Animal Feed Science and Technology 174 (2012) 190–200

Contents lists available at SciVerse ScienceDirect

Animal Feed Science and Technology

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

Development of an *in vitro* method for determination of methane production kinetics using a fully automated *in vitro* gas system—A modelling approach

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ARTICLE INFO

Article history:
Received 10 July 2011
Received in revised form 5 March 2012
Accepted 12 March 2012

Keywords:
Methane kinetics
Gas production
Rumen model
Sample size
VFA

ABSTRACT

The objective of the present study was to estimate methane production using the kinetic parameters from an automated *in vitro* gas production (GP) system in a mechanistic, dynamic rumen model. Four sample sizes [300 mg (5 g/l), 600 mg (10 g/l), 900 mg (15 g/l) and 1200 mg (20 g/l)] of timothy hay were incubated in 60 ml buffered rumen fluid in an automated *in vitro* system to determine methane and total gas production. A logarithmic model ($R^2 > 0.99$) was fitted to methane data to estimate methane concentrations at time intervals of 0.2 h. The first-order gas production rates were not different (P=0.18) with values of 0.072, 0.061, 0.061 and 0.059/h for the sample size of 300, 600, 900 and 1200 mg, respectively. The methane production rates were 0.052, 0.046, 0.046 and 0.045/h, respectively. Predicted methane production decreased linearly (PLIN < 0.01) as the sample size increased from 300 (36.9 ml/g dry matter, DM) to 1200 mg (28.2 ml/g DM). After 48 h of incubation total volatile fatty acids (VFA) production decreased (PLIN < 0.01) as the sample size increased from 300 to 1200 mg (4.41 mmol/g DM and 3.82 mmol/g DM, respectively). Neutral detergent fibre digestibility (aNDFomD), apparent organic matter digestibility (AOMD) and true organic matter digestibility (TOMD) decreased (PLIN = 0.01) as the sample size increased (0.479, 0.433 and 0.681 for 300 mg, and 0.369, 0.379 and 0.614 for 1200 mg of sample size, respectively). Actual methane production (24 and 48 h of incubation) was strongly correlated ($R^2 = 0.97$) with the methane production predicted from VFA stoichiometry (VFA measured at 24 and 48 h of incubation). It is concluded that *in vitro* GP measurements can be successfully used to estimate kinetic parameters of methane production and consequently to predict methane production. It seems that sample size did not affect the first-order production rate of methane and therefore, it is possible to use greater amounts of substrate in the *in vitro* GP system up to 1000 mg (16.6 g/l).

1. Introduction

Methane (CH_4) is the second most significant contributor to the 'greenhouse' effect by trapping 20 times more heat than carbon dioxide (CO_2) (Yan et al., 2010). Ruminants contribute to approximately one quarter of all anthropogenic methane

Abbreviations: aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; aNDFomD, aNDFom digestibility; aNDSom, neutral detergent soluble excluding ash; AOMD, apparent organic matter digestibility; CP, crude protein; DM, dry matter; GP, gas production; HS, headspace; MRT, mean rumen retention time; NDF, neutral detergent fibre; OM, organic matter; OMF, OM fermented; pdNDFD, potential digestible neutral detergent fibre digestibility; TDOM, truly digested organic matter; TOMD, true organic matter digestibility; VFA, volatile fatty acid.

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doi:10.1016/j.anifeedsci.2012.03.008

emissions (Beauchemin et al., 2008), but they also convert fibre and non-protein nitrogen to high quality human food. However, the production of useable end-products such as volatile fatty acids (VFA) and microbial biomass are associated in losses of the potent greenhouse gas methane (Van Soest, 1994).

Methane production during enteric fermentation in ruminant accounts for approximately 0.04–0.12 of the dietary gross energy intake (Johnson and Johnson, 1995). This energy is lost as eructated gas to the atmosphere and represents an energy loss to the animal (Holter and Young, 1992; DeRamus et al., 2003; Yan et al., 2010). The *in vivo* methods for measuring methane production are very laborious and expensive (respiration chambers) or possibly inaccurate (tracer methods) and difficult to standardise. Therefore, *in vitro* methods would be useful for screening the effects of diets and additives on methane production. Two types of *in vitro* methods have been used for measurements of methane production; continuous culture experiments as described by Czerkawski and Breckenridge (1977) and batch culture experiments reported by Van Nevel and Demeyer (1981).

In batch cultures, methane measurements are made with using end-point measurements, or occasionally two time points, and they do not take into account the dynamics of digestion and passage kinetics in the rumen. The automated *in vitro* gas production (GP) method is widely used to produce kinetics data of the degradation of feed or feed components (Pell and Schofield, 1993). Huhtanen et al. (2008) demonstrated that using the parameter values estimated from gas production kinetics data in a mechanistic dynamic rumen model predicted *in vivo* digestibility of potential digestible neutral detergent fibre (pdNDFD) accurately and precisely. Analysis of the kinetics of *in vitro* GP data used in the mechanistic rumen model also allowed the estimation of first-order digestion rate for pdNDF. Provided that methane measurements are made at different time points this approach could be used for predictions of methane production taking into account the dynamic nature of the rumen particle kinetics.

