Animal Feed Science and Technology 230 (2017) 96–102

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

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

F.M. Macome[a,b,*], W.F. Pellikaan[a], J. Th. Schonewille[b], A. Bannink[c], H. van Laar[d], W.H. Hendriks[a,b], D. Warner[a], J.W. Cone[a]

[a] Wageningen University & Research, Department of Animal Sciences, Animal Nutrition Group, PO Box 338, 6700 AH Wageningen, The Netherlands
[b] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, PO Box 80163, 3508 TD Utrecht, The Netherlands
[c] Animal Nutrition, Wageningen Livestock Research, Wageningen University & Research PO, Box 338, 6700 AH Wageningen, The Netherlands
[d] Trouw Nutrition, PO Box 220, 5830 AE Boxmeer, The Netherlands

ARTICLE INFO

Keywords:
Grass silage
In vitro
In vivo
Methane
Dairy cow

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.

Abbreviations: ADFom, acid detergent fibre excluding residual ash; aNDFom, neutral detergent fibre assayed with a heat stable amylase and excluding residual ash; CH_4, methane; CP, crude protein; DM, dry matter; GP, gas production; OM, organic matter; OMI, organic matter intake; DOM, degraded organic matter; DMI, dry matter intake; DOMI, digestible organic matter intake; R_{max}, maximum rate of gas or CH_4 production; A, A1, A2, is the asymptote of CH_4 and gas production in phase 1 and 2 respectively; B and B2, is the incubation time at which half of the asymptotic CH_4 or gas production in phase 2 is reached; C and C2, is the sharpness of the switching characteristics for the CH_4 profile and for that of the second phase of gas production; FPCM, fat- and protein-corrected milk; GEI, gross energy intake

* Corresponding author.
E-mail address: felicidade.macome@wur.nl (F.M. Macome).

http://dx.doi.org/10.1016/j.anifeedsci.2017.04.005
Received 29 June 2016; Received in revised form 4 April 2017; Accepted 5 April 2017

1. 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 (Moss et al., 2000; Rinne et al., 1997).

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; Dijkstra et al., 2005; Getachew et al., 1998). This *in vitro* approach however, 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). 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.

2. Materials and methods

2.1. 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 1.

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

Table 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[b]						
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.

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.

2.2. 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).

2.3. 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 heat-stable 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.

2.4. 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 $P \leq 0.05$.

3. Results

3.1. 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

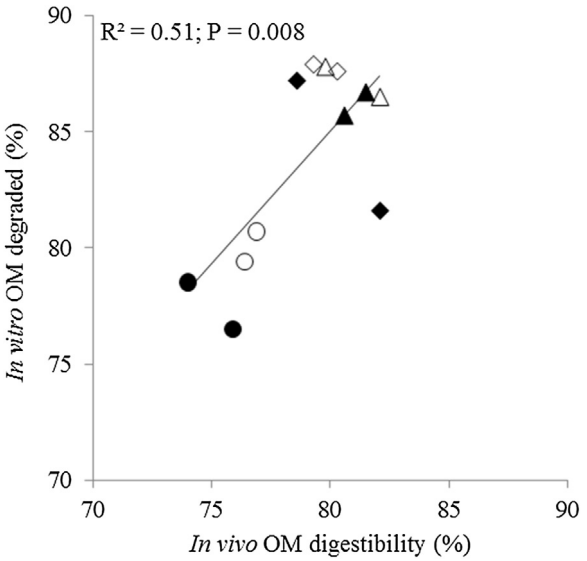

Fig. 1. Relationship *in vivo* OM digestibility and *in vitro* OM degraded (of silages of grass harvested at three stages of maturity (28, 41 and 62 days of regrowth; EM, MM and LM, respectively) and with two N fertilisation rates (65 kg of N/ha, LF; 150 kg of N/ha, HF). Δ = EM-LF; ◊ = MM-LF; O = LM-LF; ▲ = EM-HF; ♦ = MM-HF; ● = LM-HF.

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*

Table 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[b]										
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 phase 2, 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.

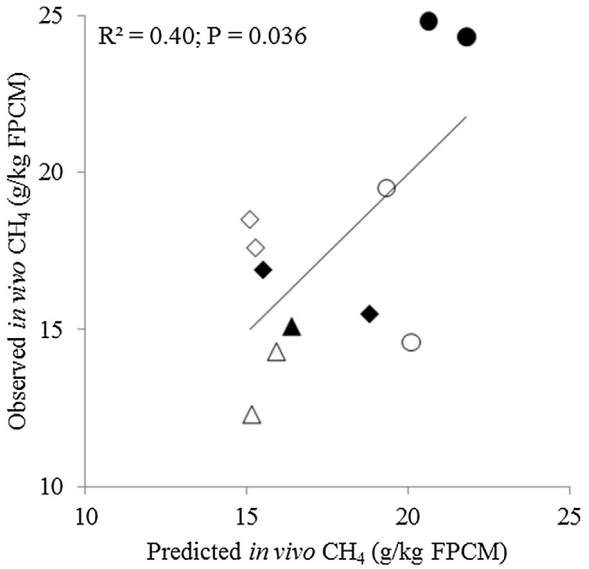

Fig. 2. Relationship between observed *in vivo* (CH_4 g/kg of fat-and protein- corrected milk (FPCM)) and predicted *in vivo* CH_4 (g/kg of FPCM) by the regression equation CH_4 g/kg FPCM = 66.87–0.589 × OMD of silages of grass harvested at three stages of maturity (28, 41 and 62 days of regrowth; EM, 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).

vitro degraded organic matter, and a positive relationship with *in vivo* OM digestibility was observed (R^2 = 0.51; P = 0.008; Fig. 1). The *in vitro* obtained GP data, being a measure for total fermentation of the different substrates are shown in Table 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.

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; Fig. 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.

3.2. *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). 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). 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).

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). Maturity of the grass silages only affected the 72-h CH_4 production when expressed as ml/g of OM (Table 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). 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) 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, that R_{max} for GP was 3.3% larger in the HF sampels, compared to LF, and for CH_4 the variation was 50.5% larger in HF compared to LF.

4. Discussion

4.1. 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 he 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.

4.2. 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 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) 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).

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). 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.

5. 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).

Conflict of interest

The authors declare to have no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the Islamic Development Bank for providing financial support to FMM for this research project, and the ministry of Economic Affairs, the Product Board Animal Feed and the Dutch Dairy Board who financed the trial of Warner et al. (2016) as part of the project Low Emission Animal Feed which provided the grass silages, adapted donor cows and *in vivo* observation on cow performance and methane emission in this paper.

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 grass and 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. Sect. A 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, 34–43.