 2, TMR control, TMR sainfoin), DAY_j = day (j= day 8, 18 and 33),

$(SUB \times DAY)_{ij}$ = interaction term and e_{ij} = residual error. Day and substrate were included in the model and day was used as a REPEATED statement. Cow was included in the SUBJECT statement and period was used as a RANDOM variable. Differences among main effects were analysed using the Tukey-Kramer's multiple means comparison procedure in the LSMEANS statement in SAS. (SAS, 2010) Linear regressions between *in vivo* and *in vitro* CH_4 production were performed using the PROGREG statement in SAS (SAS, 2010). Throughout, the level of statistical significance was pre-set at $P < 0.05$ and a trend at $0.05 \leq P < 0.10$.

RESULTS

Due to technical problems related to the storage of the data produced by the automated GP equipment, *in vitro* data of the following substrates were lost: day 25, 3 CON and 3 SAIN. Consequently, the corresponding *in vivo* data on CH_4 production were omitted when investigating the relationship between *in vitro* and *in vivo* CH_4 production.

In vivo CH_4 production

Methane production *in vivo* expressed in g/kg·day was not affected by diet treatment and day, nor by their interaction (Table 5.2). Methane production in g/kg DM intake was not affected by diet treatment ($P \leq 0.118$), but was for day ($P = 0.002$) and diet × day ($P = 0.034$). Cows allocated to the SAIN diet had a lower *in vivo* CH_4 production per kg DM intake on day 18 ($P = 0.005$) and day 33 ($P = 0.076$) compared to day 8, whilst no day effects were observed for CON ($P \geq 0.135$). Similar but less pronounced results were obtained for CH_4 production per kg OM intake. Methane production expressed in g/kg milk tended ($P = 0.074$) to be lower in the SAIN group compared to CON.

Table 5.2. Summary statistics of *in vivo* methane (CH_4) production of the six lactating dairy cows receiving a grass silage (CON) based or a sainfoin silage (SAIN) based total mixed rations (TMR), and served as donor cows of rumen fluid for *in vitro* incubations

Parameter	Treatment	Days on TMR			SEM	P-value		
		8	18	33		Treatment	Day	Treatment × Day
CH_4 g kg^{-1} day	CON	359.2	360.2	365.5	26.47	0.893	0.978	0.934
	SAIN	361.4	369.3	360.8				
CH_4 g kg^{-1} DM intake	CON	20.5	19.7	21.0	0.89	0.118	0.002	0.034
	SAIN	21.1[a]	19.1[b]	19.8[ab]				
CH_4 g kg^{-1} OM intake	CON	22.3	21.3	22.8	1.06	0.603	0.002	0.051
	SAIN	23.4[a]	21.2[b]	22.1[ab]				
CH_4 g kg^{-1} milk	CON	17.3	17.4	17.6	1.63	0.074	0.987	0.981
	SAIN	15.5	15.3	15.5				

CON, control TMR containing grass/maize silage fed to cows and used as substrate; DM, dry matter; OM, organic matter; SAIN, TMR containing sainfoin silage and grass/maize silage fed to cows and used as substrate; SEM, standard error of mean.

[a,b]Values within a row with different superscript letters differ significantly ($P \leq 0.05$).

In vitro gas and methane production

In vitro GP measured after 48 h showed no effect of day (Table 5.3), but was significantly affected by substrate ($P = 0.044$) and substrate × day interaction ($P < 0.005$). Within day, SAIN gave a lower ($P = 0.006$) GP compared to CON, but only in case rumen fluid was obtained at day 8. The asymptote GP of the soluble fraction (A1) and its associated half time (B1) were not affected by substrate and only B1 was affected by day ($P = 0.027$; Table 5.3). Both A1 and B1 tended ($P = 0.085$ and $P = 0.053$, respectively) to be affected by substrate × time. Within day, SAIN did not differ from CON ($P \geq 0.158$), but in the case of the CON substrate, B1 increased ($P = 0.027$) from day 8 to 33. The switching characteristic of the GP curve related to the soluble fraction (C1), was neither affected by substrate nor substrate × day ($P \geq 0.181$), but increased over the course of time ($P = 0.031$). The asymptote GP of the non-soluble fraction (A2) and its associated half time (B2) were neither affected by day ($P \geq 0.148$) nor by substrate × day ($P \geq 0.104$). However, the use of SAIN caused a significantly lower ($P = 0.007$) A2 and tended ($P = 0.069$) to lower B2. The switching characteristic of the GP curve related to the non-soluble fraction (C2) was similar ($P \geq 0.248$) across substrates and time. The asymptote of GP related to microbial turnover (A3) showed a tendency ($P = 0.051$) for a day effect and was significantly affected by substrate × day ($P = 0.035$). In case SAIN, but not CON, was used as substrate, A3 increased when rumen inoculum was obtained at day 33 instead of day 8 of the experimental period. Furthermore, for the two treatments combined, A3 values were found to be 22.9% higher ($P = 0.051$) at day 33 compared to day 8. In contrast, half time of maximum GP in phase 3 (B3) was only affected by substrate ($P = 0.045$) and across time, B3 values were 7.8% higher when SAIN was used as substrate. The switching characteristic of the GP curve related to microbial turnover (C3) was only affected by day ($P = 0.016$). Methane production expressed as ml/g OM incubated (Table 5.4) tended to be affected by the interaction between substrate and time ($P = 0.059$) and the values followed a pattern similar to GP. In contrast, values on relative CH_4 production (% of total GP) were similar across substrates and time ($P \geq 0.284$). In line with the results on CH_4 production (ml/g OM), the asymptotic CH_4 production (A) was only affected by substrate × time ($P = 0.014$). The values on the half time of the asymptotic CH_4 production (B) were found to be similar across treatments and time ($P \geq 0.336$). The switching characteristic of the CH_4 production curve (C) was borderline affected by time ($P = 0.051$) but these differences in C had a minor impact on the CH_4 production curve and are, therefore, considered not relevant.

Table 5.3. Gas production (GP) and curve fit parameter estimates from *in vitro* measurements using rumen fluid from donor cows receiving a grass silage (CON) based or a sainfoin silage (SAIN) based total mixed ration (TMR).

Parameter[c]	Substrate[d]	Day[d]			SEM[e]	P-value		
		8	18	33		Substrate	Day	Substrate × Day
GP (ml/ g OM[e] incubated)	CON	314.1	-	280.6	30.50	0.044	0.762	0.005
	SAIN	247.3**	262.2	297.5				
A1 (ml/ g OM incubated)	CON	106.8	-	66.1	9.32	0.938	0.219	0.085
	SAIN	86.0	89.1	88.6				
A2 (ml/ g OM incubated)	CON	171.5	-	178.9	17.35	0.007	0.148	0.104
	SAIN	134.4**	144.2	167.5				
A3 (ml/ g OM incubated)	CON	35.8	-	35.7	2.65	0.595	0.051	0.035
	SAIN	26.9[b]	28.9[b]	41.4[a]				
B1 (h)	CON	0.7	-	1.6	0.32	0.784	0.027	0.053
	SAIN	1.0	1.5	1.2				
B2 (h)	CON	6.9	-	6.7	2.48	0.069	0.881	0.568
	SAIN	5.9	5.7	6.1				
B3 (h)	CON	22.6	-	24.7	2.68	0.045	0.4	0.867
	SAIN	25.0	24.8	26.7				
C1	CON	0.8	-	1.6	0.19	0.343	0.031	0.181
	SAIN	0.9	1.1	1.2				
C2	CON	2.6	-	2.7	0.15	0.248	0.805	0.602
	SAIN	2.6	2.5	2.6				
C3	CON	3.9	-	4.5	0.54	0.816	0.016	0.107
	SAIN	4.8[a]	3.3[b]	3.9[ab]				

[a,b] Values within a row with different superscript letters differ significantly ($P \leq 0.05$).

[c] GP, gas production at 48 h of incubation; A1, A2 and A3 are the asymptotic of gas production in phase 1, phase 2 and phase 3 respectively; B1, B2, and B3 are the incubation at which time half of asymptotic of gas has been formed; C1, C2 and C3 are the sharpness of the curve.

[d] Day, refers to the experimental day numbers at which rumen fluid sample was collected for *in vitro* incubations.

[e] CON, control TMR containing grass/maize silage fed to cows and used as substrate; OM, organic matter; SAIN, TMR containing sainfoin silage and grass/maize silage fed to cows and used as substrate; SEM, standard error of mean. ** Significantly ($P \leq 0.01$) different from corresponding CON value.

Table 5.4. Methane (CH_4) production and curve fit parameters from *in vitro* measurements after 48 h of incubation using rumen fluid from donor cows receiving a grass silage (CON) based or a sainfoin silage (SAIN) based total mixed ration (TMR).

