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Evaluation of a gas in vitro system for predicting methane production in vivo

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ABSTRACT

Methane production from ruminant livestock varies with the diet as a result of factors such as dry matter intake, diet composition, and digestibility. To estimate the effect of dietary composition and feed additives, CH_4 production can be measured in vitro as a first step because large numbers of samples can be incubated and analyzed at the same time. This study evaluated a recently developed in vitro method for prediction of in vivo CH_4 production by examining the relationship between predicted and observed CH_4 production values. A total of 49 different diets (observations), used in previous 13 in vivo studies, were selected to include diets varying in nutrient composition. Methane production was measured in all in vivo studies by respiration chambers or the GreenFeed system (C-Lock Inc., Rapid City, SD). Overall, the in vitro system predicted CH_4 production well ($R^2 = 0.96$), but the values obtained were slightly underestimated compared with observed in vivo values (mean 399 L/d compared with 418 L/d: root mean square prediction error = 51.6 L/d or 12.3% of observed mean). Further analysis of the effect on residuals showed no significant relationship between CH_4 production and most factors known to affect CH_4 production such as dry matter intake, digestibility, and dietary concentrations of fat and starch. However, some factors included in the model were not well predicted by the system, with residuals negatively related to neutral detergent fiber concentration and positively related to concentrate proportion. The in vitro system can thus be useful for screening diets and evaluation of feed additives as a first step that can be best interpreted when feeding cows at maintenance level.

Key words: in vitro, in vivo, predicting methane production

INTRODUCTION

Methane is one of the major greenhouse gases. Dairy cows contribute CH_4 to the atmosphere due to microbial fermentation of feed in the rumen and hindgut. The production of CH_4 by ruminants also causes energy losses for the animal, corresponding to 2 to 12% of gross energy (**GE**) intake (Johnson and Johnson, 1995). The total amount of CH_4 released is dependent on several factors, such as DMI, type of feed, feed quality, and OM digestibility (Johnson and Johnson, 1995; Ramin and Huhtanen, 2013). Different strategies have been evaluated with the aim of reducing enteric CH_4 production and interesting possibilities are offered by feed supplements [e.g., dietary fat, ionophores, plant compounds, and enzymes (Beauchemin et al., 2009; Hook et al., 2010; Knapp et al., 2014)]. To evaluate the effect of dietary composition and feed additives, reliable measurement of CH_4 production is essential. Some common in vivo measurement techniques are available, all of which have advantages and disadvantages in terms of, for example, accuracy and cost. The most consistent is the respiration chamber technique, where the concentrations of CH_4 and CO_2 in air flux (L/min) are measured (Johnson and Johnson, 1995; Yan et al., 2010), but this technique is very costly and is not suitable for measurements on many animals at the same time. Another technique that can be used for on-farm measurements is the sulfur hexafluoride (SF_6) tracer technique (Johnson et al., 1994), which is based on the ratio of SF_6 to CH_4. Results obtained using this technique show higher variation than chamber values (Hammond et al., 2009). Yet another technique that can be used to estimate total daily CH_4 emissions of individuals is based on spot sampling over several days of breath in feed troughs in automatic milking systems or in concentrate feeders, such as the GreenFeed system (C-Lock Inc., Rapid City, SD) and the spot sampling method used by Madsen et al. (2010). These techniques may give higher variation than chamber techniques, but this can be compensated for by a large number of animals for measurements.

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However, in vivo studies are expensive and to reduce costs and effects on animals, various in vitro systems have been developed. Continuous culture experiments as described by Czerkawski and Breckenridge (1977) and batch culture experiments as reported by van Nevel and Demeyer (1981) are commonly used for evaluating the effects of diets and additives on enteric CH_4 production. Recently, Ramin and Huhtanen (2012) developed an in vitro method for prediction of CH_4 production in the rumen of cows using the kinetic parameters from an automated in vitro gas production (**GP**) system in a 2-compartment rumen model. This approach takes rumen dynamics (digestion kinetics) into account and may have advantages compared with single time point batch culture systems. However, in vitro techniques are applied to predict treatment effects in vivo, and it is therefore important that these techniques are reliable and well validated. In the review of Yáñez-Ruiz et al. (2016) where the designs, implementation, and interpretation of in vitro batch systems were reviewed, it was proposed that studies with direct comparisons between in vitro and in vivo systems would allow better interpretation of data and treatments suitable for evaluating the in vitro GP system. The aim of this study was to investigate the potential of the in vitro GP system for ranking different type of diets according to predicted CH_4 production, compared with in vivo CH_4 production values.

MATERIALS AND METHODS

Studies and Treatments

The diets for which in vitro GP and CH_4 production were predicted were selected from 13 different in vivo studies and consisted of 49 diets in total (Appendix). The majority of these diets were formulated based on a larger data set previously used to develop prediction equations for methane production (Ramin and Huhtanen, 2013). They were selected to include different dietary composition: feeding levels, proportion of concentrate, carbohydrate composition of concentrates, protein and fat supplementation, forage type, and maturity of forage (Table 1). Measurement of CH_4 production in the original in vivo studies was mainly performed in respiration chambers, with the exception of the study by Gidlund et al. (2015) where the GreenFeed system was used (C-Lock Inc.). In 4 cases, the original ingredients used in the in vivo studies (21 diets) were also used for the in vitro system. The in vitro diets for the remaining 9 of the 13 in vivo studies (28 diets) were formulated to be as similar as possible to the original feeds used in vivo in terms of ingredient composition and concentrations of ME, CP, and NDF. Digestibility of silage samples was determined either in vivo in sheep or in vitro. Ingredients for those 9 studies were provided by the Swedish University of Agricultural Sciences Research Centers in Umeå and Uppsala (Sweden), LUKE National Resources Institute (Finland), and the feed companies Raisio Ltd. (Raisio, Finland) and Teknosan (Vänersborg, Sweden).

