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In vitro gas and methane production of silages from whole-plant corn harvested at 4 different stages of maturity and a comparison with in vivo methane production

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ABSTRACT

The current study investigated the relationship between in vitro and in vivo CH_4 production by cows fed corn silage (CS)-based rations. In vivo CH_4 production was measured in climate respiration chambers using 8 rumen-cannulated 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 2 blocks, and randomly assigned to 1 of the 4 total mixed rations (TMR) that consisted of 75% experimental CS, 20% concentrate, and 5% wheat straw [dry matter (DM) basis]. The experimental CS were prepared from whole-plant corn that was harvested at either a very early (25% DM), early (28% DM), medium (32% DM), or late (40% DM) stage of maturity. The 4 experimental TMR and the corresponding CS served as substrate in 2 separate in vitro runs (each run representing 1 block of 4 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 gas production (GP) and CH_4 production parameters was influenced by an interaction between substrate and origin of rumen fluid. In vitro measured 48-h GP was not affected by the maturity of whole-plant corn, irrespective whether CS alone or as part of TMR was incubated in adapted rumen inoculum. Incubation of the experimental TMR did not affect the kinetics parameters associated with gas or CH_4 production, but when CS alone was incubated the asymptote of GP of the soluble fraction was slightly decreased with increasing maturity of CS at harvest. In vitro CH_4 production expressed as a percent of total gas was not affected by the maturity of whole-plant corn 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 CS-based rations.
Key words: methane, corn silage, maturity, in vitro, in vivo

INTRODUCTION

Whole-plant corn silage (**CS**) 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 CS largely depends on the content and degradability of the starch. The starch content, as well as the vitreousness of corn kernels, increases with maturity, and the fractional rate of ruminal starch degradation of corn decreases with maturity (Philippeau and Michalet-Doreau, 1997). The stage of maturity of the corn plant at harvest, therefore, has a significant effect on the nutritive value of CS, feed intake, milk yield (Johnson et al., 1999; Cammell et al., 2000; Warner et al., 2013) and 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 (Pellikaan et al., 2011; Holtshausen et al., 2012; Hatew et al., 2015). Currently, only a limited number of studies are available reporting in vivo CH_4 production of cattle upon changes in maturity of whole-plant corn at harvest (Cammell et al., 2000; Mc Geough

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et al., 2010; Hatew et al., 2016), and a dearth of direct in vitro-in vivo comparisons exist 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 our knowledge, however, a lack of studies have addressed the issue of adaptation of rumen inoculum with CS-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 corn harvested at 4 different stages of maturity. We hypothesized that in vitro CH_4 production is related to the in vivo CH_4 production when 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 of the 28 cows in the latter study (Hatew et al., 2016) had a permanent rumen cannula, and these 8 animals served as donors of rumen fluid for the current in vitro incubations. Housing, animals, feeding regimens, 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 1 of the 4 TMR that consisted of 75% experimental CS, 20% concentrate, and 5% wheat straw (DM basis). The experimental CS were harvested in 2013 at 4 different stages of maturity; that is, very early (September 20, 25% DM; **CS25**), early (September 28, 28% DM; **CS28**), medium (October 9, 32% DM; **CS32**), or late (October 31, 40% DM; **CS40**). Five days before the start of the adaptation period, cows received a high-CS diet containing a nonexperimental corn silage. The 4 experimental TMR and the corresponding CS (Table 1) served as substrate in 2 separate in vitro runs (each run with 1 block of 4 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 between the 2 in vitro runs was 1 wk.

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 CS (n = 4) were used as sole substrates and incubated separately with each of the rumen fluid inocula types (n = 4). Furthermore, the CS-based TMR (n = 4) were used as substrate and incubated with rumen fluid from cows adapted to the

Table 1. Chemical composition of corn silages differing in maturity at harvest and of TMR; data are adopted from Hatew et al. (2016)

	Corn silage (CS)[1]				TMR[2]			
Parameter	CS25	CS28	CS32	CS40	TMRCS25	TMRCS28	TMRCS32	TMRCS40
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 of 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 corn was harvested at targeted DM contents of 25, 28, 32, or 40% for CS25, CS28, CS32, and CS40, respectively.