Parameter[a]	Substrate[b]	Day[c]			SEM[b]	P-value		
		8	18	33		Substrate	Day	Substrate × Day
CH_4 (ml/g OM[b] incubated)	CON	57.8	-	51.6	5.74	0.98	0.318	0.059
	SAIN	48.7*	49.6	60.8				
CH_4 (% of total gas)	CON	18.4	-	18.4	0.73	0.321	0.475	0.284
	SAIN	19.7	18.9	20.4				
A (ml/g OM incubated)	CON	70.2	-	57.4	4.27	0.873	0.543	0.014
	SAIN	55.5	58.1	70.5				
B (h)	CON	11.3	-	11.3	0.88	0.912	0.336	0.805
	SAIN	11.4	9.8	10.9				
C	CON	0.7	-	1.6	0.13	0.335	0.051	0.152
	SAIN	1.0	1.5	1.2				

[a] A, asymptotic of methane; B, incubation at which time half of asymptotic of methane has been formed; C, sharpness of the curve.
[b] CON, control TMR containing grass/maize silage fed to cows and used as substrate; OM, organic matter; SAIN, TMR containing sainfoin silage and grass/maize silage fed to cows and used as substrate; SEM, standard error of mean.
[c] Day, refers to the experimental day numbers at which rumen fluid sample was collected for *in vitro* incubations.
* Significantly ($P \leq 0.05$) different from corresponding CON value.

Fermentation end products

Total VFA concentrations (Table 5.5), measured after 48 h of *in vitro* incubations in the rumen fluid, were neither affected by substrate nor by day of sampling of rumen fluid ($P \geq 0.321$), but were significantly ($P = 0.006$) affected by a substrate × day interaction. In case SAIN was used as substrate, the concentration of total VFA was found to be lower ($P = 0.003$) when rumen fluid was used that was collected at day 8 of the experimental period. The molar proportions of HAc and HPr were affected by substrate ($P = 0.001$; $P = 0.001$, respectively) and day ($P = 0.049$; $P = 0.051$, respectively), but showed no substrate × day interaction ($P \geq 0.490$). The HAc molar proportions at day 8 were lower ($P = 0.003$) for SAIN compared to CON substrate, whilst for HPr the molar proportions were higher ($P = 0.039$) compared to CON. The concentration of BCVFA was not affected by the main effects but was affected by the substrate × day interaction ($P = 0.014$). The NGR showed a substrate ($P = 0.002$) and day ($P = 0.012$) effect, with NGR being increased in response to the SAIN silage substrate on day 8 ($P = 0.008$) compared to the CON substrate.

Relationship between *in vitro* and *in vivo* CH_4 production

In vitro CH_4 production at 24 and 48 h of incubation, expressed in g/kg OM incubated, as measured on day 8, 18 or 33 showed a non-significant relationship with *in vivo* CH_4 production expressed in g/kg^{-}OM intake ($R^2 = 0.03$, $P = 0.450$; $R^2 = 0.02$, $P = 0.481$, see Figure 5.1 A and B). This relationship could not be improved using different *in vitro* time points (2, 4, 8, 12, 28 and 36 hours of incubations) with R^2-values ≤ 0.12 and P-values ≥ 0.103 (data not shown).

Table 5.5. Total volatile fatty acid (TVFA), molar proportion of volatile fatty acid from *in vitro* fermentations, using rumen fluid from donor cows receiving a grass silage (CON) based or a sainfoin silage (SAIN) based total mixed ration (TMR).

Parameter[a]	Substrate[b]	Day[c]			SEM[b]	P-value		
		8	18	33		Substrate	Day	Substrate × Day
TVFA (mmol/l)	CON	102	-	87.6	3.35	0.321	0.396	0.006
	SAIN	85.4**	97.8	97.1				
Acetic acid (% of TVFA)	CON	66.2	-	62.3	0.74	0.001	0.049	0.49
	SAIN	63.4**	65.3	64.5				
Propionic acid (% of TVFA)	CON	18.3	-	19.9	1.57	0.001	0.051	0.699
	SAIN	20.4*	19.7	22.3				
Butyric acid (% of TVFA)	CON	11.3	-	10.5	0.33	0.419	0.058	0.705
	SAIN	11.4	10.3	10.9				
BCVFA (% of TVFA)	CON	3.5	-	3.2	0.11	0.867	0.988	0.014
	SAIN	3.2	3.4	3.5				
NGR	CON	3.8	-	3.4	0.12	0.002	0.012	0.725
	SAIN	4.4**	3.9	3.9				

[a]BCVFA is branched chain volatile fatty acid; NGR is non-glucogenic to glucogenic ratio.
[b]CON, control TMR containing grass/maize silage fed to cows and used as substrate; SAIN, TMR containing sainfoin silage and grass/maize silage fed to cows and used as substrate; SEM, standard error of mean.
[c]Day, refers to the experimental day numbers at which rumen fluid sample was collected for *in vitro* incubations.
*Significantly ($P \leq 0.05$) and ** ($P \leq 0.01$) different from corresponding CON value.

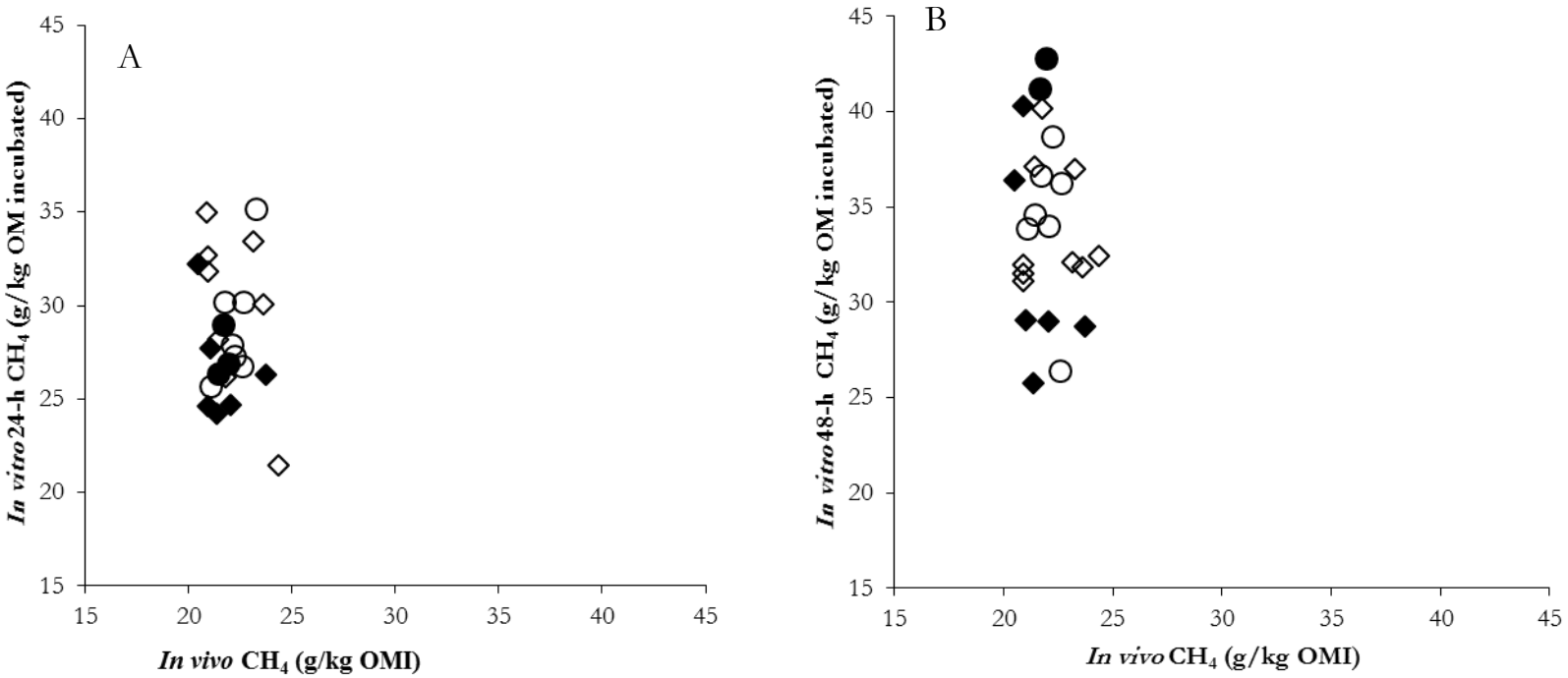

Figure 5.1. Relationship between (A) 24-h *in vitro* methane (CH_4) (R^2 = 0.03; P = 0.450), or (B) 48-h *in vitro* CH_4 (R^2 = 0.02; P = 0.481) and *in vivo* CH_4 production from cows fed a control diet (○ in period 1, ● in period 2) or a sainfoin silage based diet (◊ in period 1, ♦ in period 2) at day 8, 18 and 33, respectively. Each data point represents an individual cow at different experimental periods. OM = organic matter, OMI = OM intake.