Animals, Experimental Design, and Laboratory Procedures

The study was performed at the Swedish University of Agricultural Sciences in Umeå, Sweden. All handling of animals was approved by the Umeå Ethics Committee for Animal Research, Sweden. Three dairy cows of the Swedish Red breed in late lactation, fed a TMR (grass silage/concentrate ratio 600/400 g/kg on a DM basis) ad libitum, were used as donors of ru-

Table 1. Description of diets and type of animal used in in vivo studies for which original[1] or constructed diets were analyzed in vitro

Diet	Reference	Forage	Animal type[2]
1–4	Ferris et al., 1999	Grass silage and different proportions of concentrate	1
5–7	Keady and Mayne, 1998	Grass silage and different energy sources for concentrate	1
8–11	Beever et al., 1988	Grass silage cut at different date and barley substitution	3
12–15	Kirkpatrick et al., 1997	Different types of grass silage and different levels of concentrate	2
16–17	Gordon et al., 1995	Grass silage and different types of concentrate	1
18–20	Jentsch et al., 1972	Hay, different levels of added canola oil	1
21–23	Moss et al., 1995	Grass silage and different levels of barley	4
24–26	Moss and Givens, 2002	Grass silage and supplementation with soybean meal	4
27–28	Tyrrell et al., 1992	Direct-cut alfalfa or orchard grass ensiled	3
29–32	Brask et al., 2013a	Corn and grass silage and different physical forms of canola	1
33–38	Brask et al., 2013b	Early/late grass silage or corn silage, with/without canola oil supplement	1
39–45	Gidlund et al., 2015	Grass silage and soybean meal or canola meal	1
46–49	Hellwing et al., 2013	Grass silage and different types of treated wheat	1

[1]Original diets were also used in vitro for diets 29 to 49.

[2]1 = dairy cows; 2 = beef cattle; 3 = growing cattle; 4 = sheep.

Table 2. Nutritional content of replacement diets for constructed diets 1 to 28 (g/kg of DM unless otherwise stated)

		Forage					Concentrate					
Feed	n	DM	Ash	ME[1]	CP	NDF	DM	Ash	NDF	CP	Starch	Ether extract
1–4	4	—	—	10.6	156	563	905	59.2	214	255	109	4.28
5	1	369	107	10.6	187	427	892	66.9	276	227	15	29.0
6	1	369	107	10.6	187	427	882	49.1	194	223	95	22.7
7	1	369	107	10.6	187	427	867	31.4	112	224	191	13.9
8–9	2	—	—	10.6	156	563	—	—	—	—	—	—
10–11	2	537	63.9	9.5	101	568	891	32.1	145	130	589	—
12	1	389	103	10.4	154	413	—	—	—	—	—	—
13	1	389	103	10.4	154	413	893	36.7	142	183	518	2.4
14	1	537	63.9	9.5	101	568	894	38.7	140	207	486	3.5
15	1	537	63.9	9.5	101	568	893	36.1	142	177	526	2.1
16	1	389	103	10.4	154	413	884	45.8	123	280	201	35.7
17	1	389	103	10.4	154	413	862	62.3	323	184		57.8
18	1	—	—	12.2	179	380	897	46.2	133	294	369	7.4
19	1	—	—	12.2	179	380	904	50.8	121	357	246	57.6
20	1	—	—	12.2	179	380	912	58.0	109	451	96	95.9
21–24	4	335	102	10.9	130	424	891	32.1	146	130	589	—
25–26	2	335	102	10.9	130	424	907	69.7	112	567	—	19.7
27	1	367	130	8.0	170	414	—	—	—	—	—	—
28	1	436	136	9.0	211	507	—	—	—	—	—	—

[1]MJ/kg of DM.

men inoculum. Rumen fluid was collected 2 h after the morning feeding. The rumen fluid from these cows was strained separately through a double layer of cheesecloth into pre-warmed thermos flasks that had been flushed with CO_2, transported to the laboratory within 10 min after collection, and pooled on an equal volume basis. The pooled rumen fluid was strained through 4 layers of cheesecloth and mixed (20:80 vol/vol) with buffered mineral solution (Menke and Steingass, 1988) supplemented with peptone (pancreatic digested casein, Merck, Darmstadt, Germany) at 39°C under constant stirring and continuous flushing with CO_2.

Samples of silage and concentrate were dried at 60°C in a forced air oven for 48 h and milled through a 1-mm screen using a Retsch mill (Retsch, SM 2000, Rheinische, Haan, Germany). The samples were then stored in sealed glass jars at room temperature and used for chemical analysis. Conventional chemical analyses for DM, NDF assayed with heat-stable amylase and expressed exclusive of residual ash [amylase-treated neutral detergent fiber organic matter (**aNDFom**)], CP, starch (for concentrate), ash, and calculated ME were performed using standard methods as described by Bertilsson and Murphy (2003). Nutritional content for each replacement feed is presented in Table 2, and average nutrient composition for all replacement feeds used to reconstitute diets in Table 3.

Prior to in vitro incubation, 1,000-mg portions of substrate in accordance with the reference diet composition were weighed into serum bottles (250 mL, Schott, Mainz, Germany). All bottles were filled with 60 mL of buffered rumen fluid and placed in a water bath at 39°C for 48 h under continuous agitation. Each diet was randomly distributed in 3 runs out of a total of 5 runs. Two bottles with no substrate added were included as blanks in each run.