Usually, substrate to rumen fluid ratios are much smaller in the *in vitro* systems than *in vivo*, which can result in depletion of substrate leading to degradation of microbes. Methane measured *in vitro* may therefore not only represent methane production from the substrate. Therefore, the aims of the present study were to develop an application of the automated *in vitro* GP system in order to measure methane production over time for estimating kinetic parameters of gas production and secondly, to use the derived kinetic parameters in a mechanistic rumen model based on dynamics of digestion and digesta passage for predicting methane production at different sample sizes.

2. Materials and methods

2.1. Sample preparation

Sample of a low quality timothy hay (*Phleum pratense*) was dried at 60 °C in a forced air oven for 48 h, and milled through a 1-mm screen using a Retsch mill (Retsch, SM2000, Rheinische, Haan, Germany). The hay sample was then stored in sealed glass jars until required. The dry matter (DM) concentration of the hay was 956 g/kg and the DM contained (g/kg): ash 68.3, organic matter (OM) 932, crude protein (CP) 104, neutral detergent fibre (NDF) 570 and indigestible NDF (iNDF) 188. The concentration of iNDF was determined by a 12-d ruminal *in situ* incubation in Swedish Red cows (Huhtanen et al., 1994).

2.2. In vitro gas production measurements

Prior to the incubation, 300 mg (5 g/l), 600 mg (10 g/l), 900 mg (15 g/l) and 1200 mg (20 g/l) samples were weighed into serum bottles (Schott, Mainz, Germany, 250 ml). Four serum bottles were assigned as blanks without substrate and incubations were completed with five replicates for the first run and two replicates for the second run. In the second run (with two replicates for each sample size) samples were only drawn for the predictions of methane and total gas production. Whereas, in the first run samples were collected for the predictions of methane and total gas production, VFA (24 and 48 h of incubation) and digestibility measurements. For both runs the incubation was terminated after 48 h. One sample from gas production, four samples from VFA (2 for 24 h and 2 for 48 h) measurements, and one sample from digestibility measurements were lost of reasons (failure in gas recordings and double amount of buffer) not related to treatment, and not used for further analysis.

The study was conducted with the permission of the Swedish Ethical Committee on Animal Research. Rumen fluid was collected 2 h after morning feeding from three cannulated Swedish Red cows in early lactation fed on a grass silage based diet. The fluid was kept in pre-warmed thermos flasks that were previously flushed with CO_2. Rumen fluid was transported to the laboratory within 10 min, pooled, filtered through four layers of cheesecloth and flushed with CO_2. Filtered rumen fluid was then mixed with buffered mineral solution (Menke and Steingass, 1988) (20:80 v/v) supplemented with peptone (pancreatic digested casein) (Merck, Darmstadt, Germany) at 39 °C with constant stirring and continuous flushing with CO_2. All bottles were then filled with 60 ml buffered rumen fluid and placed in a water bath at 39 °C whilst gently and continuously agitated. The gas production measurement was conducted by a fully automated system, readings were done every 12 min and corrected to the normal air pressure (101.3 kPa) (Cone et al., 1996). The gasses are released from the system by opening of the electric gas valve. More details of the system were described by Hetta et al. (2003). Since we aimed to study methane production at different time points (2, 4, 8, 24, 32 and 48 h) and the collection of fluid from the fermentation unit over time for VFA determination, the modified tubes method described by Karlsson et al. (2009) was used in the system (T-tube). To facilitate the collection of samples from the headspace for methane measurements, an additional metal three-way valve was

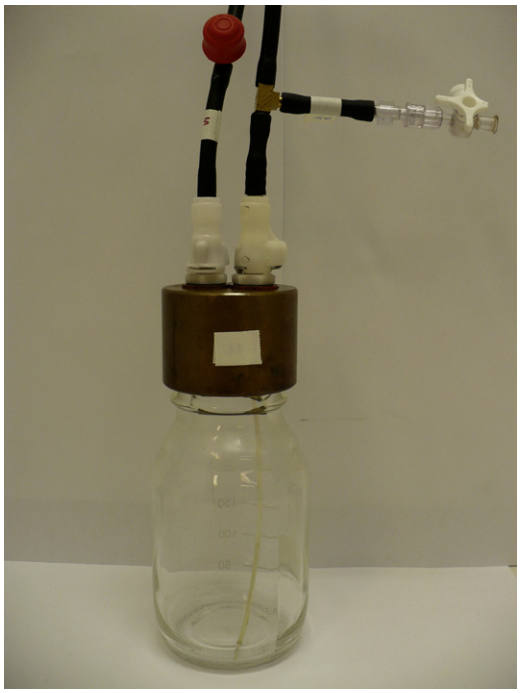

Fig. 1. Fermentation unit. The T-tube was used for liquid sampling (volatile fatty acids determination); the red rubber suba seal was used for gas sampling with a gas tight syringe. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

connected on the way of the other pressure tube leaving the serum bottle with a rubber suba seal septa (Z124567-100EA, 13, Sigma–Aldrich) attached to the third port (Fig. 1).