[2]Total mixed rations had corn silage:wheat straw:concentrate ratio of 75:5:20 (DM basis). TMRCS25, TMRCS28, TMRCS32, and TMRCS40 contained either CS25, CS28, CS32, or CS40, respectively.

[3]Number of days from planting until harvesting of the whole plant for ensiling.

[4]NA = not applicable.

corresponding TMR. The CS 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.

Gas and Methane Production

Gas production profiles of the experimental TMR and CS 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 prewarmed insulated flasks flushed with CO_2. Rumen fluid was then filtered through cheese cloth and subsequently mixed (1:2 vol/vol) 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 preflushed 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 microliters 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 GC 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 nonlinear least squares regression procedure was used (SAS, 2010) and the data were fitted according to the equation

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

where Y = cumulative gas or CH_4 production (mL/g of 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 triphasic 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, whereas phase 3 is assumed to be related to 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. Methane concentrations at each individual valve opening were then calculated, and cumulative CH_4 was calculated as the sum of the increase in headspace CH_4 concentration between 2 successive valve openings and the amount of CH_4 vented from the bottle.

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

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

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

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 of OM) was calculated as CH_4 (L/kg of 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

Table 2. Summary statistics of in vivo CH_4 production of the 8 lactating dairy cows that served as donors of rumen fluid for in vitro incubations[1]

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

[1]The data are extracted from Hatew et al. (2016).
[2]OMD = OM degraded.

of gas (L/mol) was calculated using the ideal gas law; that is, 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 CS was the sole substrate available for in vitro fermentation, data were analyzed by ANOVA using the PROC MIXED procedure (SAS, 2010) using the model:

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

where Y = response variable (CH_4 or GP), μ = overall mean, RUN_i = run (i = 1 to 2), SUB_j = substrate (j = 1 to 4; CS with maturity stage of whole-plant corn 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 1 of the 4 corn silages), $(SUB \times RF)_{jk}$ = interaction term, and e_{ijk} = residual error term. When TMR instead of CS was used as a substrate, the factors RF_k and $(SUB \times RF)_{jk}$ were excluded from the aforementioned 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, 2010). Linear regression analysis between in vitro CH_4 production (g/kg of OM incubated) and in vivo CH_4 production (g/kg of OM intake) 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, 2010). Throughout, the level of statistical significance was preset at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

RESULTS

Chemical Composition of the CS

Upon maturation of whole-plant corn, 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 of DM and were found to be negatively related to the NDF and ADF contents (Table 1). The contents of CP and ADL were similar across the different corn silages. The aforementioned changes in chemical composition of the different CS are mirrored by the corresponding TMR.

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 2). The greatest coefficients of variation of CH_4 production were calculated when in vivo CH_4 production was expressed either as grams per kilogram DMI or grams per kilogram of OM intake (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.

Corn Silage and In Vitro Gas Production

In vitro gas production (**GP 48-h**) and the related kinetics parameters (Table 3) were not affected ($P \geq 0.131$) by the interaction between maturity of corn plant at harvest and the dietary background of the rumen fluid (i.e., rumen fluid obtained from cows fed either TMR of CS25, CS28, CS32, or CS40). The maturity of corn plant at harvest did not affect the measured 48-h GP (Table 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 CS28 instead of CS40. 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 CS at harvest ($P = 0.009$), whereas the half time of maximum GP of the soluble fraction (B1) was found to be significantly shorter when CS25 and CS28 instead of CS32 and CS40 were incubated. The latter observations are in line with the greater ($P = 0.001$) rate of maximum gas produc-

tion of the soluble fraction (R_{max1}) after the incubation of 2 immature CS. 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 CS 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 CS at harvest ($P = 0.227$ and $P = 0.111$, respectively), but the maximum rate of GP related to the nonsoluble fraction (R_{max2}) increased with increasing maturity of CS ($P = 0.013$). The dietary background of rumen fluid had a significant effect on A2, B2, and R_{max2}. The time at which the maximum gas production of the nonsoluble fraction (TR_{max2}) was reached was not affected by the maturity of CS ($P = 0.625$), but the dietary background of rumen fluid had a significant effect on TR_{max2} ($P = 0.024$). The switching characteristic of the GP curve related to the nonsoluble fraction (C2) tended ($P = 0.062$) to be affected by the maturity of CS at harvest, whereas 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 effect on the curve of total GP.