Total GP in the in vitro system was recorded with an automated system (Cone et al., 1996) with readings ev-

Table 3. Average nutrient content in replacement diets (g/kg of DM unless otherwise stated), n = 28

	Silage				Concentrate			
Item	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum
DM	401	76.8	335	537	893	12.5	862	912
Ash	98.2	20.49	63.9	136	47.5	14.25	31.4	69.7
ME	10.4	0.86	8.0	12.2	—	—	—	—
CP	150	29.2	101	211	165	56.4	109	323
NDF	474	73.8	380	560	248	130.6	130	567
Starch	—	—	—	—	321	231.7	15.0	589
Ether extract	—	—	—	—	21.3	25.93	2.10	95.9

ery 12 min, and corrected to normal air pressure (101.3 kPa). Mean blank GP within each run was subtracted from the sample GP. To analyze CH_4 production, gas samples were collected using a gas-tight syringe (1 mL, Hamilton, Bonaduz, Switzerland) from the headspace (**HS**) of each bottle after 2, 4, 8, 24, 32, and 48 h of incubation. Methane concentration was measured for each bottle by injecting 0.2 mL of HS gas into a gas chromatograph (Varian Chromatography, Palo Alto, CA). The gas chromatograph (Varian Chromatography) was equipped with a thermal conductivity detector. Separation was achieved using a 1-m-long stainless steel column packed with Haysept T (80 to 100 mesh), argon as the carrier gas with a flow rate of 32 mL/min, and an isothermal oven temperature of 32°C. The injector and detector temperature were set to 110 and 135°C, respectively. Calibration gas was completed using a standard mixture of CO_2 and methane (100 mmol/mol) prepared by AGA Gas (AGA Gas AB, Sundbyberg, Sweden). Peaks were identified by comparison with the standard gas. Methane production in vivo was predicted using a modeling approach described by Ramin and Huhtanen (2012). In brief, based on the kinetic data on CH_4 production obtained from in vitro GP, the cumulative CH_4 production (mL) at each time point (0.2 h) was calculated as

$$\begin{aligned}\text{total } CH_4 \text{ production (mL)} &= \text{HS volume (mL)} \\ \times \text{ HS } CH_4 \text{ concentration (mL/mL)} &+ \text{total GP (mL)} \\ \times \text{ A} \times \text{HS } CH_4 \text{ concentration (mL/mL)}&, \qquad [1]\end{aligned}$$

where the total HS volume per bottle and connected pressure tubes was 265 mL, and A is the ratio of CH_4 concentration in outflow gas (= measured GP) to CH_4 concentration in HS gas. Because the outflow gas could not be collected in the system, A was predicted using a mechanistic model, as described by Ramin and Huhtanen (2012), using stoichiometric principles and assuming that 1 mol of VFA released 1 mol of CO_2. Methane concentration at time intervals of 0.2 h was estimated by a logarithmic model of time versus CH_4 concentration. Kinetic parameters such as volume (V, mL/g of DM), lag (l, h), and rate (k, /h) for rapid and slow were estimated by fitting the 2-pool Gompertz function model to the CH_4 data (Schofield et al., 1994), using the NLIN procedure in the SAS program (version 9.3, SAS Inst. Inc., Cary, NC). The parameters were then subjected to a dynamic, mechanistic 2-compartment rumen model described by Huhtanen et al. (2008), which estimated the proportion of asymptotic CH_4 production at infinite time ($V_1 + V_2$) produced during the residence of feed in the rumen for a given time (CH_4-prop). Methane production (mL/g of DM) was calculated as CH_4-prop × asymptotic CH_4 production (mL/g of DM). The effective first-order CH_4 production rate was estimated by solving the 2-compartment equation described by Allen and Mertens (1988) for kd when digestibility (here proportion) and passage kinetic parameters are known. Passage rate (k_p) for each diet was calculated from NDF intake per kilogram of BW based on the empirical relationship between NDF intake as a proportion of BW and NDF passage rate derived from rumen evacuation data (Krizsan et al., 2010):

$$\begin{aligned}k_p \text{ (1/h)} = 0.0177 &+ \text{NDF intake (g/kg of BW)} \\ &\times 0.00076. \qquad [2]\end{aligned}$$

According to Cannas et al. (2003), the passage rate of concentrate particles is 1.6 times faster than the passage rate of forage particles. The passage rate for forage and concentrate can then be calculated from the passage rate of the whole diet and known proportions of forage (F-) and concentrate (C-) NDF in dietary NDF:

$$\begin{aligned}\text{forage } k_p \text{ (1/h)} = k_p \times (\text{C-NDF} + 1.6 \times \text{F-NDF}) \,/ \\ [1.6 \times (\text{C-NDF} + \text{F-NDF})],\end{aligned}$$

$$\text{concentrate } k_p \text{ (1/h)} = 1.6 \times \text{forage } k_p \text{ (1/h)}. \qquad [3]$$

Total retention time (**RT**; $1/k_p$) was divided between a nonescapable (large particles) and escapable pool (small particles) in the ratio of 20:80 for concentrates and 30:70 for forage (Danfær et al., 2006). Passage kinetic parameters for concentrates were used for the fast pool (V_1) and those for forage for the slow pool (V_2). Predicted mean RT for total diets in this study varied from 34.2 to 51.1 h. The model is programmed in Excel (Microsoft Corp., Redmond, WA): the simulations were run for 120 h with a 0.05-h integration step and using the model described by Huhtanen et al. (2008).

Statistical Analysis

Before analyzing the relationship between observed and predicted values, outliers were identified by PROC MIXED in SAS (version 9.3, SAS Institute Inc.) by comparing predicted values with values produced by the statistical model, taking into account feed effects and random run effects. Observations with residuals smaller or greater than 2 standard deviation units of the mean were removed from the data set. Least

squares means for each diet were estimated in PROC MIXED by the model:

$$\text{predicted } CH_4 = \text{run}_i + \text{diet}_j + e_{ijk}, \quad [4]$$

where $i = 3$ for run, $j = 49$ for diet, and e_{ijk} is a random error term. The relationship between predicted and observed CH_4 emissions was assessed using the linear regression technique (FIXED model). The relationship between predicted and observed values was also evaluated for a subset of data where original feeds from in vivo experiments were used in vitro ($j = 21$). The performance of the in vitro technique in predicting in vivo CH_4 emissions was further evaluated using the MIXED regression model procedure of SAS (Littell et al., 1996) with random study effect. The relationship between independent and dependent variables was estimated using the following model:

$$Y_{ijk} = B_0 + B_1X_{1ij} + b_0 + b_1X_{1ij} + e_{ijk}, \quad [5]$$

where B_0 and B_1X_{1ij} are fixed effects (intercept and effects of independent variables) and b_0 (intercept), b_1 (slope), and e_{ij} are random experiment effects ($i = 1, \ldots, 13$ studies and $j = 1, \ldots, n_i$ values). Root mean square prediction error (**RMSPE**) was calculated as

$$\text{RMSPE} = \sqrt{[\Sigma\ (\text{observed} - \text{predicted})^2/n]}. \quad [6]$$