2.3. Gas sampling and methane measurement

Gas samples were drawn from each bottle by a gas tight syringe (Hamilton, Bonaduz, Switzerland) at 2, 4, 8, 24, 32 and 48 h of incubation through the rubber suba seal. Methane was determined by injecting 0.2 ml of gas into a star 3400 (CX series) gas chromatograph (Varian Chromatography, USA) equipped with a thermal conductivity detector (TCD). Separation was achieved using a 1.8 m long stainless steel column packed with Haysept T (80–100 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 temperatures were set to 110 °C 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.

2.4. VFA sampling and determination

One ml of rumen fluid was collected at 24 and 48 h of incubation from the bottles, mixed with 200 μl of 22 M formic acid (Cottyn and Bouque, 1968) and stored at −18 °C until processed for VFA determination. Sampled amount of CO_2 gas was flushed through the T-tube to each bottle in order to empty the tube to the content of the bottles. The VFAs were determined by high performance liquid chromatography (HPLC) (Ericson and Andre, 2010). The acids were separated with a packet ReproGel H column (Ammerbuch, Germany), and detected with a RI 2414 detector (Waters Assoc, USA).

2.5. In vitro apparent digestibility

At the end of the incubation (48 h of incubation), the bottles were put on ice in order to terminate the fermentation. The whole content of each bottle was transferred to pre-weighed plastic cups and freeze dried by Edwards freeze dryer (Crawley Sussex, England). Dry matter of residues of the whole bottles content after freeze drying were measured by drying at 105 °C for 16 h (Method 925.10, AOAC, 1990).

Apparent organic matter digestibility (AOMD) was calculated as mg organic matter fermented (OMF)/organic matter of substrate (mg) (Demeyer, 1991).

$$\mathrm{OMF(mg)} = (0.5\mathrm{AA} + 0.5\mathrm{PA} + \mathrm{BA} + \mathrm{iVA} + \mathrm{VA}) \times 162$$

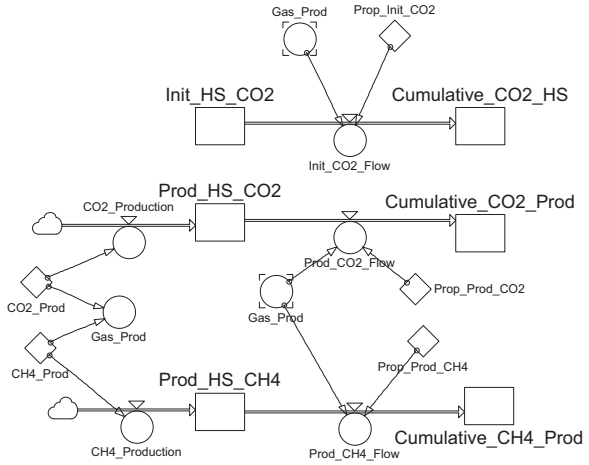

Fig. 2. A simplified model describing the prediction of methane concentration in gas outflow from the *in vitro* gas system. Init_HS_CO_2 = initial headspace CO_2 pool [1], Prod_HS_CO_2 = produced headspace CO_2 pool [2], Prod_HS_CH_4 = produced headspace CH_4 pool [3], Cumulative_CO_2_HS = cumulative outflow pool of initial headspace CO_2 [4], Cumulative_CO_2_Prod = cumulative outflow pool of produced CO_2 [5], Cumulative_CH_4_Prod = cumulative outflow pool of produced CH_4 [6], CO_2_Prod and CH_4_Prod = production of CO_2 and CH_4 from substrate fermentation according to stoichiometric principles, Gas_Prod = total gas production (CO_2_Prod + CH_4_Prod), Prop_Init_CO_2 = proportion of initial CO_2 in headspace, Prop_Prod_CO_2 = proportion of produced CO_2 in headspace, Prop_Prod_CH_4 = proportion of produced CH_4 in headspace, Init_CO_2_Flow = flow of initial headspace CO_2 from headspace, Prod_CO_2_Flow = flow of produced CO_2 from headspace, Prod_CH_4_Flow = flow of produced CH_4 from headspace. At the beginning of fermentation the fractional CO_2 concentration in pool [1] is 1.00, whereas in the other pools fractional gas concentrations are 0.00. The volume of headspace ([1] + [2] + [3]) = 265 ml during the whole incubation. Relative CH_4 concentration (outflow/head space) is calculated as [6/(4 + 5 + 6)]/[3/(1 + 2 + 3)].

where 162 = molar weight (mg/mmol) of carbohydrate polymer unit, AA, PA, BA, iVA and VA are the production of acetate, propionate, butyrate, isovalerate and valerate (mmol) after 48 h of incubation corrected for the blanks.