The asymptote of gas production in phase 3 (A3) was not affected by maturity of whole-plant corn 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 corn 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 CS, 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 CS ($P = 0.029$), 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 CS nor the dietary background of the rumen fluid ($P \geq 0.127$).

CS 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 ($P \geq 0.381$) by the interaction between maturity of corn plant at harvest and the dietary background of the rumen fluid (Table 4).The 48-h CH_4 production expressed as milliliters per gram of OM tended ($P = 0.054$) to be affected by the maturity of whole-plant corn 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 corn at harvest. Moreover, the various kinetics parameters (A, B, C, R_{max}, and TR_{max}) related to CH_4 production did not differ ($P \geq 0.328$) among substrates. The dietary background of rumen fluid tended to influence CH_4 production, either expressed as milliliters per gram of OM or as a percent of total GP ($P = 0.071$ and $P = 0.087$, respectively), and the lowest values were found when CS was inoculated with rumen fluid from cows fed late-maturity CS. The asymptotic CH_4 production (A) and its associated half time (B) were not influenced ($P \geq 0.182$) by the dietary background of the rumen fluid. Likewise, R_{max} and TR_{max} were not affected ($P \geq 0.204$) 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 had minor effect on the CH_4 production curve and are therefore considered not relevant.

TMR and In Vitro Gas and Methane Production

Incubation of the experimental TMR (Table 5) neither affected ($P \geq 0.406$) 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 3 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 CS. In all 3 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 milliliters per gram of OM or as a percent of total GP, and the associated kinetics parameters were found to be similar ($P \geq 0.143$) among the experimental TMR (Table 5).

Relationship Between 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 6, Figure 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 ($R^2 = 16\%$) when

Table 3. In vitro gas production and associated kinetics parameter estimates for main effect of substrate (SUB; corn 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 corn silage in question)

	SUB[2]				Dietary background of RF[3]					*P*-value		
Variable[1]	CS25	CS28	CS32	CS40	TMRCS25	TMRCS28	TMRCS32	TMRCS40	SEM	SUB	RF	SUB × RF
mL/g of 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
Hour												
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 of OM per hour												
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
Hour												
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

[a–c]Values within a row and within SUB and RF with different superscript letters differ significantly at $P \leq 0.05$.

[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, 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 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, CS25, CS28, CS32, and CS40 = corn silage made of whole-plant corn harvested at a targeted DM content of 25, 28, 32, and 40%, respectively.

[3]Main effect of rumen fluid, TMRCS25, TMRCS28, TMRCS32, and TMRCS40 = rumen fluid from donor cows fed TMR containing 75% (DM basis) of CS25, CS28, CS32, and CS40, respectively.

Table 4. In vitro methane production and associated kinetics parameter estimates for main effect of substrate (SUB; corn 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 corn silage in question)

	SUB[2]				Dietary background of RF[3]					*P*-value		
CH_4 48-h[1]	CS25	CS28	CS32	CS40	TMRCS25	TMRCS28	TMRCS32	TMRCS40	SEM	SUB	RF	SUB × RF
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 of 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 of OM per hour)	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 h of incubation (mL/g of OM) and relative methane production expressed as a percent of GP 48-h, the cumulative gas production measured after 48 h 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, CS25, CS28, CS32, and CS40 = corn silage made of whole-plant corn harvested at a targeted DM content of 25, 28, 32, and 40%, respectively.

[3]Main effect of rumen fluid, TMRCS25, TMRCS28, TMRCS32, and TMRCS40 = rumen fluid from donor cows fed TMR containing 75% (DM basis) of CS25, CS28, CS32, and CS40, respectively.

12-h in vitro incubation results were regressed against in vivo CH_4 production, but the relationship (Figure 1B) was found to be nonsignificant ($P = 0.321$).

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 a dearth of direct in vitro-in vivo comparisons exist 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 CS-based rations.

