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

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Summary

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 four 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/day vs. 15.4 kg/day). 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 oesophageal 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. The in vitro CH_4 production after 48 hr 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 = .00–.18, $p \geq .287$). 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.

KEYWORDS
gas production, grass maturity, in vitro, methane

1 | 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 & Johnson, 1995) and contributes to greenhouse gas emissions (Moss, Jouany, & Newbold, 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, Van Gelder, Visscher, & Oudshoorn, 1996; Dijkstra, Kebreab, Bannink, France, & Lopez, 2005; Getachew, Blümmel, Makkar, & Becker, 1998). This in vitro

approach can also be used to evaluate different feeding strategies for their potential to mitigate CH_4 production (Hatew et al., 2015; Holtshausen et al., 2012; Pellikaan et al., 2011). 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, Weisbjerg, Nørgaard, & Heringstad, 2012; Warner, Bannink, Hatew, Van Laar, & Dijkstra, 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 (FPCM). 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 this 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.

2 | MATERIAL AND METHODS

2.1 | 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 four cows, cows were assigned to an early lactation or a late lactation group, and within groups, cows were randomly assigned to one of four 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/day respectively). The experimental setup was designed for the in vivo measurements of CH_4 production by Warner et al. (2017). Cows in three periods with four cows in early lactation and three periods with four cows in late lactation were used as rumen fluid donors for the in vitro experiments.

Grass swards were harvested in 2013 at four different dates as grass maturity advanced: May 6th (G1), May 25th (G2), July 1st (G3) and July 8th (G4). Swards were composed of diploid perennial rye grass (*Lolium perenne*) cultivars of intermediate and late heading type (36% each) and timothy (*Phleum pratense*; 28%) (BG Superplus, Barenbrug, Oosterhout, the Netherlands) (Warner et al., 2017). Each grass was wilted for 2 or 3 days and ensiled in bales of approximately 500 kg using 12 layers of stretch plastic without addition of inoculants. Just before the in vitro incubations samples were taken with a hollow drill from different places in the bales, to make sure that a representative sample was obtained. 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.

2.2 | 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 four cows, provided that cows with the largest feed intake were never restricted to <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 four donor cows within each period for the in vitro measurements, resulting in four different types of rumen fluid per period. So, in total rumen fluid was taken from 24 cows and used for the in vitro experiments. Each grass silage was incubated with all four 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 three rations. Mixed 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 oesophageal tubing. A recent paper suggested that the rumen bacterial community composition does not differ between oesophageal tubing and rumen cannula sampling (Paz, Anderson, Muller, Kononoff, & Fernando, 2016).

2.3 | 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 mixed ration) was weighed into 250-ml fermentation bottles (Schott, Mainz, Germany) and 60 ml of buffered rumen fluid was added. Each grass silage or mixed ration was analysed in triplicate. In vitro incubation measurements lasted for 48 hr. Rumen fluid was obtained from four 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 flask, filled with CO_2. 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 three cows adapted to one of the three other grass silage rations (MRGS1 through MRGS4). Gas production curves were fitted with a multiphasic model as described by Cone et al. (1996) and Groot, Cone, Williams, Debersaques, and Lantinga (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 nine unknown parameters often gives non-physiological results, the values for A1 and A2 were fixed. The GP after 3 hr is assumed to be A1 and the GP between 3 and 20 hr is assumed to be A2, as described by Van Gelder et al. (2005). As the rations were comparable to each other, fixing the values of A1 and A2 was justified, with in vivo most probably identical passage rates. During the GP incubations, 10 μl of headspace gas was collected from the bottles at nine timepoints (0, 2, 4, 8, 12, 24, 28, 36 and 48 hr), 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 performed in triplicate over three different in vitro runs performed on different weeks. The in vitro CH_4 production data were fitted with a monophasic model (Groot et al., 1996). The maximum rate of the GP of the non-soluble fraction (R_{max2}, ml/hr) and of CH_4 production (R_{max}, ml/hr) was calculated as described by Bauer, Williams, Voigt, Mosenthin, and Verstegen (2001).