2.6. In vitro true digestibility

Neutral detergent fibre (aNDFom), assayed with a heat stable amylase and expressed exclusive of residual ash, was determined as approximately 500 mg of each air-dry sample was placed in pre-weighed filter bags and heat sealed. The bags were then subjected to the ANKOM^{200} fibre analyser (A200I, Macedon NY 14502, USA) at 100 °C for 60 min with addition of sodium sulphite and α-amylase to the neutral detergent solution (Mertens, 2002). The ash content in the residues was determined by weighing them after incinerating the bags at 550 °C for 3 h (Nabertherm, Germany). Digestibility of aNDFom (aNDFomD) and true organic matter digestibility (TOMD) were calculated using the aNDFom residues after the 48 h *in vitro* incubation. For TOMD it was assumed that neutral detergent soluble fraction is completely digestible.

2.7. Gas production kinetics

Cumulative methane production (ml) at each time point (0.2 h) was calculated according to the formula as: total methane production (ml) = headspace (HS) volume (ml) × HS methane concentration + gas production (ml) × A × HS methane concentration. The headspace volume in the system is 265 ml (the volume is for bottles and pressure tubes connected to the gas reader box). The total gas volume is automatically recorded by the system and corrected for the normal air pressure. Coefficient A is the ratio of methane concentration in outflow gas to HS.

Because the outflow of gas cannot be collected in our system, the ratio of the methane concentration in the outflow (=measured gas production) to the methane concentration in the headspace (Ratio A) was predicted using a mechanistic model (Fig. 2). In the model the total gas production was calculated using stoichiometric principles as outlined by Van Soest (1994) and assuming that one mole of VFA production releases one mole of CO_2 from the buffer. Molar proportions of 650, 200 and 150 mmol/mol acetate, propionate and butyrate, respectively, were assumed. In the model simulations, a digestion rate of 0.06/h, corresponding to the observed first order gas production rate, was used. The model is based on the following assumptions: (i) CO_2 and methane are in equilibrium in the headspace, *i.e.* the proportions of gasses in the outflow are the same as in the headspace, (ii) the total volume of initial headspace CO_2 (Init_HS_CO_2) + produced headspace CO_2 (Prod_HS_CO_2) + produced headspace methane (Prod_HS_CH_4) is constant (265 ml) and (iii) that the outflow of each of the three gas phases is relative to their concentrations in the headspace. Relative methane concentration (A in the above formula) in outflow gas to that in headspace gas at each time point was calculated using the following equation: A = [Cumulative_CH_4 Prod/(Cumulative_CH_4_Prod + Cumulative_CO_2_Prod + Cumulative_CO_2_HS)]/[Prod_HS_CH_4/(Prod_HS_CH_4 + Prod_HS_CO_2 + Init_HS_CO_2)]. The simulations were made by POWERSIM® 2.5 Software, (Powersim AS, Istaldsø, Norway) graphical modelling software (Powersim, 1996) using Runge–Kutta 4 fixed step integration method and 0.0625 h time step.

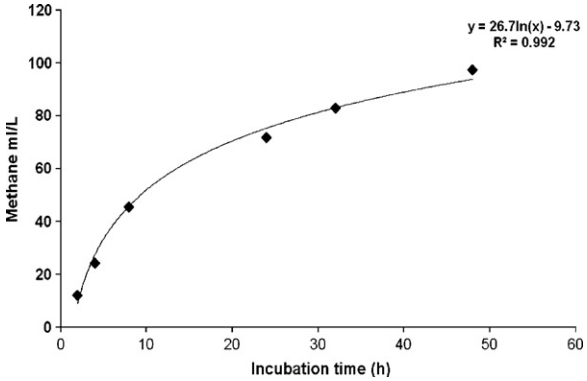

Fig. 3. Logarithmic model fitted to observed methane concentration data to obtain values at time intervals of 0.2 h.

Methane concentration at time intervals of 0.2 h was estimated by a logarithmic model of time *versus* methane concentration. The fit of the equation was with R^2 values >0.99 (an example is given in Fig. 3). Methane production (ml) at each time point was calculated as total gas×calculated methane concentration.