DISCUSSION

Relationship between *in vitro* and *in vivo* CH_4 production

In the current *in vitro* experiment, we used rumen fluid from donor cows adapted to two dietary treatments, a SAIN silage-based and grass silage-based diet, which is generally not the case for the *in vitro* studies reported in literature. Despite the *in vitro* measurements being performed simultaneously with adapted rumen fluid from the donor cows used in the *in vivo* experiment, (Huyen et al., 2016a), our observation showed a non-significant relationship between *in vitro* measured CH_4 production expressed in g/kg OM incubated and the corresponding *in vivo* CH_4 production expressed in g/kg OM intake measurements at different time points of incubation. This was unlike a previous study (Hatew et al., 2015) where a significant positive relationship was found, but similar to the study of Macome et al. (2017) who also observed a non-significant relationship (R^2 = 0.01-0.08) between *in vitro* (ml/g of OM) and *in vivo* (g/kg DM intake) measured CH_4 production. The absence of a relationship between *in vitro* and *in vivo* CH_4 production could have been influenced by the fact that passage rate and hindgut fermentation are not simulated in the *in vitro* measurements (Hatew et al., 2015)

In vitro gas and methane production

Partial replacement of grass silage by sainfoin silage in the TMR of the cows affected some kinetic parameter and the extent of *in vitro* GP (Table 5.3), whereas no effects were observed for CH_4 *p*roduction (Table 4). On day 8 of the experimental period, SAIN silage substrate resulted in a slight reduction of GP compared to CON, which could be a result of CT decreasing the attachment of rumen microorganisms to plant cell walls which is essential for their degradation (Makkar et al., 1989; Tavendale et al., 2005). The formation of CT complexes with dietary protein makes the protein more resistant to microbial degradation. Furthermore, CT may reduce fibre digestion by complexing with lignocellulose and preventing microbial digestion (McSweeney et al., 2001). After 25 days of adaptation of rumen microbes to the SAIN diet (day 33; Table 5.3), the *in vitro* GP parameters between substrates were not affected. This indicates that rumen microbiota adapted to the dietary conditions and returned to their initial level at the onset of the experiment. Similar transient effects on *in vitro* fermentation characteristics have been observed in dairy cows receiving medium chain fatty acids and essential oils, (Klop et al., 2016) or lactic acid bacteria provided as silage inoculants or as direct fed microbials (Ellis et al., 2016)

In the present study, reduced *in vitro* CH_4 production from the SAIN substrate compared to the CON substrate was observed only at the first day after cows were allocated to their SAIN

based diet (day 8 of the experiment). Reduction of *in vitro* CH_4 production using tanniniferous material has been reported by others researchers (Pellikaan et al., 2011b; Theodoridou et al., 2011; Hatew et al., 2016). Methane production is generally related to the acetate and propionate ratio because of their different metabolic pathways where acetate is a producer and propionate is a consumer of hydrogen (Martin et al., 2010). However, in the present study, no reduction in CH_4 production after long term diet adaptation was observed. This could be due to lower fibre degradation caused by the formation of tannin-lignocellulose complexes preventing microbial fermentation (McSweeney et al., 2001; Waghorn et al., 2003). Huyen et al. (2016b) reported a reduction in *in vitro* CH_4 production and CH_4 concentration in all their CT sources compared to a control substrate. A similar observation was reported by Huyen et al. (2016a) for *in vivo* CH_4 production expressed per kg DM intake in sainfoin silage-fed cows compared to control-fed cows. The authors attributed this effect to a decrease in fibre digestibility in the rumen and subsequent a reduction in CH_4 production. Carula et al. (2005) proposed that inhibition of methanogenesis by CT is the result of suppressed fibre degradation limiting hydrogen derived from the synthesis of acetate. Depressed fibre degradation could be due to a reduction in the number of cellulolytic bacteria or the formation of tannin-cellulose complexes decreasing the absolute amount of CH_4. Reduced digestibility of diets containing CT is commonly observed, (Waghorn, 2008; Patra and Saxena, 2011) thereby negatively affecting voluntary feed intake. (Waghorn et al., 1994; Frutos et al., 2004). Hatew et al. (2016) reported a reduction in CH_4 production and differences in the antimethanogenic activity among CT extracts which was attributed to differences in the CT polymer size (mDP values). Similarly, Huyen et al. (2016b) concluded that CT extracted from different plants had diverse effects on the extent of rumen fermentation and CH_4 production.

Fermentations end products

Sainfoin silage substrate showed a numerical decrease in total VFA which agrees with the lower GP at day 8 for the SAIN substrate compared to the CON substrate. The molar proportion of propionic acid was higher in the SAIN substrate compared to the CON substrate at the first day of diet allocation (day 8) but was not significant by day 25. This increase in propionic acid resulted from the associated decrease in the molar proportion of acetic acid also observed on day 8. A non-significant reduction in total VFA was observed with the SAIN silage substrate compared to the CON substrate on day 1. Other studies reported a decrease in total VFA and ammonia in incubations containing CT. (Bhatta et al., 2009; Huyen et al., 2016b). The amount of BCVFA produced *in vitro* did not change by replacing the grass silage diet by SAIN silage. Condensed

tannins are a binding agent to protein to form insoluble complexes and hence reduce protein degradation in the rumen fluid (Patra and Saxena, 2011).

CONCLUSIONS

A non-significant correlation between *in vitro* CH_4 expressed in g/kg OM incubated measured after 24 and 48-h of incubation in rumen fluid and *in vivo* CH_4, expressed in g/kg OM intake was observed. Sainfoin silage incubated in rumen fluid from cows adapted to this forage did not reduce *in vitro* gas and CH_4 production by day 25 compared to CON substrate. The *in vitro* study was able to identify short terms effects of diet change but not the long term effects up to 25 days. Molar proportion of acetic acid, propionic acid and NGR were affected by substrate on day 1. The results of the present study show that *in vitro* CH_4 production cannot predict the *in vivo* CH_4 production.

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REFERENCES

Bhatta, R., Uyeno Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512-5522.

Carulla, J.E., Kreuzer, M., Machmüller, A., Hess. H.D., 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agric. Res. 56, 961-970.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., Oudshoorn. L., 1996. 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.

Dal Pizzol, J.G., Ribeiro-Filho, H.M.N., Quereuil, A., Le Morvan, A., Niderkorn, V., 2017. Complementarities between grasses and forage legumes from temperate and subtropical areas on *in vitro* rumen fermentation characteristics. Anim. Feed Sci. Technol. 228, 178-185.

Ellis, J.L, Bannink, A., Hindrichsen, I.K., Kinley, R.D., Pellikaan, W.F., Milora, N., Dijkstra, J., 2016. The effect of lactic acid bacteria included as a probiotic or silage inoculant on *in vitro* rumen digestibility, total gas and methane production. Anim. Feed Sci. Technol. 211, 61-74.

Frutos, P., Hervás, G., Giráldez, F.J., Mantecón, A.R., 2004. Review. Tannins and ruminant nutrition. Spanish J. Agric. Res. 2, 191-202.

Getachew, G., Blümmel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. 72, 261-281.

Getachew, G., Robinson, P.H., DePeters, E.J., Taylor, S.J., Gisi, D.D., Higginbotham, G.E., 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.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77-89.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta , S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Hatew, B., Stringano, E., Mueller-Harvey, I., Hendriks, W.H., Carbonero, C.H., Smith, L.M.J. Pellikaan, W.F., 2016. Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on *in vitro* ruminal methane production and fermentation characteristics. J. Anim. Physiol. Anim. Nutr. 100, 348-360.

Hayot Carbonero, C., Mueller-Harvey, I., Brown, T.A., Smith, L., 2011. Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. Plant Genet. Resour. C 9, 70-85.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci. 62, 40-45.

Hristov, A.N., Oh J., Firkins J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B., Tricarico, J.M., 2013. Special topics: Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci .91, 5045-5069.

Huyen, N.T., Desrues, O., Alferink, S.J.J., Zandstra, T., Verstegen, M.W.A., Hendriks, W.H., Pellikaan, W.F., 2016a. Inclusion of sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations affects nutrient digestibility, nitrogen utilization, energy balance, and methane emissions. J. Dairy Sci. 99, 3566-3577.

Huyen, N.T., Fryganas, C., Uittenbogaard, G., Mueller-Harvey, I., Verstegen, M.W.A., Hendriks, W.H., Pellikaan, W.F., 2016b. Structural features of condensed tannins affect *in vitro* ruminal methane production and fermentation characteristics. J. Agric. Sci. 154, 1474-1487.

ISO 6496, 1999. Animal Feeding Stuffs. Determination of Moisture and other Volatile Matter Content. Geneva, Switzerland.

ISO 5984, 2002. Animal Feeding Stuffs. Determination of Crude Ash. Geneva, Switzerland.