The error was also expressed as a proportion of the observed mean to give an estimate of the overall prediction error. To center the predicted values, the overall predicted mean value was subtracted from each predicted value. This made the slope and intercept estimates orthogonal, and thereby independently assessable. Residual analysis was conducted as described by St-Pierre (2003) for CH_4 emissions, by regressing the centered predicted values against the residuals (observed-predicted CH_4 emissions). To estimate sources of bias, a regression analysis was conducted between the residuals of CH_4 emissions and variables identified as affecting CH_4 emissions, such as total DMI, OM digestibility, and dietary concentrations of NDF, starch, CP, and ether extract.

RESULTS

Diets

All diets tested in the study were assumed to cover differing dietary compositions, see Table 1. The mean DMI was 13.4 kg of DM/d and the concentration of NDF and CP was 396 and 182 g/kg of DM, respectively.

Prediction of CH_4 Emissions from the In Vitro System

All diets were run in 3 or 4 replicate incubations, resulting in a total of 167 observations for the 49 diets. Six of the observations were identified as outliers and removed from the data set, 4 values were single observations in a diet and 2 values were from the same diet.

The in vitro system slightly underestimated CH_4 production compared with observed in vivo values (399 L/d compared with 418 L/d; Table 4). The relationship between predicted and observed CH_4 emissions is shown in Figure 1. The following linear relationships were developed with the fixed regression model:

$$\text{observed } CH_4 \text{ (L/d)} = 0.92(\pm 0.034) \times \text{predicted } CH_4 \text{ (L/d)} + 49(\pm 15.6) \quad [7]$$

with $R^2 = 0.94$ and RMSPE = 51.6 L/d (12.3% of observed mean). When using the mixed regression model, the linear relationship was as follows:

$$\text{observed } CH_4 \text{ (L/d)} = 0.88(\pm 0.050) \times \text{predicted } CH_4 \text{ (L/d)} + 63(\pm 21.3) \quad [8]$$

with $R^2 = 0.96$ and RMSPE = 40.1 L/d (9.5% of observed mean). When predicted values were centered, the intercept was significant ($P \leq 0.01$) with the fixed model, but not ($P = 0.19$) with the mixed model. Slope bias was significant with both the fixed and mixed model ($P = 0.03$ and $P \leq 0.01$, respectively). When only the original feeds used in vivo were incubated in vitro and were evaluated separately by fixed regression model (Figure 2), the linear relationship was as follows:

$$\text{observed } CH_4 \text{ (L/d)} = 0.81(\pm 0.172) \times \text{predicted } CH_4 + 127(\pm 94.7) \quad [9]$$

with $R^2 = 0.54$ and RMSPE = 53.0 L/d (9.3% of observed mean).

Input Variables

Variables that affect CH_4 production the most, such as total proportion of concentrate, DMI, OM digestibility, and dietary concentrations of NDF, starch CP, and ether extract were tested against residuals of CH_4 emissions (Figure 3). For all variables tested, the proportion of concentrate ($P = 0.005$) and NDF ($P = 0.021$) was significantly related to residuals of CH_4 emissions, whereas other variables were not related to residuals.

DISCUSSION

The performance of the in vitro system examined in this study was evaluated by investigating the relationship between observed CH_4 emissions in vivo and predicted CH_4 emissions in a gas in vitro system. Predicted CH_4 correlated highly with observed in vivo values ($R^2 = 0.94$). The 49 diets used in this study represented differing dietary compositions and covered several feeding parameters that varied in terms of proportion of concentrate, starch, protein, fat, and maturity of forage, to investigate the effect of NDF, digestibility, and level of intake (see Tables 2 and 3).

In Vitro CH_4 Prediction

The major advantage of the in vitro system is that a large number of diets can be tested for predicted CH_4 emissions and ranked according to CH_4 production. The most promising diets or additives can then be selected for in vivo studies. Predicted CH_4 data from in vitro tests on different feedstuffs can also be used in calculations such as a life cycle assessment. Moreover, feed additives can be tested as a first step to evaluate potential effects on CH_4 production. In the study by Ramin and Huhtanen (2012), where GE concentration of the feed was assumed to be 18.5 MJ/kg of DM, it was found that the predicted in vitro CH_4 production, calculated as energy yield (**CH_4-E**), decreased from 7.8 to 6.0% of total GE intake with an increased amount of substrate (timothy hay) from 300 to 1,200 mg. These values are close to the observed in vivo values at maintenance and production levels of intake in dairy cows (Yan et al., 2000). Based on the predicted CH_4 production in the current study, it was possible to calculate the energy loss using the assumption that GE concentration was 18.5 MJ/kg of DM. This resulted in predicted CH_4 within the range 3.9 to 8.8% GE, which was expected.

When only original diets were analyzed, the prediction error was smaller compared with when reconstituted diets were also included in the analysis. The optimal approach would probably be to use only original diets from in vivo studies for the in vitro test, but it was difficult to get sufficient samples from in vivo respiration chamber studies. Even though more than half of the diets (28/49) had to be reconstituted, a robust relationship was still present between predicted and observed CH_4 production (R^2 for mixed model = 0.96) when all diets were analyzed.