Kinetic parameters of fermentation (from methane and total gas production at each time point (0.2 h), separately) were estimated by fitting the data to the two pool Gompertz function (Schofield et al., 1994) by the NLIN procedure (SAS Inst. Inc., Cary, NC) as follows:

$$V_t = V_1 \times \exp(-\exp(1 + k_1 \times e \times (L_1 - t))) + V_2 \times \exp(-\exp(1 + k_2 \times e \times (L_2 - t))),$$

where V_t = the measured total gas or methane volume at time t; V_1, k_1, and L_1 = the asymptotic cumulative gas volume (ml/g DM), rate (/h), and lag (h) parameters for the first pool (rapid), and V_2, k_2, and L_2 = the corresponding parameters for the second pool (slow) and t = incubation time. This model was chosen because it fitted the data better than commonly used one-pool models and it also predicted *in vivo* data accurately (Huhtanen et al., 2008).

The parameters were then subjected to a dynamic, mechanistic two-compartment rumen model described by Huhtanen et al. (2008). The model was originally developed to predict the digestibility of pdNDF from the gas kinetic data. Here the model was applied to estimate the proportion of asymptotic methane production at infinitive time ($V_1 + V_2$) that will be produced during the residence of feed in the rumen. Methane production (ml/g DM) was calculated as = proportion × asymptotic methane production (ml/g DM). The effective first-order methane production rate was estimated by solving the two-compartment equation described by Allen and Mertens (1988) for k_d when digestibility (here proportion) and passage kinetic parameters are known. A mean retention time (MRT) of 50 h (20 h in the first compartment and 30 h in the second compartment) corresponding to the maintenance level of feed intake was used in model simulations. The simulations of methane production were also made using a 35 h mean retention corresponding dairy cows fed approximately 20 kg DM/d (Krizsan et al., 2010). The model is programmed in POWERSIM® 2.5; the simulations were run for 120 h with a time step of 0.0625 h integration step and using the Euler integration method.

2.8. Stoichiometrical calculations

Methane production (blank corrected) was predicted according to VFA stoichiometry equations (Wolin, 1960), the equation used was as below:

$$\text{Predicted methane (ml)} = 22.4 \times (0.5 \times \text{AA} - 0.25 \times \text{PA} + 0.50 \times \text{BA} - 0.25 \times \text{VA})$$

where AA, PA, BA, and VA are the production (mmol) of acetate, propionate, butyrate and valerate, and 22.4 is gas volume (ml/mmol gas). The VFA production data was corrected for blanks.

2.9. Statistical analyses

Predicted values of the kinetic parameters, methane and total gas production (n = 27) were subjected to the general linear model (GLM) procedure of the SAS program (SAS, 2002) using the model:

$$Y_{ij} = \text{SL}_i + \text{Run}_j + (\text{SL} \times \text{Run})_{ij} + e_{ijk},$$

where SL_i is substrate level (i = 4); Run (j = 2; first run 5 replicates, second run 2 replicates) and e_{ijk} is random error term. The SL effects were tested using SL×Run as an error term (df = 3). The sum of squares of SL effect was further partitioned into linear and quadratic effects of the substrate level using orthogonal polynomial contrasts.

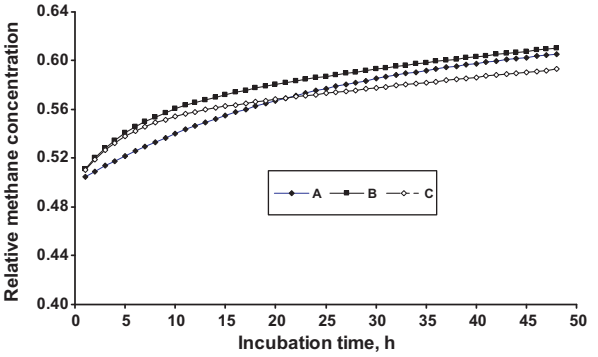

Fig. 4. Results from simulations with the gas *in vitro* model (Fig. 2.) showing cumulative values for relative methane concentration in released gas compared with headspace gas where 'A' has similar digestion rate (0.05/h) for neutral detergent fibre and neutral detergent solubles (aNDFom and aNDSom, respectively) with volatile fatty acids (VFA; acetate: propionate: butyrate) ratios 65:20:15; 'B' has digestion rates 0.05 (aNDFom) and 0.20/h (aNDSom) and VFA ratios 70:18:12 and 60:25:15 for aNDFom and aNDSom, respectively; 'C' has digestion rates as in 'B' and VFA ratios 75:18:10 and 55:30:15 for aNDFom and aNDSom, respectively.