ISO 15914, 2004. Animal Feeding Stuffs. Enzymatic Determination of Total Starch Content. Geneva, Switzerland.

ISO 5983-1, 2005. Animal Feeding Stuffs. Determination of Nitrogen Content and Calculation of Crude Protein Content-part 1. Kjeldahl Method. Geneva, Switzerland.

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

Karnezos, T.P., Matches, A.G., Brown, C.P., 1994. Spring lamb production on alfalfa, sainfoin, and wheatgrass pastures. Agron. J. 86, 497-502.

Klop, G., van Laar-van Schuppen, S., Pellikaan, W.F., Hendriks, W.H., Bannink. A., Dijkstra, J., 2016. Changes in *in vitro* gas and methane production from rumen fluid from dairy cows during adaptation to feed additives *in vivo*. Animal 11, 591-599.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., Tricarico, J.M., 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97, 3231-3261.

Macome, F.M., Pellikaan, W.F., Schonewille, J.T., Bannink, A., van Laar, H., Hendriks, W.H., Warner, D., Cone, J.W., 2017. *In vitro* rumen gas and methane production of grass silages differing in plant maturity and nitrogen fertilisation, compared to *in vivo* enteric methane production. Anim. Feed Sci. Technol. 230, 96-102.

Makkar, H.P.S., Singh, B., Negi, S.S., 1989. Relationship of rumen degradability with biomass accumulation, cell wall constituents and tannin levels in some tree leaves. Anim. Prod. 49, 299-303.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351-365.

McMahon, L.R., Majak, W., McAllister, T.A., Hall, J.W., Jones, G.A., Popp, J.D., Cheng, K.J., 1999. Effect of sainfoin on *in vitro* digestion of fresh alfalfa and bloat in steers. Can. J. Anim. Sci. 79, 203-212.

McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O., 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91, 83-93.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Dev. 28, 7-55.

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

Parker, R.J., Moss, B.R., 1981. Nutritional value of sainfoin hay compared with alfalfa hay. J. Dairy Sci. 64, 206-210.

Patra, A.K., Saxena, J., 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91, 24-37.

Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011a. A novel method to determine simultaneously methane production during *in vitro* gas production using fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Pellikaan, W.F, Stringano, E., Leenaars. J., Bongers, D.J.G.M., van Schuppen-van Laar, S., Plant, J., Mueller-Harvey, I., 2011b. Evaluating effects of tannins on extent and rate of *in vitro* gas and CH_4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166-167, 377-390.

Pinares-Patiño, C.S., Waghorn, G.C., 2014. Technical manual on respiration chamber designs, Eds. Ministry of Agriculture and Forestry, Wellington, New Zealand, p 119.

SAS, SAS Institute Inc. Statistical Analysis Software. SAS/STAT 9.3 User's Guide, Cary, NC. (2002-2010).

Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Technol. 123-124, 403-419.

Theodoridou, K., Aufrère, J., Niderkorn, V., Andueza, D., Le Morvan, A., Picard, F., Baumont, R., 2011. *In vitro* study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetation cycles. Anim. Feed Sci. Technol. 170, 147-159.

Van Gelder, A.H., Hetta, M., Rodrigues, M.A.M., de Boever, J.L., Den Hartigh, H., Rymer, C., van Oostrum, M., van Kaathoven, R., Cone, J.W., 2005. Ranking of *in vitro* fermentability of 20 feedstuffs with an automated gas production technique: Results of a ring test. Anim. Feed Sci. Technol. 123, 243-253.

Van Soest P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Waghorn, G.C., Shelton, I.D., McNabb, W.C., 1994. Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 1. Non-nitrogenous aspects. J. Agric. Sci. 123, 99-107.

Waghorn, G.C., McNabb, W.C., 2003. Consequences of plant phenolic compounds for productivity and health of ruminants. Proc. Nutr. Soc. 62, 383-392.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. Anim. Feed Sci. Technol. 147, 116-139.

Yáñez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., O'Kiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z., Hristov, A.N., 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants - a review. Anim. Feed Sci. Technol. 216, 1-18.

Chapter 6

General discussion

BACKGROUND AND OUTLINE

In vitro techniques are routinely used to evaluate the nutritional quality of feeds and feed ingredients as they have an advantage of being less expensive, less laborious and allow maintenance of more precise experimental conditions than *in vivo* studies (Getachew et al., 2005; Bhatta et al., 2007). It is well known that gas in the gas production (GP) technique is produced after the fermentation of the substrate where CO_2 and methane (CH_4) are produced as direct gas (Getachew et al., 1998), and indirect gas is produced from the buffering of short chain fatty acids (SCFA). The molar proportion of SCFA (acetate, propionate and butyrate) produced is dependent of the type of substrate. For instance, rapidly fermentable carbohydrates yield more propionate compared to slowly fermentable. The use of the GP technique also allows estimation of CH_4 production from different feeds, feed ingredients or additives (Getachew et al., 2005; Pellikaan et al., 2011a; Hatew et al., 2015).

Studies investigating the relationship between *in vitro* and *in vivo* CH_4 production by cows fed the same feed as substrate used in *in vitro* incubations are rare in the literature. It is important to have an accurate prediction on CH_4 production of rations to be fed in practice. Therefore, the Dutch government financed a large program to determine the actual CH_4 emission from dairy cattle *in vivo*, using climate respiration chambers (CRCs). This offered the unique opportunity to conduct parallel *in vitro* experiments with the aim to derive accurate *in vitro* prediction equations for CH_4 production. Such equations can subsequently be used in practice to reduce CH_4 emission. Methane is a by-product of the microbial fermentation of carbohydrates in the forestomaches of ruminants and in the hindgut as well. However, the hindgut digestion can be essential in ruminants when substrate degradability in the rumen decreases, particularly after feeding ground or pelleted diets and diets containing high starch (Hoover, 1978).

This chapter provides a discussion of the results obtained and a synthesis of the data from the experiments reported in this thesis. In addition, new regression equations are derived across diets fed to the cows in **Chapter 3**, **4** and **5** to ascertain the accuracy of the *in vitro* method to predict *in vivo* CH_4 production.

UNIT OF EXPRESSION OF METHANE PRODUCTION

Methane production measured in the CRCs can be expressed in different units such as CH_4 in g or l/day, CH_4 yield or as CH_4 intensity (CH_4 Ei) which is CH_4 per unit of product (meat, milk).

For example, with improved management CH_4 will be reduced. An increase in milk production per year will reduce CH_4 production per kilo of milk produced but not necessarily the total amount of CH_4 (l/day). Thus, CH_4 Ei per energy correct milk fat and protein corrected milk or milk production should be considered if farmers want to follow strategies on CH_4 mitigation and should focus on the efficiency of the production (Knapp et al., 2014).

Methane expressed as g or l/day is routinely used in the CRCs. The next unit most often used to express CH_4 production is per unit of organic matter intake (OMI) or per unit of dry matter intake (DMI) as a measure of CH_4 yield. Methane is positively correlated to OMI and DMI and has a large effect on enteric CH_4 emission from ruminants. Dry matter intake is a principal factor limiting CH_4 and is related to forage quality, less digestible forage produce more CH_4/kg of digested OM (DOM). Expressing CH_4 per unit DMI provides an indication of the amount of CH_4 emitted per unit of feed. In spite of the level of DMI influencing the production of CH_4 due to the fact that ash is not fermented, it seems opportune to express CH_4 as g/kg of OMI. However, only material that is digested is related to CH_4 production, thus CH_4 per unit of DOM intake is more appropriate. Lastly, only feed components that are fermented are related to CH_4 production. In this manner, CH_4 expressed per unit of fermentable organic matter (FOM) would be the best method to compare effects of mitigation strategies between studies. However, a drawback of expressing it per unit of FOM is the fact that it does not take into account the contribution of fermentation in the large intestine.

In vitro CH_4 production is commonly expressed as ml, g or mmol/g of OM incubated (Bodas et al., 2008), although it can also be expressed in ml/g DOM. During the fermentation of feed, CO_2, CH_4 and volatile fatty acids (VFA) are produced. In the various chapters of this thesis, the unit used to express *in vitro* CH_4 production is g/kg OM incubated. In order to compare *in vivo* and *in vitro* results, CH_4 production reported in *in vivo* studies were expressed per unit OMI as not in all of the studies CH_4 per unit of FOM was determined with corresponding *in vitro* values expressed per unit of OM incubated.

DIRECT RELATIONSHIP BETWEEN *IN VITRO* AND *IN VIVO* METHANE PRODUCTION WITHIN FEEDS

It was hypothesized that *in vitro* CH_4 production as measured in the different experiments will correlate with *in vivo* CH_4 production as measured in climate respiration chambers. In **Chapter 3**, there was no significant direct relationship between *in vivo* (g/kg of OMI) and 8, 12, 24, and 72-

h *in vitro* CH_4 (g/kg OM incubated) production. Similarly, in **Chapter 4***,* no direct relationship was found between *in vivo* and *in vitro* CH_4 production measured at various time points between 2 and 48 h. And again in **Chapter 5**, *in vitro* measurement of CH_4 production using rumen fluid from adapted dairy cows did not predict their *in vivo* CH_4 production. These results are highly consistent and it can be concluded that the *in vitro* GP technique cannot directly predict *in vivo* CH_4 production within specific feeds differing in individual feed ingredients.