2.4 | Chemical analyses

Grass silage samples were dried at 70°C for 48 hr 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°C overnight (ISO 6496; ISO, 1999), and ash content was determined by incineration for 3 hr at 550°C (ISO 5984; ISO, 2002). Crude fat was determined according to ISO 6492 (ISO, 1999), and nitrogen was measured by the Kjeldahl method (AOAC, 1990), and crude protein (CP) was calculated as $N \times 6.25$. Neutral detergent fibre (NDF) was analysed according to Van Soest, Robertson, and Lewis (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, Van der Koelen, Valk, and De Visser (1993) with modifications by Abrahamse, Dijkstra, Vlaeminck, and Tamminga (2008), using 40% ethanol.

2.5 | Statistical analyses

Triplicate in vitro data were pooled per cow and substrate. All triplicate results for each substrate (i.e., grass silage and mixed 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 (four stages of grass maturity × four rumen inocula) by the mixed model procedures of SAS (2010). Substrate ($n = 4$; either grass silage or mixed 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 mixed rations were used as substrate for the in vitro measurements, two statistical models were used:

$$Y_{ijk} = \mu + \mathrm{SUB}_i + \mathrm{RF}_j + \mathrm{Block}_K + (\mathrm{SUB} \times \mathrm{RF})_{ij} + e_{ijk}$$

where Y_{ijk} = response variable (e.g., CH_4, GP, fermentation kinetics parameters), μ = overall mean, SUB_i = grass silage substrate ($i = 4$; maturity stages GS1 through GS4), RF_j = rumen fluid ($j = 4$; samples from four cows receiving GS1 through GS4), (SUB × RF) = interaction term and e_{ijk} = residual error.

$$Y_{ij} = \mu + \mathrm{SUB}_i + e_i$$

where Y_i = response variable (e.g., CH_4, GP, fermentation kinetics parameters), μ = overall mean, SUB_i = mixed ration substrate ($i = 4$; maturity stages MRGS1 through MRGS4)), and e_i = residual error.

Differences between treatment means were compared by the least squares means procedure and 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 was declared significant at $p \leq .05$. The PROC REG statement was used in SAS to predict the in vivo CH_4 production. 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 $p \leq .05$ and a trend at $.05 < p \leq .10$.

TABLE 1 Chemical composition of grass silages of increasing maturity stage at harvest (GS1 to GS4), maize silage, wheat straw and compound feed

	Grass silages*						
Component (g/kg DM)	GS1	GS2	GS3	GS4	Maize silage	Wheat straw	Compound feed†
DM (g/kg)	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
Neutral detergent fibre	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).

TABLE 2 In vitro gas production (GP) after 48 hr of incubation and curve fit parameters of grass silages made from grass differing in maturity at harvest (GS1 to GS4) and incubated with four different types of rumen fluid (RFGS1 to RGS2)

	Variables†					
Item*	GP (ml/g OM)	*A*1 (ml/g OM)	*A*2 (ml/g OM)	*B*2 (hr)	*C*2	R_{max2} (ml/hr)
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	<.0001	.001	<.0001	<.0001	<.0001	<.0001
RF	<.0001	<.0001	.001	<.0001	<.0001	.168
SUB × RF	.193	.972	.311	.962	.918	.031

*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 mixed 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.
Values within a column and item with different superscript (a, b, c) differ significantly ($p < .05$).

3 | RESULTS

3.1 | 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 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 also affected the

TABLE 3 In vitro methane (CH_4) production after 48 hr of incubation and curve fit parameters of grass silages differing in maturity at harvest (GS1 to GS4) and incubated with four different types of rumen fluid (RFGS1 to RFGS4)

	Variables†					
Item*	CH_4 (ml/g OM)	*A* (ml/g OM)	*B* (hr)	*C*	R_{max} (ml/hr)	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	.004	.186	<.0001	<.0001	.030	.071
RF	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
RF × SUB	.854	.894	.920	.990	.759	.363

*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 mixed 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; C is the sharpness of the curve; R_{max} is the maximum rate of CH_4 production.
Values within a column and item with different superscript (a, b, c) differ significantly ($p < .05$).

chemical composition of the different mixed rations (data not shown).

3.2 | 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 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 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 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. 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.

3.3 | In vitro gas and CH_4 production of the mixed rations

The in vitro GP, CH_4 production and fermentation characteristics of the incubated mixed ration samples are shown in Tables 4 and 5. Contrary to the grass silages, the mixed 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 (Tables 4). Parameter A1 decreased upon progressing grass maturity, indicating that less soluble sugars and 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 ($p < .0001$), showing a decreased R_{max2} as grass matured.