The total number of observations for VFA measurements was 36 ($n = 18$ for 24 h and $n = 18$ for 48 h) and $n = 19$ for digestibility. Because VFA and digestibility measurements were made only for the first run (5 replicates) the effects of SL were evaluated by linear regression analysis after averaging the replicates using the following model:

$$Y_{ij} = \mathrm{SL}_i + e_{ij},$$

where SL_i is substrate level and e_{ij} is random error term. Methane production estimated from VFA stoichiometry was plotted against the measured methane production. There were 18 data points at each time point (36 in total for 24 and 48 h). Relationships between predicted and observed methane were estimated by REG procedures of SAS (SAS, 2002) and residual analysis was made as described by St-Pierre (2003) by regressing the centred predicted values against residuals (observed – predicted).

3. Results

Simulated relative methane concentration in gas outflow *versus* headspace is given in Fig. 4 when the parameter values of digestion rates and VFA proportions were changed. Faster digestion rate of aNDSom compared with aNDFom fraction increased the initial rise in the relative methane concentration in gas outflow due to the faster initial rate of total gas production and the replacement of initial headspace CO_2 with CO_2 and methane produced from substrate fermentation. Fermentation pattern had a relatively small effect on the relative concentration of methane in outflow gas.

The effects of sample size on gas and methane production are shown in Table 1. The two-pool Gompertz model fitted well ($R^2 \geq 0.9999$) to methane production data. The decrease, both in methane and total gas production, took place in the rapidly digestible pool whereas the differences were rather small in the slowly digestible pool. Asymptotic methane production decreased linearly (PLIN < 0.01) as the sample size increased from 300 to 1200 mg (Table 1). The results of the first-order production rate were not different (P=0.12) with values of 0.052, 0.046, 0.046 and 0.045/h for the sample size of 300, 600, 900 and 1200 mg, respectively (Table 1). Predicted methane production decreased linearly (PLIN < 0.01) as the sample size increased from 300 to 1200 mg (Table 1). Asymptotic values of total gas production decreased (PLIN = 0.02) from 241 to 212 ml/g DM for 300 mg and 1200 mg sample size, respectively. Predicted values of the total gas production decreased (PLIN < 0.01) with increasing sample size (Table 1). On average, predicted methane production was proportionally 0.14 lower with 35 h MRT compared with 50 h MRT.

Total VFA production after 48 h of incubation decreased (PLIN = 0.01) as the sample size increased (Table 2). On the contrary, the molar proportions of acetate after 48 h of incubation decreased (PLIN = 0.01) as the sample size increased (Table 2). Instead the molar proportion of propionate increased (PLIN = 0.03) as the sample size increased, whereas the changes in isovalerate and valerate were inconsistent.

Apparent organic matter digestibility calculated based on the production of VFAs after 48 h of incubation decreased (PLIN = 0.01) from 0.433 to 0.379 with increasing sample size (Table 3). Similar results (PLIN = 0.01) were obtained for aNDFomD and TOMD.

Besides measurements, production of methane was also predicted stoichiometrically using VFA production data at 24 and 48 h of incubation. There was a high correlation ($R^2 = 0.97$) between predicted *versus* observed values. As the intercept was close to 0.0 and the slope close to 1.0 (Fig. 5), there was no mean or linear bias (Fig. 6). Random variation accounted for most of the mean squared prediction error of the stoichiometric model (0.946).

Table 1
The effects of sample size on predicted values of methane production and the kinetic parameters of methane and total gas production (least square means).

Item		Sample size (mg per 60 ml culture)				SEM[b]	Contrast[a]	
		300	600	900	1200		*L*	*Q*
Methane	Asymptotic CH_4 (ml/g DM)	45.6	40.9	39.7	36.3	0.36	<0.01	0.18
	Rate (/h)	0.052	0.046	0.046	0.045	0.0021	0.12	0.40
	Predicted CH_4 (50 h)[c] (ml/g DM)	36.9	32.0	31.1	28.2	0.47	<0.01	0.15
	Predicted CH_4 (35 h)[d] (ml/g DM)	32.5	27.7	26.7	24.2	0.53	<0.01	0.15
	V_1 (ml/g DM)	18.6	15.0	13.3	11.4	0.44	<0.01	0.17
	k_1 (/h)	0.087	0.087	0.089	0.094	0.0019	0.11	0.31
	L_1 (h)	0.2	0.6	0.7	0.7	0.10	0.05	0.24
	V_2 (ml/g DM)	27.0	25.8	26.3	24.8	0.46	0.08	0.76
	k_2 (/h)	0.025	0.023	0.024	0.024	0.0010	0.81	0.51
	L_2 (h)	6.0	6.6	6.7	6.8	0.38	0.27	0.72
Total gas	Asymptotic gas (ml/g DM)	241	223	223	212	4.30	0.02	0.51
	Rate (/h)	0.072	0.061	0.061	0.059	0.0045	0.18	0.46
	Predicted gas (ml/g DM)	209	187	188	177	2.38	<0.01	0.81
	V_1 (ml/g DM)	82.6	73.9	59.5	60.1	2.41	<0.01	0.17
	k_1 (/h)	0.139	0.119	0.160	0.154	0.010	0.18	0.57
	L_1 (h)	−0.70	−0.82	−0.12	−0.04	0.11	0.01	0.47
	V_2 (ml/g DM)	158	149	164	152	4.14	0.82	0.80
	k_2 (/h)	0.031	0.027	0.029	0.028	0.0017	0.41	0.46
	L_2 (h)	2.1	2.6	2.0	2.9	0.42	0.46	0.62
CH_4[e]/gas		0.177	0.171	0.165	0.159	0.002	0.01	0.93