DIRECT RELATIONSHIP BETWEEN *IN VITRO* AND *IN VIVO* METHANE PRODUCTION ACROSS FEEDS

Across the experiments conducted in **Chapters 3** to **5**, 48-h *in vitro* CH_4 production per unit of incubated OM, a trend was observed for a correlation with *in vivo* CH_4 production expressed per unit of OMI ($R^2 = 0.17$, P = 0.051, see Figure 6.1*)*. Although the relationship could be improved using different in vitro time points (8, 12, 18, 24, 30 and 36-h of incubations) in terms of level of significance ($P \geq 0.028$), R^2 values of ≤ 0.09 were obtained (Table 6.1) indicating that the goodness of fit was less compared to the 48-h equation.

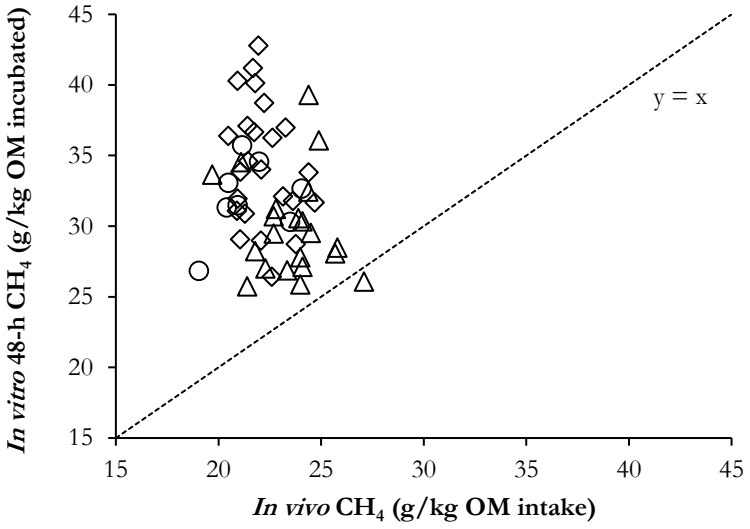

Figure 6.1. Relationship between *in vivo* CH_4 expressed as (g/kg OMI) and *in vitro* CH_4 in g/kg OM of TMR incubated) from cows fed various rations used in (**Chapter 3** = Δ, **Chapter 4** = ○ and **Chapter 5** = ◊, respectively).

The lack of a relationship within feeds and the weak correlation across feeds could be due to the small range in CH_4 production observed in the *in vivo* experiments, ranging from 19.0 to 27.1 (g/kg OMI). A large number of factors with considerable influence on the results obtained by the

in vitro technique have been reported (Rymer et al., 2005). The activity and source of rumen microbial inoculum represent the largest source of variation on the *in vitro results* (Cone et al., 2002). In many *in vitro* studies, rumen inoculum is obtained from donor cows fed a basal diet which is different from the substrate used in the *in vitro* incubations. However, in all research Chapters in this thesis, the substrates incubated were the same total mixed rations as those fed to the cows with the exception of substrates used in **Chapter 2**.

Table 6.1. Slope, intercept and the coefficient of determination (R^2) of the linear relationships between *in vivo* CH_4 production (g/kg OMI) and *in vitro* CH_4 production (g/kg OM incubated) measured at different time points from cows fed different rations (**Chapter 3**, **4** and **5**).

Incubation time (h)	Slope	Intercept	R^2	P-value
8	-0.359	22.60	0.04	0.141
10	-0.467	27.78	0.05	0.092
12	-0.536	31.39	0.06	0.065
18	-0.667	39.01	0.08	0.039
24	-0.780	44.29	0.09	0.028
30	-0.807	46.26	0.08	0.043
36	-0.496	41.12	0.03	0.183
48	-1.754	70.93	0.17	0.051

OM = organic matter; OMI = OM intake.

In addition, factors such as *in vivo* transit time, passage rate, absorption and removal of nutrients, large intestinal fermentation are not simulated by the *in vitro* methodology, thereby, potentially contributing to the poor relationship. In addition, the *in vitro* system is a batch culture system and the microbial profile of the rumen fluid may deviate from that which is present in the rumen after the same period of time after incubating a substrate. Although the *in vitro* system does not predict CH_4 production *in vivo*, the *in vitro* GP system is routinely used for screening feedstuffs and feeds for their fermentation characteristics by many researchers (Cone et al., 1996; Getachew et al., 1998; Dijkstra et al., 2005).

RELATIONSHIP *IN VITRO* AND *IN VIVO* METHANE PRODUCTION USING FEED COMPOSITION

With the aim to improve the relationship between *in vitro* and *in vivo* CH_4 production across feeds, a stepwise multiple regression analysis was performed using the composition of the diets as fed to the animals in **Chapters 3**, **4** and **5**, and *in vitro* GP parameters at any time point (Table 6.2).

This analysis resulted in an equation where *in vitro* CH_4 production was not selected but only OM, crude protein (CP) and NDF content of the diet. This model (no 2, Table 6.2) explains 41.4% of the variation. Also a "full" model was run to predict *in vivo* CH_4 expressed as g/kg OMI where all parameters within the model are selected to predict *in vivo* CH_4 production (Slentry P = 1.0). When all variables were included, an R^2 was obtained of 0.626. Using the 48-h *in vitro* CH_4 production and associated kinetics parameters as well as feed composition data, again, no *in vitro* data were selected by the model and only OM, DM and NDF were selected. Again it is clear that none of the parameters which are related to *in vitro* CH_4 production were selected indicating again that across feeds, *in vitro* measurement of CH_4 or its kinetics parameters make no contribution to predicting *in vivo* CH_4 production. Figure 6.2 provides a schematic representation of the observed *in vivo* CH_4 and predicted CH_4 production using the equation of model 2.

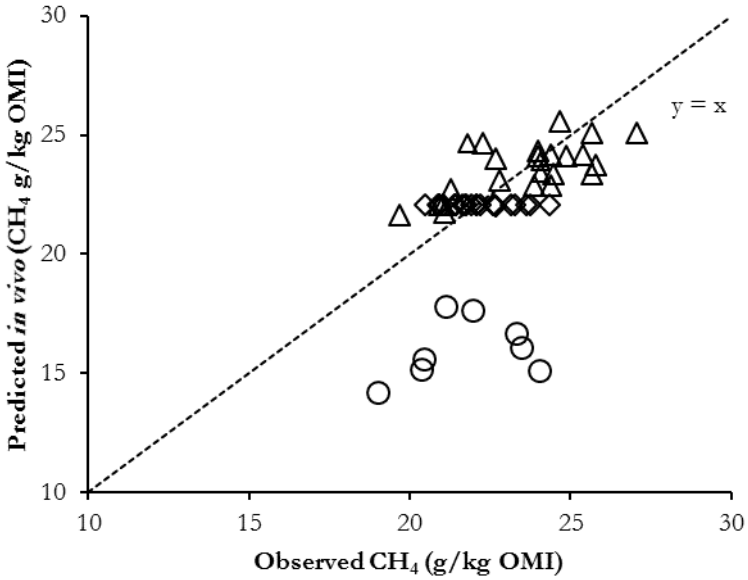

Figure 6.2. Relationship between observed *in vivo* methane (CH_4) and predicted CH_4 expressed as g/kg OMI, by the regression equation: CH_4 (g/kg OMI) = 61.96 - 0.0785 × OM (g/kg DM) + 0.0336 × CP (g/kg DM) + 0.0675 × NDF (g/kg DM). Data from **Chapter 3** = Δ, **Chapter 4** = ○ and **Chapter 5** = ◊. DM = dry matter, CP = crude protein, NDF = neutral detergent fibre OM = organic matter and OMI = OM intake.

IMPROVING QUALITY OF GRASS SILAGE

Grass silage is a principal component of rations in intensive and extensive ruminant production systems. It is well established that factors such as chemical composition, rumen degradation and digestibility of diets greatly influence CH_4 production (Hristov et al., 2013). However, its nutritional value varies with stage of maturity at harvest. It is well known that forage quality decreases with advancing maturity of grass, because of the chemical and physical changes in grass. Grass harvested at an advanced maturity stage has a decreased CP, increased NDF and acid detergent fiber (ADF) content (Rinne et al., 1997; Cone et al., 1999). The data show that the nutritional value of grass silage has a large effect on animal performance and requirement for supplementation of concentrates (Randby et al., 2012). In line with this, the nutritional composition can be changed (**Chapter 2**, Table 2.1) with nitrogen (N) and fiber contents mainly affected by N fertilisation rate and stage of grass at harvest, respectively.