Study Effect

The fixed and mixed model regression analyses were used to estimate the relationship between predicted CH_4 values from the in vitro system and observed CH_4 values. The RMSPE was smaller when the mixed model was used compared with the fixed model [9.5% (40.1 L/d) compared with 12.3% (51.6 L/d) of observed mean, respectively]. Furthermore, the mean bias was not significant when the mixed model was used, but with the fixed model the mean bias effect was significant ($P = 0.01$). All these findings suggest that the mixed model, where the random study effect was removed from the residual variance, should be used. This random study effect includes differences in calibration of chambers. In a recent study in the United Kingdom, Gardiner et al. (2015) analyzed the accuracy of respiration chambers and concluded that considerable errors in CH_4 measurement can occur unless appropriate validation is performed on a regular basis. Similarly, Ramin and Huhtanen (2015) found significant between-laboratory differences in the residuals of both CH_4 emissions and OM digestion in the mechanistic model Karoline predictions. Karoline is a dynamic and mechanistic model describing digestion and metabolism in dairy cows.

Table 4. Average concentration of dietary components and methane production in observed and predicted data

Item	n	Mean	SD	Minimum	Maximum
Diet composition, g/kg of DM					
CP	45	182	48	115	405
Ether extract	41	40.1	19.3	19	109
Starch	41	121	85	3.7	324
NDF	49	396	80	254	585
Feed intake, kg of DM/d	49	13.4	6.9	0.87	21.3
BW, kg	45	511	130	89.3	637
Diet digestibility, g/kg					
OM digestibility	49	767	53	591	910
Methane emissions, L/d					
Observed	49	418	207	35.8	664
Predicted	49	399	217	21	734

DMI

Residual analysis indicated a negative slope bias (i.e., observed CH_4 increased less than predicted). The slope bias can be due to increased efficiency of microbial protein synthesis with increased DMI (Broderick et al., 2010), which was not taken into account in predicted values. Simulations with the Karoline model have indicated that increased efficiency of microbial protein synthesis (g of microbial N per kg of OM truly digested in the rumen) markedly contributes to decreased CH_4 yield (g/kg of DMI) with increased DMI (Ramin and Huhtanen, 2015). Including the changes in microbial protein synthesis in the prediction model would probably reduce the slope bias between observed and predicted CH_4 emissions. Increases in the efficiency of

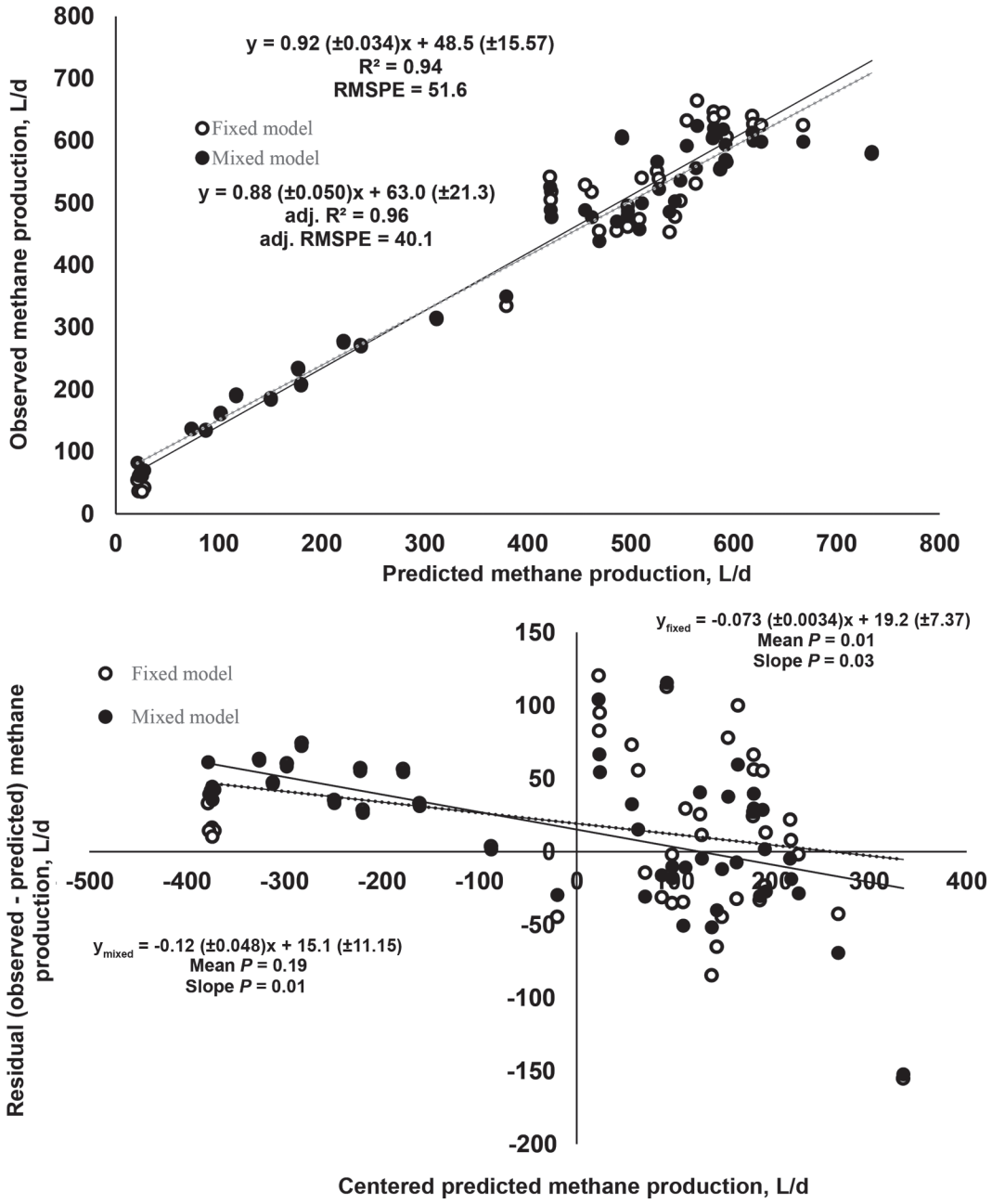

Figure 1. (Above) Relationship between predicted methane production in vitro, based on 48-h incubation, and observed methane production in vivo (L/d; n = 49), with fixed and mixed model regression analysis and (below) centered predicted values and residuals (observed − predicted) of methane production (L/d; n = 49). adj. = adjusted; RMSPE = root mean square prediction error.

microbial protein synthesis partition more fermented carbon to microbial cells, and consequently, less carbon is available for VFA and GP (Blaxter and Clapperton, 1965).