TABLE 4 In vitro gas production (GP) after 48 hr of incubation and curve fit parameters of mixed rations (MRGS1-MRGS4), containing grass silage made from grass differing in maturity at harvest

	Variables†					
Item*	GP (ml/g OM)	*A1* (ml/g OM)	*A2* (ml/g OM)	*B2* (hr)	*C2*	R_{max2} (ml/hr)
MRGS1	303.4^{a}	86.4^{a}	179.5	5.9^{b}	2.5	22.3^{a}
MRGS2	302.4^{a}	65.4^{ab}	194.9	7.1^{a}	2.7	21.1^{a}
MRGS3	268.8^{b}	50.8^{b}	177.8	7.4^{a}	2.6	18.1^{b}
MRGS4	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	<.0001	.009	.105	<.0001	.024	<.0001

*MRGS1-MRGS4 are the mixed 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.
Values within a column with different superscript (a, b, c) differ significantly ($p < .05$).

TABLE 5 In vitro methane (CH_4) production after 48 hr of incubation and curve fit parameters of mixed rations (MRGS1-MRGS4), containing grass silage differing in maturity

	Variables†					
Item*	CH_4 (ml/g OM)	*A* (ml/g OM)	*B* (hr)	*C*	R_{max} (ml/hr)	CH_4 (% of total gas)
MRGS1	49.3^{b}	52.4^{b}	8.2^{c}	1.6	4.1^{a}	16.2^{b}
MRGS2	55.0^{a}	60.8^{a}	10.3^{b}	1.5	3.7^{a}	18.2^{a}
MRGS3	45.5^{bc}	51.1^{b}	10.8^{b}	1.4	3.2^{b}	16.9^{b}
MRGS4	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	<.0001	.002	<.0001	.221	.002	.001

*MRGS1-MRGS4 are the mixed 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.
Values within a column with different superscript (a, b, c) differ significantly ($p < .05$).

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 5). In particular, parameter B increased, indicating a slower CH_4 synthesis with progressing grass maturity at harvest.

3.4 | In vivo CH_4 production

The in vivo data presented in Table 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 ($p = .083$).

4 | DISCUSSION

4.1 | 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 (Cone, Van Gelder, Soliman, De Visser, & Van Vuuren, 1999; Rinne, Huhtanen, & Jaakkalo, 1997). A decline in GP with progressing maturity using both grass silage and mixed 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 mixed 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

TABLE 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. Data extracted from Warner et al. (2017)

	Variables†			
Item*	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	.001	.001	.004	.002
SL	.389	.809	.837	.083

*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 organic matter intake (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.
Values within column with different superscript (a, b) differ significantly ($p < .05$).

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 mixed ration samples was affected by the grass maturity. An increase in fibre content of the more mature grass silages could increase the acetic acid production during fermentation in rumen fluid, making more hydrogen available for methanogens. However, a decreased total fermentation could also result in a lower total CH_4 production of the more mature grasses. 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, O'Brien, Boland, O'Donovan, and O'Kiely (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). More mature grass could lead to more methane during fermentation, caused by an increased NDF content, or to less methane because of a decreased total fermentation. We observed a small decrease in 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 and in vitro residues at the end of the incubation may reveal which hypothesis is correct in the current study.

In the current study, the origin of the rumen fluid showed an effect on the in vitro CH_4 production of the different grass samples. Total gas production varied 8.4% and methane production 21%, depending on the origin of the rumen fluid. This effect was visible even for the less extreme combinations, such as when a grass silage was incubated with rumen fluid from a donor cow not previously adapted to that diet, but adapted to a diet with a chemical composition coming close to it. These results suggest that adaptation to the respective diet is essential for in vitro GP and CH_4 fermentation studies.

4.2 | 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 ml/g OM incubated, was observed for any different incubation timepoint. The strongest correlation between in vitro and in vivo CH_4 production (g/kg OMI) was observed at 12 hr of in vitro incubation ($R^2 = .23$, $p = .226$).