Management factors such as N fertilisation rate and maturity of grass at harvest have an influence on rumen degradation characteristics (Heeren et al., 2014), chemical composition (fiber content, protein and sugars), digestibility and nutritional value (Warner et al., 2015). It is important to determine the effects of grass quality at harvest on CH_4 emissions in order to provide suggestions on grass harvest strategies and grassland management. Due to changes in the nutrient composition of grass, the nutrient digestibility and the profile of fermentation end-products in the rumen will be influenced. Methane production in ruminants tends to increase with maturity of grass. Recently, Warner et al. (2016) quantified the *in vivo* CH_4 production of dairy cows fed grass silages differing in maturity and produced from grasslands with differences in N fertilisation. At the same time, the current *in vitro* experiment was performed with the same fisulated animals used in the *in vivo* experiment (**Chapter 2**). I observed that maturity of grass at harvest did not affect *in vitro* CH_4 synthesis expressed in ml/g of DOM and CH_4 expressed as % of the total gas, whereas N fertilisation decreased the *in vitro* CH_4 production, expressed in any unit (**Chapter 2**, Table 2.2).

In line with this, feeding of rye-grass herbage harvested at an early maturity reduced CH_4 Ei. Warner et al. (2015) reported that improving grass digestibility shows the potential to reduce CH_4 Ei and may be an effective strategy for CH_4 mitigation. Warner et al. (2016) reported an interaction between maturity of grass silage at harvest and N fertilisation when CH_4 was expressed per unit of DOM. This finding indicates that agronomic factors such as maturity at harvest and N fertilisation must be considered for grassland management in light of CH_4 mitigation. However, comparative studies dealing with grass silage quality *in vivo* vs *in vitro*

Table 6.2. Regression equations between *in vivo* CH_4 production of cows fed different rations, and *in vitro* gas production and ration chemical composition parameters.

Model number and variables included Regression model	Model P-value	R^2 of slentry[a] P < 0.05	 P < 1.0
1. Linear regression *in vitro* CH_4 at any time point			
CH_4 (g/kg OMI) = 44.29 - 0.780 × $_{24h}CH_4$ (g/kg OM incubated)	0.028	0.09	-
2. Stepwise regression *in vitro* CH_4 at any time point and chemical composition			
CH_4 (g/kg OMI) = 61.96 - 0.0785 × OM (g/kg DM) + 0.0336 × CP (g/kg DM) + 0.0676 × NDF (g/kg DM)	<0.0001	0.414	0.507
3. Stepwise regression all *in vitro* $_{48h}CH_4$ parameters and chemical composition			
See model 2	<0.0001	0.414	0.650

[a] Probability value as a selection entry for stepwise regression in SAS.

CH_4, methane; CP, crude protein; DM, dry matter; OM, organic matter; OMI, OM intake; NDF, neutral detergent fibre.

using the same substrates as fed to cows on CH_4 production are limited. Some modelling approaches predicted that decreased maturity at harvest and increased N fertilisation decreases CH_4 Ei and CH_4 yield (Boadi et al., 2004; Bannink et al., 2010). In **Chapter 2**, it was concluded that grass silage quality had an effect on *in vitro* CH_4 production expressed in ml/g OM and N fertilisation had an effect on CH_4 expressed in ml/g OM, ml/g DOM or as a % of total gas.

In **Chapter 3**, an experiment is reported using cows in two distinct stages of lactation (early and late lactation stage) fed grass silages varying in quality. A decrease in *in vitro* total gas and CH_4 production, expressed in ml/g OM, was observed with advancing maturity of the ensiled grass. It was expected that *in vitro* CH_4 production per gram of DOM would increase as grass maturity at harvest increased. Nevertheless, the evidence on chemical composition with high NDF in more mature grass, and reduction in CP shows that digestibility decreases with advancing maturity of grass at harvest. Instead of an increase in CH_4 production expressed in ml/g OM incubated or per unit of FOM for more mature grass, a decrease in CH_4 production with advancing maturity was observed. Because precise data on digestibility were not measured in this experiment, it is difficult to draw conclusions.

MAIZE SILAGE QUALITY ON METHANE PRODUCTION

The nutritional value of maize silage depends mainly on the content and degradability of the starch. The starch content as well as the vitreousness of maize kernels increases with maturity, and the fractional rate of ruminal starch degradation of maize decreases with maturity (Philippeau and Michalet-Doreau, 1997; Ettle et al., 2001). The changes in nutritional components of maize silage with increasing maturity of the maize plant at harvest are well-documented (Cammell et al., 2000; Phipps et al., 2000; Warner et al., 2013). Nevertheless, a limited number of studies have assessed the effect of maturity of maize plant at harvest on CH_4 emissions in lactating cows (Cammell et al., 2000; Mc Geough et al., 2010; Hatew et al., 2016). The increasing starch content with the progression of maturity of the maize plant at harvest on the ruminant's diet allows an alternative way of decreasing CH_4 emission intensity (Hristov et al., 2013).

In **Chapter 4** in this thesis (data from Hatew et al., 2016), a relationship between CH_4 Ei or CH_4 yield and starch content was not observed. Hassanat et al. (2013) observed a decrease on enteric CH_4 production with increasing maize silage in the diet due to starch content in the diet. Benchaar et al. (2014), evaluating the effect of replacing barley silage with maize silage, reported a tendency for a decrease in CH_4 yield expressed in g/kg DMI. Based on the *in vivo* CH_4 data reported

in **Chapter 4** and data from other studies (Hassanat et al., 2013; Benchaar et al., 2014; Pirondini et al., 2015), linear regression analysis of CH_4 Ei expressed in g/kg FPCM and CH_4 yield expressed in g/kg DMI on level of starch were developed (Figure 6.3). The following linear regressions were obtained:

In vivo CH_4 (g/kg DMI)

= 20.77 – 0.0032 × starch content (g/kg DM), P = 0.643, R^2 = 0.014

In vivo CH_4 (g/kg FPCM)

= 16.82 – 0.012 × starch content (g/kg DM), P = 0.015, R^2 = 0.317

It can be observed that CH_4 does not decrease when expressed per unit DMI with increasing starch content in the diet. When expressed per unit FPCM there is a significant decrease with increasing starch content in the diet, although the decrease is only minor (slope = 0.012).

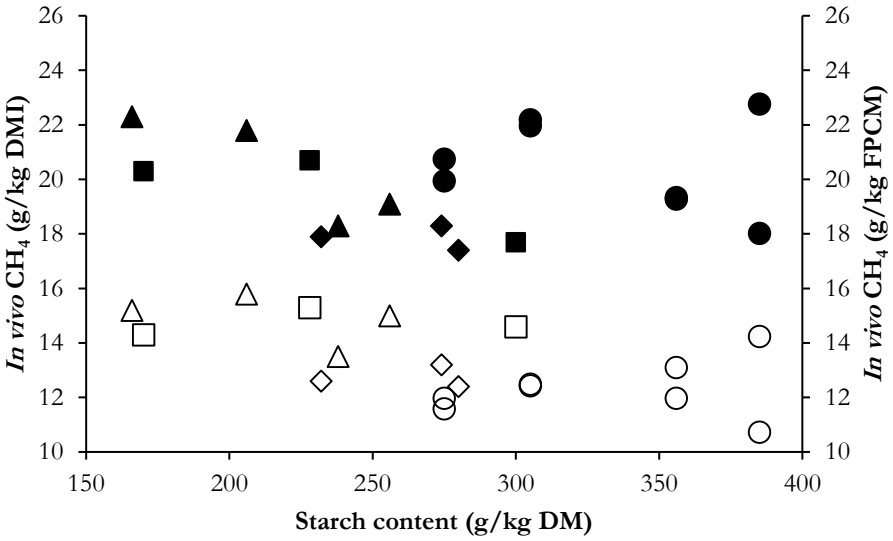

Figure 6.3. Effects of dietary starch content on methane (CH_4) emissions of dairy cows expressed in g/kg dry matter intake (DMI, closed symbols) or in g/kg fat and protein corrected milk (FPCM, open symbols). Source of data: **Chapter 4** of this thesis (circle), Hassanat et al. (2013, square), Benchaar et al. (2014, triangle) and Pirondini et al. (2015, diamond).