Increased DMI usually increases total CH_4 production (Johnson and Johnson, 1995; Ellis et al., 2007; Hristov et al., 2013; Ramin and Huhtanen, 2013), but CH_4 yield (g/kg of DMI) decreases with increased DMI (Blaxter and Clapperton, 1965; Yan et al., 2009; Ramin and Huhtanen, 2013). This decrease in CH_4 yield at higher feeding levels seems to be related to various factors, one of which may be that an increase in feeding level leads to increased passage rate, which reduces the digestibility of diets and the amount of H_2 produced per unit intake, which reduces CH_4 yield (Jentsch et al., 2007). In addition, in a recent modeling exercise, Huhtanen et al. (2016) indicated that improved efficiency of microbial synthesis was associated with lower predicted CH_4 yield with increased passage rate (shorter RT).

The RT of forage and concentrate in the present study was based on calculations by Cannas et al. (2003), assuming a 1.6-fold longer passage rate for forage than for concentrate. An increase in feeding level is accompanied by an increase in passage rate and therefore rumen RT for microbes is lowered. This increases the efficiency of microbial synthesis and the microbial cell yield (Janssen, 2010) and because microbial cells are more reduced than dietary carbohydrates (Hungate et al., 1971; Czerkawski, 1986), less H_2 is available for CH_4 production (Russell et al., 1992; Janssen, 2010). An alternative explanation is that when passage rate increases, the H_2 concentration in the rumen may increase at first, which in turn gives negative feedback to H_2-producing microbes, and thus H_2 production and in turn CH_4 production are lowered (Janssen, 2010).

An increase in feeding level has also been shown to change the fermentation pattern in the rumen [e.g., causing a decreased acetate to propionate ratio (Sveinbjörnsson et al., 2006)], accompanied by reduced formation of CH_4 due to the lower H_2 production from fermentation (Moss et al., 2000). Matching the sample size to intake could reduce the slope bias between observed and predicted CH_4 production because CH_4 yield decreases as the amount of substrates increases.

Residual Analysis

Residual analysis was used to obtain information on model performance by estimating the relationships between the residuals of CH_4 production and the most important input variables known to affect CH_4 production. A significant relationship between residuals and input variables indicates errors in the model structure for the corresponding object or linear bias in input data. The significant relationship observed between NDF (g/kg) and the residuals of CH_4 emissions may have arisen

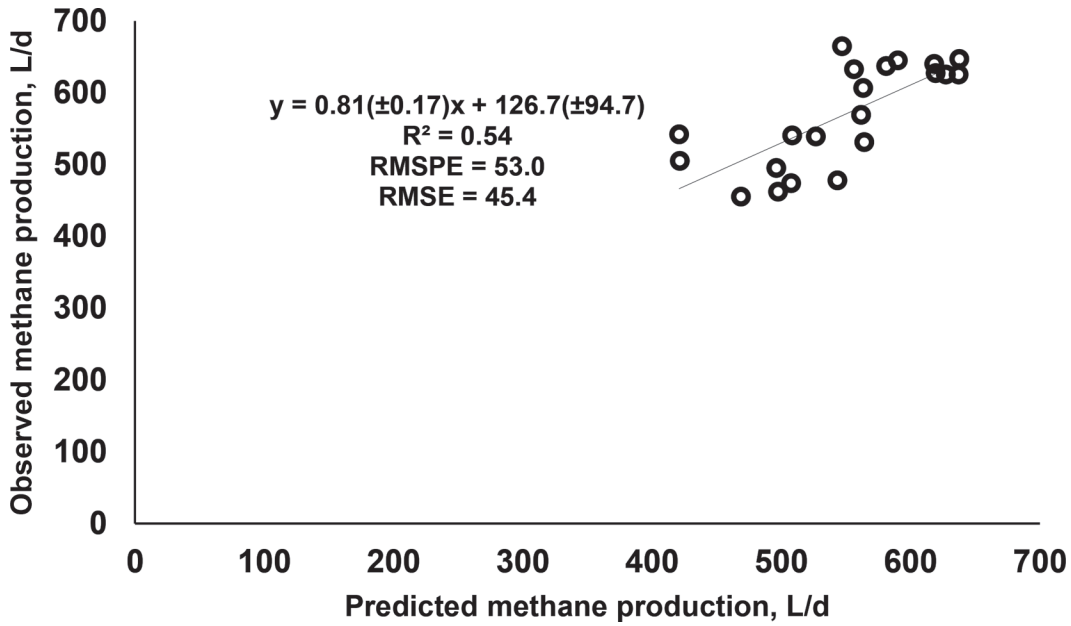

Figure 2. Relationship between predicted methane production in vitro, based on 48-h incubation, and observed methane production (L/d) for original diets from in vivo studies also used for in vitro tests (n = 21), based on fixed model regression analysis. RMSE = root mean square error; RMSPE = root mean square prediction error.