Relationship Between In Vitro and In Vivo CH_4 Production

In contrast to expectations, the current data clearly showed a lack of relationship between in vivo and in vitro CH_4 productions despite the fact that identical dietary material was used and rumen fluid was obtained from fully adapted animals. Similarly, Hatew et al. (2015) showed that in vitro CH_4 production was not related to in vivo CH_4 production (both expressed in mL of CH_4/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 between 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 was, at least potentially, overestimated under in vitro conditions at 48 h. This is of particular interest because Hatew et al. (2016) reported that the effective rumen degradability of starch decreases with advancing maturity of the corn 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 unrealistic under practical feeding conditions. Using ^{13}C-labeled corn silage, Warner et al. (2013) reported rumen retention times (reciprocal of fractional passage rate) of 19 to 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

Table 5. In vitro gas production and fermentation parameters incubation of a TMR, incubated with inoculum obtained from cows fed a TMR with an identical ingredient composition. Rations were based on corn silages differing in maturity at harvest

	Treatment[2]					
Variable[1]	TMRCS25	TMRCS28	TMRCS32	TMRCS40	SEM	*P*-value
GP 48-h (mL/g of OM)	311.3	300.8	309.0	289.8	8.31	0.406
A1 (mL/g of OM)	63.1	55.7	48.2	44.3	9.18	0.570
A2 (mL/g of OM)	188.3	189.6	199.9	193.3	1.95	0.070
A3 (mL/g of 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 of OM per hour)	26.1	17.9	25.2	14.9	8.06	0.695
R_{max2} (mL/g of OM per hour)	16.6	20.4	18.9	20.5	0.87	0.128
R_{max3} (mL/g of OM per hour)	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 of 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 of 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 of OM per hour)	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 h 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 phase 1, phase 2, and phase 3, respectively; R_{max1}, R_{max2}, and 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; CH_4 48-h = absolute cumulative methane production measured after 48 h of incubation (mL/g of OM) and relative methane production expressed as a percent 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]TMRCS25, TMRCS28, TMRCS32, and TMRCS40 = TMR containing corn silage (75% on DM basis) made from whole-plant corn harvested at targeted DM contents of either 25, 28, 32, or 40% of DM of corn silage, respectively.

vitro incubation times may provide better fits with in vivo CH_4 production. However, the relationship between in vivo and in vitro CH_4 production after 12 h of in vitro incubation of the substrates was also found to be nonsignificant, 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-Patino 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 between in vitro and in vivo CH_4 production.

Table 6. Slope, intercept, and R^2 of the linear relationships between in vivo CH_4 production (g/kg of OM intake) and in vitro CH_4 production measured at different time points (g/kg of 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