USE OF PLANTS CONTAINING CONDENSED TANNINS

The use of plants which contain secondary compounds such as condensed tannins (CT) in dairy cattle has been reported to have anti-methanogenic properties (Beauchemin et al., 2007; Martin et al., 2010). Tannins can be divided into two groups, i.e. CT and hydrolysable tannins (HT). Hydrolysable tannins contain a carbohydrate core, often glucose, esterified with phenolic acids (gallic and hexahydroxdiphenic) and can be hydrolysed by heating with a weak acid (Makkar, 2003). It has been suggested by Hristov et al. (2013) that tannins have the potential for CH_4 mitigation up to 20%, but the anti-methanogenic effect depends on the dietary concentration (Jayanegara et al., 2009). However, next to the dietary dose of tannins, also the origin of tannins needs to be considered in relation to the efficacy of tannins to reduce CH_4 production (Pellikaan et al., 2011b). In this thesis, the effect of replacing grass silage (a CT-free forage) by sainfoin silage (a CT-containing forage) on *in vitro* CH_4 and compared to *in vivo* CH_4 data was evaluated (**Chapter 5**). Huyen et al. (2016) reported an effect of sainfoin silage containing CT on CH_4 production expressed as g/kg of DMI. Carula et al. (2005) reported that inhibition of methanogenesis by CT is mainly due to the suppressed fiber degradation which is limiting H_2 derived from the synthesis of acetate. Depressed fiber degradation could be due to a reduction in the number of cellulolytic bacteria or formation of tannin-cellulose complexes. Results on CH_4 reduction were reported by others researchers (Pellikaan et al., 2011b; Theodoridou et al., 2011; Hatew et al., 2015). It has been shown that diets rich in CT tended to reduce ruminal methanogenesis and decrease ruminal protozoa. However, in **Chapter 5**, a slight reduction in *in vitro* CH_4 production was observed on the first day after animals were changed to their SAIN silage-based diet. From the results obtained in **Chapter 5**, the recommendations in practice on CH_4 mitigation using SAIN silage-based diet fed to animals should be considered with care.

CONCLUSIONS

Based on the data in the thesis, the following conclusions can be drawn:

- *In vivo* CH_4 production of dairy cows cannot be predicted by *in vitro* CH_4 production using the automated batch culture gas production technique, even if the cows are adapted to the substrate under investigation,
- The main predictors of CH_4 production by dairy cattle are related to diet composition (NDF, CP and OM),

- *In vitro* CH_4 production varies based on whether donor cows are adapted to a respective ration or not although the differences observed in thesis were minor.

REFERENCES

Bannink, A., Smits, M.C.J., Kebreab, E., Mills, J.A.N., Ellis, J.L., Klop, A., France, J., Dijkstra, J., 2010. Simulating the effects of grassland management and grass ensiling on methane emission from lactating cows. J. Agric. Sci. 148, 55-72.

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci .85, 1990-1996.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement. Aust. J. Exp. Agric. 48, 21-27.

Beever, D.E., 1993. Rumen function. In: J. M. Forbes and J. France (eds.), Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford. 187-215.

Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., Petit, H.V., D. I. Massé, D.I., 2014. Methane production, digestion, ruminal fermentation, nitrogen balance, and milk production of cows fed corn silage-based diets. J. Dairy Sci. 97, 961-974.

Bhatta, R., Tajima, K., Takusari, N., Higuchi, K., Enishi, O., Kurihara, M., 2007. Comparison of *in vivo* and *in vitro* techniques for methane production from ruminant diets. Asian-Aust. J. Anim. Sci. 7, 1049-1056.

Boadi, D., Benchaar, C., Chiquette, J., Massé, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows update review: Can. J. Anim. Sci. 84, 319-335.

Bodas, R., Lopez, S., Fernandez, M., Garcia-González, R., Rodriguez, A.B., Wallace, R.J., Gonzalez, J.S., 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Anim. Feed Sci. Technol. 145, 245-258.

Cammell, S.B., Sutton, J.D., Beever, D.E., Humphries, D.J., Phipps, R.H., 2000. The effect of crop maturity on the nutritional value of maize silage for lactating dairy cows. 1. Energy and nitrogen utilization. Anim. Sci. 71, 381-390.

Carula, J.E., Kreuzer, M., Machmuller, A., Hess, H.N., 2005. Supplementation of *acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-feed sheep. Aust. J. Agric. Res. 56, 961-970.

Chaban, B., Ng, S.Y., Jarrell, K.F., 2006. Archaeal habitats - from the extreme to the ordinary. Can. J. Microbiol. 52, 73-115.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. 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.

Cone, J.W., van Gelder, A.H., Soliman, I.A., de Visser, H., van Vuuren, A.M., 1999. Different techniques to study rumen fermentation characteristics of maturing grassland grass silage. J. Dairy Sci. 82, 957-966.

Cone, J.W., van Gelder, A.H, Bachmann, H., 2002. Influence of inoculum source on gas production profiles. Anim. Feed Sci. Technol. 99, 221-231.

Dijkstra, J., Kebreab, E., Bannink, A., France, J., Lopez, S., 2005. Application of the gas production technique to feed evaluation systems for ruminant. Anim. Feed Sci. Technol. 123, 561-578.

Dijkstra, J., Elis, J.L., Kebreab, E., Strathe, A.B., Lopez, S., France, J., Bannink, A., 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol. 172, 22-33.

Ettle, T., Lebzien, P., Flachowsky, G., Schwarz, F.J., 2001. Effect of harvest date and ruminal degradability of ensiled maize grains in dairy cows. Arch. Anim. Nutr. 55, 69-84.

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds. Anim. Feed Sci. Technol. 72, 261-281.

Getachew, G., Robinson, P.H., DePeters, E.J., Taylor, S.J., Gisi, D.D., Higginbotham, G.E., 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.

Hassanat, F., Gervais, R., Julien, C., Massé, D.S., Lettat, A., Chouinard, P.Y., Petit, H.V., Benchaar, C., 2013. Replacing alfalfa silage with corn silages in dairy diets: Effects on enteric methane production, rumen fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 4553-4567.

Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., Dijkstra, J., 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. Anim. Feed Sci. Technol. 202, 20-31.

Hatew, B., Bannink, A., van Laar, H., de Jonge, L.H., Dijkstra, J., 2016. Increasing harvest maturity of whole-plant corn silage reduces methane emission of lactating dairy cows. J. Dairy Sci. 99, 354-368.

Heeren, J.A.H., Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Bannink, A., Warner, D., de Jonge, L.H., Dijkstra, J., 2014. Rumen degradation characteristics of ryegrass herbage and ryegrass silage are affected by interactions between stage of maturity and nitrogen fertilization level. Anim. Prod. Sci. 54, 1263-1267.

Holtshausen, L., Liestøl, S.H.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on *in vitro* methane production from ensiled grass. Acta Agric. Scand. A Anim. Sci., 60, 40-45.

Hoover, W.H., 1978. Digestion and absorption in the hindgut of ruminants. J. Anim. Sci. 46, 1789-1799.

Hristov, A.N., Oh, J., Frikins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B., Tricarico, J.M., 2013. Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91, 5045-5069.

Huyen, N.T., Desrues, O., Alferink, S.J.J., Zandstra, T., Verstegen, M.W.A., Hendriks, W.H., Pellikaan, W.F., 2016. Inclusion of sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations affects nutrient digestibility, nitrogen utilization, energy balance, and methane emissions. J. Dairy Sci. 99, 1-13.

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system. Anim. Feed Sci. Technol. 50, 230-237.

Johnson, L., Harrison, J.H., Hunt, C., Shinners, K., Dogget, C.G., Sapienza, D., 1999. Nutritive value of corn silage as affected by maturity and mechanical processing: A contemporary review. J. Dairy Sci. 82, 2813-2825.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., Tricarico, J.M., 2014. Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97, 3231-3261.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351-365.

Makkar, H.P.S., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins and strategies to overcome detrimental effects of feeding tannin-rich feeds- a review. Small Rum. Res. 49, 241-256.

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to methanogens. Aust. J. Exp. Agric. 4, 7-13.

McGeough, E.J., O'Kiely, P., Foley, P.A., Hart, K.J., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, and performance of finishing beef cattle offered maize silages harvest at 4 different stages of maturity. J. Anim. Sci. 88, 1479-1491.

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

Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011a. A novel method to determine simultaneously methane production during the *in vitro* gas production using a fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Pellikaan, W.F., Stringano, E., Leenaars, J., Bongers, D.J.G.M., van Laar-van Schuppen, S., Plant, J., Mueller-Harvey, I., 2011b. Evaluating effects of tannins on extent and rate *in vitro* gas and methane production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166-167, 377-390.

Philippeau, C., Michalet-Doreau, B., 1997. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. Anim. Feed Sci. Technol. 68, 25-35.

Phipps, R.H., Sutton, J.D., Beever, D.E., Jones, A.K., 2000. The effect of crop maturity on nutritional value of maize silage for lactating dairy cows. 3. Food intake and milk production. Anim. Sci. 71, 401-409.

Pirondini, M., Colombini, S., Mele, L., Malagutti, L., Rapetti, G., Galassi, G., Crovetto, G.M., 2015. Effect of dietary starch concentration and fish oil supplementation on milk yield and composition, diet digestibility, and methane emissions in lactating dairy cows. J. Dairy Sci. 98, 357-372.