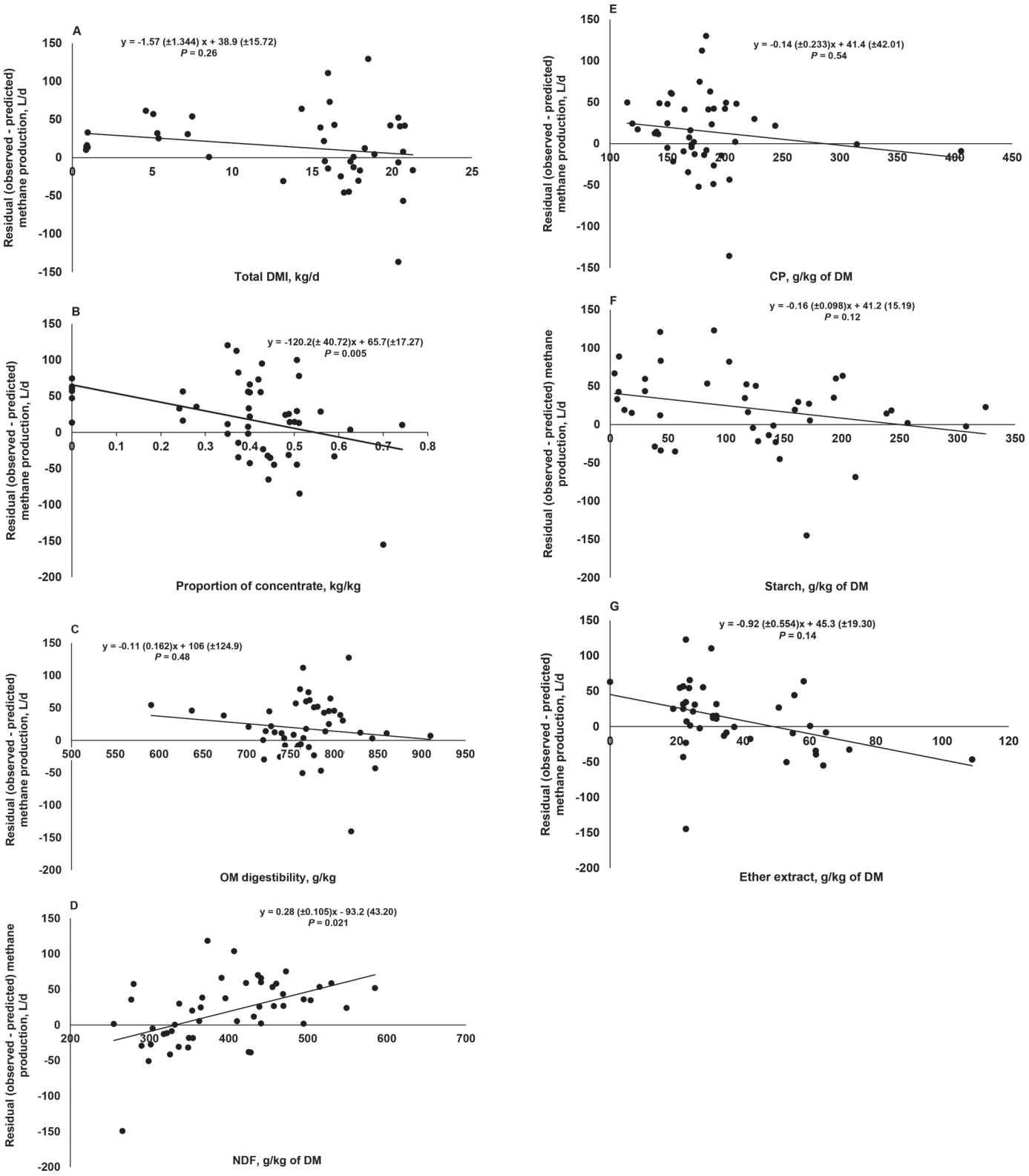

Figure 3. Relationship between input variables (proportion of concentrate, total DMI, OM digestibility, NDF, CP, starch, and ether extract) and residuals of methane production (observed methane production − predicted methane production).

because the NDF content in the feeds used in vitro was not precisely the same as in the original feed, which could have had an effect on the values.

The proportion of concentrate was significantly related to residual of CH_4 production. In addition, the significant relationship occurred because CH_4 production responses to proportion of concentrates were different between in vitro and in vivo. Decreases in CH_4 production are generally reported in vivo with higher propionate production, especially in growing cattle fed a high-concentrate (>90% on DM basis) feedlot diet (Johnson and Johnson, 1995; Beauchemin and McGinn, 2005), but some studies indicate increases. In the study by Beever et al. (1988), inclusion of barley in the diet increased CH_4 production, calculated as CH_4-E was highest with the highest proportion of barley. A recent study by Jonker et al. (2016) compared CH_4 production in vitro and in vivo in sheep fed diets in which alfalfa silage was replaced with increasing levels of starch-rich corn silage or corn grain. They found that an increase in dietary starch concentration up to around 200 g/kg in vivo (alfalfa silage:corn silage 50:50) increased CH_4 production, but thereafter higher starch concentration decreased CH_4 production. This response was not observed in vitro, where an increase in the concentration of starch linearly increased CH_4 production in that study. Those authors suggested that the differing results in vivo and in vitro might have been caused by a change in pH in vivo, whereas in vitro systems are highly buffered (Muetzel et al., 2014) and therefore less sensitive to changes in pH (Jonker et al., 2016). Furthermore, there seemed to be an interaction between concentrate proportion and feeding level in the study by Moss et al. (1995), where increased proportion of concentrate increased CH_4-E/GE numerically more at lower levels of intake. An increase in the concentrate proportion from 0 to 73% increased CH_4-E/GE from 8.3 to 11.3% at a feeding level around maintenance and from 7.4 to 8.4% when the level was 1.5 times maintenance (Moss et al., 1995). Differences in CH_4 responses with increasing concentrate levels between studies can be related to the effects of type of starch or concentrates on rumen fermentation pattern (Hristov et al., 2013; Guyader et al., 2016). Concentrates typically increase the proportion of propionate, but in the study by Moss et al. (1995) propionate actually decreased numerically and butyrate increased significantly. In addition, Murphy et al. (2000) observed no effect on propionate proportion of increasing concentrate proportion from 50 to 70%. Moreover, Patel et al. (2011) observed no difference in CH_4 production between diets with forage:concentrate ratio 50:50, 70:30, and 90:10, with the only observed difference in VFA proportions being in the proportion of butyrate, which was highest in the 50:50 diet. It appears that VFA pattern is considerably influenced by the degree of silage fermentation in grass silage diets (Huhtanen, 1998). Moreover, Friggens et al. (1998) added various supplements to grass-silage-based diets and found that increased starch level seemed to increase butyrate proportion rather than propionate proportion.