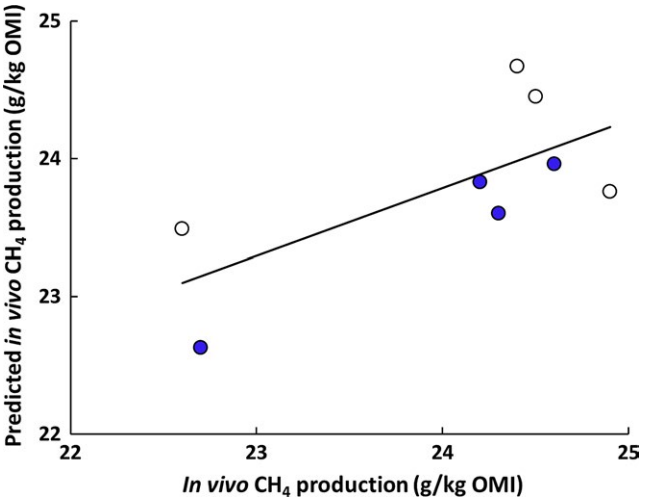

FIGURE 1 Relationship between observed in vivo CH_4 production and predicted CH_4 production, expressed in g/kg organic matter intake (OMI). Open symbols are for cows in early lactation (96 days in milk) and closed symbols for cows in late lactation (218 days in lactation). In vivo CH_4 (g/kg OMI) = − 9.68 − 0.233 × in vitro CH_4 + 1.88 × CH_4% + 8.55 × CH_4C (R^2 = .48), where in vitro CH_4 is total methane in ml/g OM incubated, CH_4% is the percentage CH4 in the total gas production, and CH_4C is the sharpness of the monophasic curve fitted to the in vitro CH_4 production [Colour figure can be viewed at wileyonlinelibrary.com]

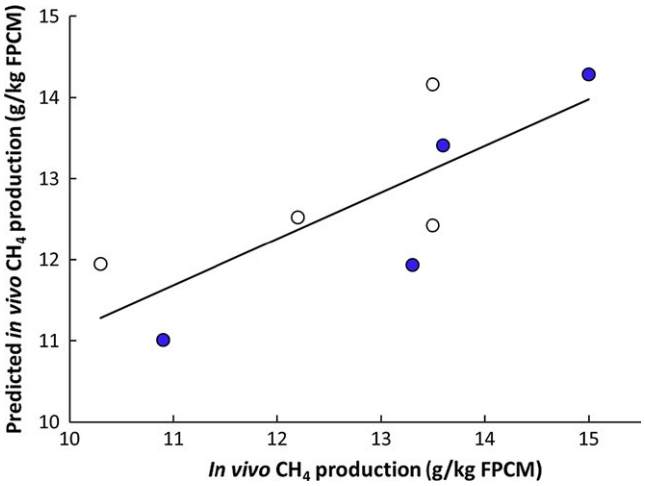

FIGURE 2 Relationship between observed in vivo CH_4 production and predicted CH_4 production, expressed as g/kg fat and protein corrected milk production (FPCM). Open symbols are for cows in early lactation (96 days in milk) and closed symbols for cows in late lactation (218 days in milk). In vivo CH_4 (g/kg FPCM) = 1.29 + 1.98 × in vitro CH_4% − 0.261 × CH_4A − 0018 × dry matter content (DM) (R^2 = .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 [Colour figure can be viewed at wileyonlinelibrary.com]

A relationship between 48 hr in vitro and in vivo CH_4 production (g/kg of OMI or of DMI), using a single linear correlation, was lacking (R^2 = .00–.18, $p \geq .287$). Also, when the in vivo CH_4 production was expressed in g/kg FPCM, there was no correlation (R^2 = .06; p = .529) with the in vitro CH_4 production (ml/g 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 timepoint 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 timepoints (GS1 = 6.91 hr; GS2 = 8.01 hr; GS3 = 10.23 hr and GS4 = 10.46 hr), suggesting an effect of grass maturity on the timepoint 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 (Figures 1 and 2). The equations used to predict in vivo CH4 production based on in vitro parameters and the chemical composition of the grass silages were as follows:

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 = .48)

In vivo CH_4 (g/kg FPCM) = 1.29 + 1.98 × in vitro CH_4% − 0.261 × CH_4A − 0018 × DM (R^2 = .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 CH4% is the percentage of CH_4 in the total gas 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.

5 | 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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