Randby, A.T., Weisbjerg, M.R., Nørgaard, P., Heringstad, B., 2012. Early lactation feed intake and milk yield responses of dairy cows offered grass silages harvested at early maturity stages. J. Dairy Sci. 95, 304-317.

Rinne, M., Huhtanen, P., Jaakkola, S., 1997. Grass maturity effects on cattle fed silage-based diets. 2. Cell wall digestibility, digestion and passage kinetics. Anim. Feed Sci. Technol. 67, 19-35.

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

Theodoridou, K., Aufrére, J., Niderkon, V., Andueza, D., Le Morvan, A., Picard, F., Baumont, R., 2011. *In vitro* study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetation cycles. Anim. Feed Sci. Technol. 170, 147-159.

Warner, D., Dijkstra, J., Hendriks, W.H., Pellikaan, W.F., 2013. Passage kinetics of ^{13}C-labeled corn silage components through the gastro intestinal tract of dairy cows. J. Dairy Sci. 96, 5844-5858.

Warner, D., Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Bannink, A., Dijkstra, J., 2015. Effect of nitrogen fertilisation rate and regrowth interval of grass herbage on methane emission of zero-grazing lactating cows. J. Dairy Sci. 98, 3383-3393.

Warner, D., Hatew, B., Podesta, S.C., Klop, G., van Gastelen, S., van Laar, H., Dijkstra, J., Bannink, A., 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10, 34-43.

Yáñez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., O'Kiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z., Hristov, A.N., 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants — a review. Anim. Feed Sci. Technol. 216, 1-18.

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Felicidade

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Felicidade Margarida Macome was born on April 26, 1970 in Maputo, Mozambique where she attended elementary and high school. She obtained a BSc in Veterinary Medicine from Eduardo Mondlane University (Maputo, Mozambique) in 1995. From 1998 to 2006, she worked at the Angonia Research Station in Tete Province as a researcher. In February 2007, she was the recipient of a Brazilian International Research Centre Scholarship (CNPq) and obtained her MSc degree specializing in Animal Nutrition from Universidade Federal da Bahia in Salvador, Brazil. As part of her degree, she investigated the inclusion of palm kernel meal from biodiesel production in diets for lambs. In 2012 she joined a three month training course and obtained an international diploma in animal feeds from PTC+, Barneveld, the Netherlands which was financed by the Dutch organisation for internationalisation in education (NUFFIC). She resumed work at her home institution (Agricultural Research Institute, Mozambique at Directorate of Animal Science) and in 2013 she started her PhD studies at the Veterinary Faculty of Utrecht University in the Netherlands. During her PhD she was located at the Animal Nutrition group of Wageningen University & Research where she conducted studies to predict *in vivo* methane production by lactating cows using the *in vitro* gas production technique. The results of her PhD studies are presented in this thesis.

List of Publications

Peer Reviewed Scientific Publications

Macome, F. M., Pellikaan, W. F., Schonewille, J. T., Bannink, A., Van Laar, H., Hendriks, W. H., Warner, D., Cone, J. W. 2017. *In vitro* rumen gas and methane production of grass silages differing in plant maturity and nitrogen fertilisation, compared to *in vivo* enteric methane production. Anim. Feed Sci. Technol. 230:96-102.

Macome, F. M., Pellikaan, W. F., Hendriks, W. H., Dijkstra, J., Hatew, Bayissa., Schonewille, J. T., Cone, J. W. 2017. *In vitro* gas and methane production of silages from whole-plant corn harvested at four different stages of maturity and a comparison with *in vivo* methane production. J. Dairy Sci. 100:8895-8908.

Macome, F. M., Pellikaan, W. F., Hendriks, W. H., Warner, D., Schonewille , J. T., Cone, J. W. 2017. *In vitro* gas and methane production in rumen fluid from dairy cows fed grass silages differing in plant maturity, compared to obtained *in vivo* data. Journal of Animal Physiology and Animal Nutrition (submitted).

Macome, F. M., Cone, J. W., Hendriks, W. H., Huyen, N.; Schonewille , J. T., Pellikaan, W. F. 2017. Methane production of sainfoin (*Onobrychis viciifolia*) silage-based and grass silage-based total mixed rations *in vitro* compared to that of lactating cows. to Journal of Science of Food and Agriculture (submitted).

Conference and Symposia Proceedings

Macome, F. M., Cone, J. W., Pellikaan, W. F., Schonewille, J. T., Hendriks, W. H. 2014. *In vitro* research on the adaptation of the rumen microflora to grass silages differing in maturity. The 39th Animal Nutrition Research Forum, 3th of April, Utrecht, The Netherlands.

Macome, F. M., Hendriks, W. H., Dijkstra, J., Warner, D., Pellikaan, W. F., Cone, J. W., Schonewille, J. T. 2015. *In vitro* gas and methane production from grass silages differing in maturity and N-fertilisation levels using adapted and mixed rumen fluid. WIAS Science Day, 5th of February, Wageningen, The Netherlands.

Macome, F. M., Hendriks, W. H., Dijkstra, J., Warner, D., Pellikaan, W. F., Cone, J. W., Schonewille, J. T. 2015. Effect of grass silage differing in maturity and nitrogen fertilisation, on *in vitro* methane production. The 66th EAAP Innovation in livestock production from ideas to practice, 31st of August to 4 September, Warsaw, Poland.

Macome, F. M., Hendriks, W. H., Dijkstra, J., Warner, D., Pellikaan, W. F., Cone, J. W., Schonewille, J. T. 2015. Effect of grass silage maturity and level of feed intake, on *in vitro* gas and methane production. The 66th EAAP Innovation in livestock production from ideas to practice, 31st of August to 4 September, Warsaw, Poland.

Macome, F. M., Hendriks, W. H., Dijkstra, J., Warner, D., Pellikaan, W. F., Cone, J. W., Schonewille, J. T. 2016. *In vitro* fermentation of methane production of maize silages harvested at different maturities in rumen fluid, adapted to the maize silages or not. WIAS Science Day, 4th of February, Wageningen, The Netherlands.

Macome, F. M.; Dijkstra, J., Hendriks, W. H., Cone, J. W., Pellikaan, W. F., Schonewille, J. T. 2016. Relationship between *in vitro* and *in vivo* methane production measured from donor cows fed maize silage at different stages of maturity. The 41st Animal Nutrition Research Forum, 15th of April, Wageningen, The Netherlands.

Training and Supervision Plan

The Basic Package (3 ECTS)

WIAS introduction course	2013
Course on Philosophy of science and/or ethics	2014

Scientific Exposure (9 ECTS)

International conferences

66th EAAP Innovation in livestock production from Ideas to practise, Warsaw, Poland	2015

Seminars and workshops

WIAS Science Day, Wageningen, the Netherlands	2014
WIAS Science Day, Wageningen, the Netherlands	2015
WIAS Science Day, Wageningen, the Netherlands	2016
Animal nutrition research forum, Utrecht	2014
Animal nutrition research forum, Belgium	2015
Animal nutrition research forum, Belgium	2016

Presentations

In vitro research on adaptation of the rumen microflora to grass silages differing in maturity, Utrecht	2014
In vitro gas and methane production from grass silages differing in maturity and N-fertilisation levels using adapted and mixed rumen fluid, Wageningen poster presentation	2015
Effect of grass silage maturity and level of intake, on *in vitro* gas and methane production, Warsaw, Poland oral presentation	2015
Effect of grass silages, differing in maturity and nitrogen fertilisation, on *in vitro* methane production, Warsaw, Poland poster presentation	2015

In vitro Fermentation and methane production of maize silages harvested at different maturities in rumen fluid, adapted to maize silages or not, Wageningen, the Netherlands, poster presentation 2015

In vitro gas and methane production on adaptation of microflora to silages of maize harvested at different stages of maturity, Wageningen, the Netherlands, oral presentation 2016

In-Depth Studies (11 ECTS)

Disciplinary and interdisciplinary courses

PhD course in Ruminant nutrition and Forage Chemistry 2016

Advanced Statistics courses

Advanced statistics course design of experiment 2014

Advanced statistics of the life sciences 2015

Statutory Courses (3 ECTS)

Use of Laboratory Animals, Utrecht, the Netherlands 2013

Professional Skills Support Courses (4 ECTS)

Data management, Wageningen, the Netherlands 2013

Techniques for writing and presenting a scientific paper, Wageningen, the Netherlands 2014

Project and Time management, Wageningen, the Netherlands 2015

Information Literacy including Endnote, Wageningen, the Netherlands 2014

Research Skills Training (6 ECTS)

Preparing own PhD research proposal 2013

Supervising Practical and Excursions

Practical rumen evacuation for BSc 2014

Supervising Thesis (2 ECTS)

Major MSc thesis 2014

TOTAL: 38 ECTS

Colophon

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