The overall increase in CH_4 production related to concentrate levels observed in the current study has also been observed in other in vitro studies; for example, Cattani et al. (2014) observed higher CH_4 production from concentrates than from forage. Similarly, in the study by Ramin and Huhtanen (2013), barley grain, oat grain, and sugar beet pulp produced more CH_4 than the 4 forages tested. The findings in the present study suggest that the in vitro GP method does not predict the effects of concentrate proportion of CH_4 production accurately (when the diets are fed at production level, in general, with a much higher level of feeding compared with feeding for maintenance), whereas a good relationship between in vivo and in vitro CH_4 production was found at feeding levels around maintenance, as also found by Moss et al. (1995).

Other Effects on Type of Feed In Vivo

The effect of diet on VFA pattern and CH_4 production is complex and it may not always be possible to predict CH_4 production accurately by in vitro methods because a particular type of diet may give different effects in different studies. For example, in studies evaluating the proportion of corn silage (increase in dietary starch content) in relation to CH_4 production and VFA pattern, the results vary widely, as reviewed by Guyader et al. (2016). In a study by Arndt et al. (2015), replacement of corn silage with alfalfa silage decreased the proportion of propionate without significant effects on CH_4 production. However, Brask et al. (2013) found that propionate proportion was much higher in corn silage diets than in grass silage diets, whereas the opposite was shown for acetate proportion, and that this effect on VFA was reflected in lower CH_4 production with corn silage diets. In another study examining gradual replacement of grass silage with corn silage, CH_4 production varied quadratically and was highest at intermediate levels of corn silage (van Gastelen et al., 2015). No difference was observed in the proportions of acetate and propionate, but the butyrate proportion increased with the level of corn silage inclusion, contradicting expectations. These differing results make it difficult to interpret whether increased levels of starch

reduce CH_4 production in a linear way, or whether there is a threshold for starch inclusion (Sauvant and Giger-Reverdin, 2009).

Fat supplementation of ruminant diets generally has an inhibitory effect on CH_4 production, but the degree of inhibition depends on type of diet, source of fat, type of fatty acids, and level of inclusion (Beauchemin et al., 2007; Beauchemin et al., 2008). Because fat is not fermented in the rumen, using fat instead of other fermentable sources reduces the amount of H_2 available for use in CH_4 production (Johnson and Johnson, 1995). Addition of fat to the diet increases the energy density, which may give less CH_4 per kilogram of product of milk or meat. Fat also has an antimicrobial action against protozoa and some cellulolytic bacteria, and the inhibition of cellulolytic microbes shifts in microbial populations, which may increase propionate production (Martin et al., 2010). Biohydrogenation of UFA can be an alternative sink for H_2, but incorporation of the H_2 produced is small compared with in methanogenesis (Czerkawski and Clapperton, 1984). In the present study, prediction of fat supplementation in vitro produced similar values to observed values.

Effect of In Vitro System

The overprediction of CH_4 production with increasing proportion of concentrate in the in vitro GP system may be due to an effect of inoculum, which may host a degree of variation within in vitro methodology (Mould et al., 2005). To minimize the bias effect of inoculum, the most appropriate method may be to use inoculum from donor animals that have been fed the same diet as tested in vitro, allowing adaptation of microbial flora. Although this may not be possible when testing new additives or many diets at the same time, it is important to be aware of the possible effect of the inoculum. In this study, the donor cows were fed a diet with a forage:concentrate ratio of 60:40, which is quite different from that in several of the diets tested and may have had an effect on the results. Another factor that influences the microbial community structure in the inoculum is the closed system in batch systems, which is different from the continuous system in vivo. The in vivo passage rate and VFA absorption also have a major effect (Dijkstra et al., 1993). In a previous study with a batch culture system, we observed that the relative abundance of *Prevotella* decreased and unclassified *Bacteriodales* increased over time in the in vitro system (Danielsson et al., 2014).

The relative abundance of the main genus *Prevotella* decreased over time and the relative abundance of unclassified *Bacteroidales* increased. Mateos et al. (2015) compared in vitro fermentation characteristics and bacterial diversity in vivo in sheep with a batch culture system and found a shift in microbial community composition, with a less diverse community in vitro. This shift in community structure may have an effect on the level of substrate degradation and fermentation pattern, which in turn may affect CH_4 production. However, the overall fermentation effect of forage was similar for both in vivo and in vitro experiments with sheep studied by Mateos et al. (2015). Composition of the medium used in vitro may affect ruminal fermentation and CH_4 production. It has been observed that highly buffered medium may increase acetate to propionate ratio compared with what happens in the rumen, which increases H_2 available for CH_4 production (Lana et al., 1998). In this study, the medium had an inclusion of 20% rumen fluid. The composition of HS gases CO_2, N_2, and H_2 may also have an effect on CH_4 production (Yáñez-Ruiz et al., 2016). In a study by Patra and Yu (2013), initial CO_2 HS was positively correlated with CH_4 production after fermentation. The higher CH_4 production may be explained by a direct and greater availability of CO_2 in the inoculum that acts as the electron acceptor for the main hydrogenotrophic methanogenesis pathway (Yáñez-Ruiz et al., 2016). This was taken into account in our modeling work (Ramin and Huhtanen 2012). The ratio of CH_4 in the outflow has been modeled to CH_4 concentration in the HS. It is suggested that further research are essential to understand the effect of a mixture of CO_2 and N_2 that best mimics rumen gas composition in vivo (Yáñez-Ruiz et al., 2016).

CONCLUSIONS

The in vitro GP system predicted CH_4 production with reasonable accuracy and precision. The residuals were not significantly related to most factors known to affect CH_4 production. However, the effect on CH_4 production of increased NDF concentration was underpredicted and the effect of increased proportion of concentrate was overpredicted. The in vitro GP system seems not to be applicable for evaluation of concentrate proportion, especially at high intake levels. The main area of application is screening of ingredients and assessing the effect of feed additives on methane production, digestibility, and digestion kinetics. However, any in vitro mitigation effect studies must be confirmed in vivo before practical application.

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APPENDIX

List of publications describing the diets used in the in vitro study.

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