V_1: volume for the rapid pool (first pool); k_1: rate for the rapid pool (first pool); L_1: lag for the rapid pool (first pool); V_2: volume for the slow pool (second pool); k_2: rate for the slow pool (second pool); L_2: lag for the slow pool (second pool).
[a] *L*: linear effect of sample size, *Q*: quadratic effect of sample size.
[b] SEM: standard error of mean ($n = 27$).
[c] Methane was predicted by using a 50 h rumen retention time in the mechanistic rumen model.
[d] Methane was predicted by using a 35 h rumen retention time in the mechanistic rumen model.
[e] Values for 50 h rumen retention time.

Table 2
The effects of sample size on volatile fatty acids (VFA) production (mmol/g DM) and molar proportions of net VFA production (mmol/mol) after 24 and 48 h incubation (least square means).

Time (h)	VFA		Sample size (mg per 60 ml culture)				SEM[b]	P-value[a]
			300	600	900	1200		*L*
24	Total VFA (mmol/g DM)		2.86	2.89	2.72	2.53	0.082	0.08
	Molar proportion (mmol/mol)	Acetate	636	614	591	584	5.94	0.02
		Propionate	273	267	281	276	6.17	0.49
		Butyrate	96	98.4	99.7	108	2.44	0.07
		Isovalerate	−2.6	−8.7	−2.9	−2.9	3.55	0.77
		Valerate	−3.7	28.6	30.7	33.5	8.67	0.14
48	Total VFA (mmol/g DM)		4.41	4.25	3.96	3.82	0.044	<0.01
	Molar proportion (mmol/mol)	Acetate	620	612	604	590	2.32	0.01
		Propionate	248	258	262	266	2.32	0.03
		Butyrate	90.6	93.0	95.2	103	2.11	0.05
		Isovalerate	5.1	8.4	6.2	4.4	2.03	0.68
		Valerate	35.4	27.2	31.5	35.1	4.66	0.88

[a] *L*: linear effect of sample size.
[b] SEM: standard error of mean, 24 h ($n = 18$), 48 h ($n = 18$).

Table 3
Least square means of neutral detergent fibre digestibility (aNDFomD), true organic matter digestibility (TOMD) and apparent organic matter digestibility (AOMD) and probability for linear (*L*) effects of sample size of timothy hay after 48 h incubation.

Item	Sample size (mg per 60 ml culture)				SEM[b]	P-value[a]
	300	600	900	1200		*L*
AOMD	0.433	0.417	0.390	0.379	0.0046	0.01
aNDFomD	0.479	0.456	0.402	0.369	0.0089	0.01
TOMD	0.681	0.667	0.634	0.614	0.0054	0.01

[a] *L*: linear effect of sample size.
[b] SEM: standard error of mean ($n = 19$).

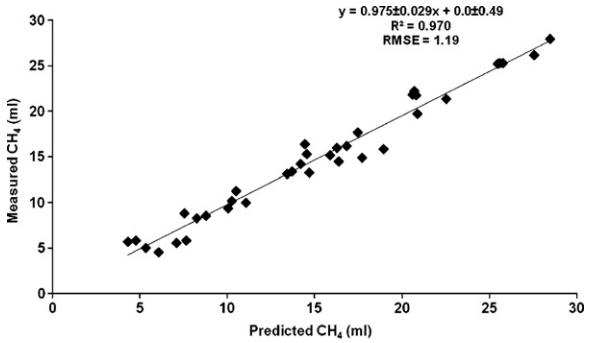

Fig. 5. Relationship between methane production predicted from volatile fatty acid stoichiometry and measured *in vitro* gas system at 24 and 48 h; $y = 0.975(\pm 0.029)x + 0.0(\pm 0.49)$ ($n = 36$, $R^2 = 0.970$ and root mean square error = 1.19).

4. Discussion

The hay used as a substrate had a low digestibility as indicated by the high concentration of iNDF (188 g/kg DM). The empirical equation of Huhtanen et al. (2006) predicted that *in vivo* organic matter digestibility (OMD) would be 0.57. Taking the metabolic faecal OM output of 100 g/kg DM intake (Huhtanen et al., 2006) into account our TOMD values at the two lower levels of substrate compared well with predicted *in vivo* values.