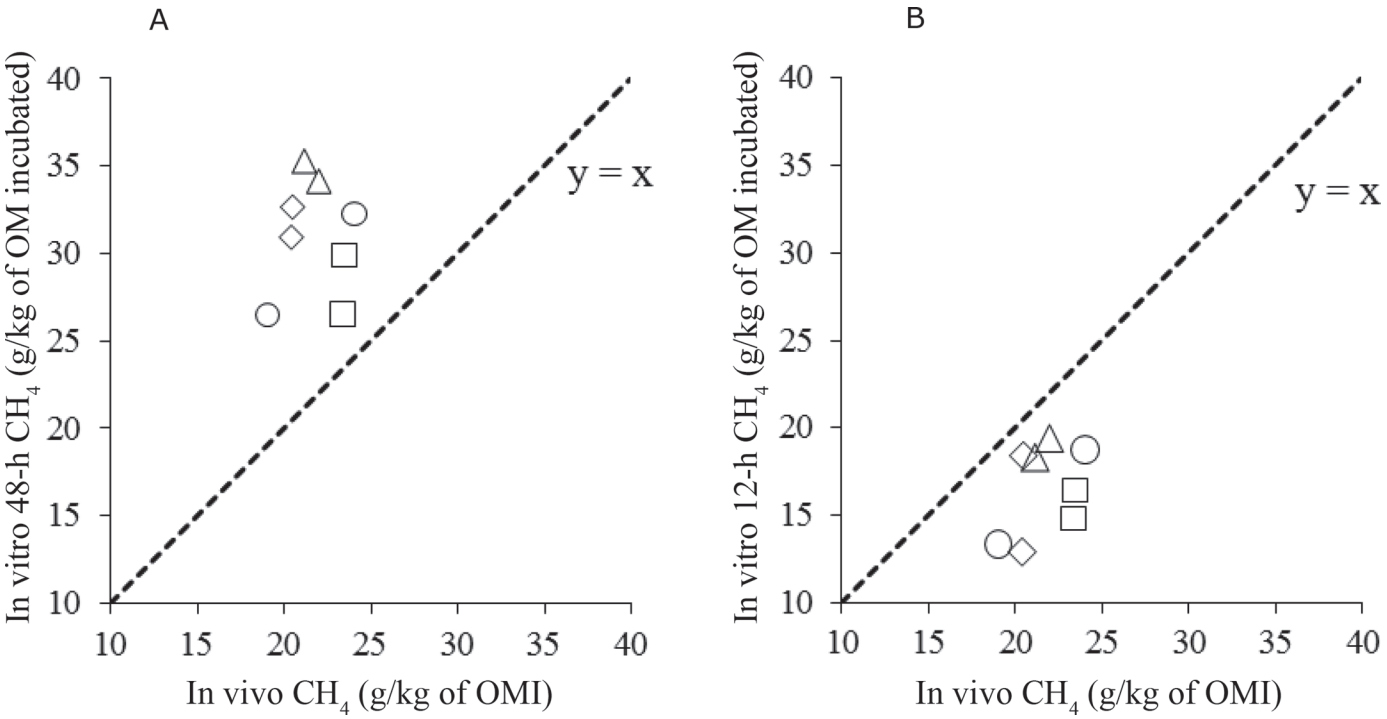

Figure 1. Relationships between in vivo [g/kg of OM intake (OMI)] and in vitro (g/kg of OM incubated) CH_4 production after 48 h (A; $R^2 < 0.01$, $P = 0.900$) and 12 h (B; $R^2 = 0.16$, $P = 0.321$) of incubation using adapted rumen fluid as in vitro inoculum and substrates identical to the rations fed during the in vivo experiment (Hatew et al., 2016). △ = TMRCS25; □ = TMRCS28; ◇ = TMRCS32; ○ = TMRCS40. The abbreviations TMRCS25, TMRCS28, TMRCS32, and TMRCS40 indicate TMR containing corn silage (75% DM basis) made from whole-plant corn harvested at targeted DM contents of 25, 28, 32, and 40%, respectively.

Maturity of Whole-Plant Corn at Harvest and In Vitro Gas and Methane Production

In line with results reported by Cone et al. (2008), the maturity of whole-plant corn at harvest did not affect cumulative 48-h GP. Thus, it appears that the in vitro degradability of the OM was not affected by the maturity of whole-plant corn at harvest. The lack of effect of maturity on GP at 48 h probably relates to the shift from NDF to starch when whole-plant corn matures. During the process of maturation of whole-plant corn, the corn kernels are filled with starch, thereby, shifting carbohydrates from NDF to starch (Bal et al., 2000; Phipps et al., 2000). Furthermore, both the digestibility of NDF and the fractional degradation rate of corn 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 corn 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, whereas the half time of maximum GP of the soluble fraction (B1) increased with increasing maturity. Furthermore, the maximum rate of gas production of the nonsoluble 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 corn 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 48-h GP) was not affected by the maturity of whole-plant CS at harvest, irrespective of CS or its corresponding TMR incubation time. 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 in-

fluenced by the maturity of whole-plant CS 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 CS.

Dietary Background of Rumen Fluid and In Vitro Gas and Methane Production

The lack of interaction between substrate (CS) 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 CS-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 when rumen fluid was adapted either to the substrate in question or not adapted. Therefore, we speculated that a sharp contrast in chemical composition among the incubated substrates 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 CS was incubated with rumen fluid that was obtained from cows adapted to TMR of CS25 and CS40.

In the current study, rumen fluid was collected 12 d after the onset of feeding the experimental TMR. This 12-d adaptation 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-CS diet for 5 d before receiving the CS of interest (Hatew et al., 2016). Thus, the rumen microbiota already had a (short) adaptation to a ration of this particular type (high CS) before the start of the experimental periods. Therefore, we anticipated that the process of adaptation to the experimental TMR was facilitated and that the results of the current in vitro measurements were not compromised by the relative short experimental periods.

CONCLUSIONS

In vitro 48-h gas and CH_4 production using adapted rumen inoculum was not affected by the maturity of whole plant corn. No statistically significant relationship was found between 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 CS-based rations.

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