4.1. Model description

In our *in vitro* system, methane could not be measured from the outflow of the *in vitro* gas system (gas released from the outlet valve at each time opening) and therefore we used a modelling approach based on stoichiometric principles to predict the methane concentration in gas outflow relative to headspace gas. Biased model assumptions can be one source of error of the total methane production. However, the proportion of total methane production estimated as outflow is relatively small compared with the headspace volume which means that a large proportion of methane remains in the headspace. Also, the deviations from default values in fermentation pattern influence similarly on the composition of headspace and outflow gas. At maximum, an error of 0.05 in the relative outflow methane concentration (Coefficient A) would generate a relative error of 0.015 in the estimates of methane production (1200 mg sample and 48 h incubation). This value is not greater than the coefficient of variation in the area under the curve of standard injections (CV = 0.01–0.015). The close relationship between observed and stoichiometrically predicted methane production indicates that the model was robust in predicting relative methane concentration in outflow gas. Recently Pellikaan et al. (2011) published a more direct method in estimating methane concentration in gas outflow. Blank corrected methane production (pooled data for 24 and 48 h) estimated by the method of Pellikaan et al. (2011) and our method was strongly correlated ($R^2 = 0.997$) with a small mean difference (0.6 ml) relative to observed CH_4 production (see Table 1).

One of the advantages of the *in vitro* GP technique in determination of methane production is that it can be automated to obtain a large number of data points allowing accurate parameter estimation. However, it is a batch culture and has some limitations compared to the *in vivo* methods; *e.g.* there is no absorbance of VFA or intake of substrate over the time. The data can be fitted by various non-first-order or some nonlinear growth models, but model parameters can be difficult to interpret and comparison of feeds is not straight forward (Pitt et al., 1999). Probably the greatest disadvantage of these models is that using the non-first-order parameters in steady state rumen models with continuous intake, digestion and passage is difficult because feed particles are of various ages and consequently have different digestion rates. To overcome this problem Pitt et al.

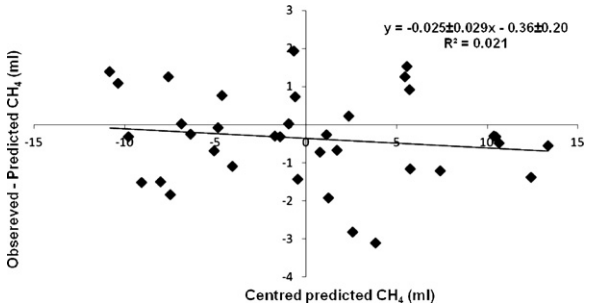

Fig. 6. Plot of residuals (observed − predicted) for methane production *versus* values predicted from volatile fatty acid stoichiometry. The regression line in the graph represents the equation $y = -0.025(\pm 0.029)x - 0.36(\pm 0.20)$, $R^2 = 0.021$. Predicted values were centred by subtracting the overall mean predicted value from each predicted value.

(1999) derived equations to estimate an effective first-order digestion rate from non first-order models (time-dependent digestion rate) to be applied in a one-compartment rumen model. However, the passage kinetics of feed particles cannot adequately be described by one-compartment rumen models ignoring selective retention of feed particles (*e.g.* Ellis et al., 1994) leading to underestimation of ruminal digestibility. Huhtanen et al. (2008) presented a two-compartment rumen model for estimating ruminal digestibility and first-order digestion rate for pdNDF from the kinetic parameters of *in vitro* GP from isolated NDF. The method predicted *in vivo* NDF digestibility of grass silages accurately suggesting that the system can also have a potential for determination of methane production. An advantage of using automated *in vitro* (GP) system for the determination of methane production from the kinetic parameters is that by taking into account rumen passage kinetics, methane production can be predicted at different intake levels. Also, in batch culture system the choice of appropriate incubation period can be difficult, since the fermentation time required to reach the *in vivo* digestibility in a dynamic rumen system depends on the digestion rate. For example, for digestion rates of 0.03 and 0.08/h fermentation times of 37 and 27 h, respectively, are needed to reach the digestibility in two-compartment rumen system with 50 h (20 + 30 h) rumen MRT. This implies that the values derived from the end-point of batch fermentations may not accurately predict total methane production or differences between the treatments. Consequently, *in vitro* end-point batch systems may only be used for qualitative ranking of treatments.

In the *in vitro* GP system part of the gas production can be derived from the degradation of microbial cells after depletion of the substrate (Cone et al., 1997) overestimating methane production based on end-point measurements. Predicting methane production from the derived *in vitro* kinetic parameters with a dynamic rumen model can, at least partly, reduce this problem. Because the lag time is rather long and the rate is slow for the degradation of the microbial pool, its contribution to predicted methane production is much less compared with only end-point measurements.

4.2. Effects of sample size on digestibility

The sample size is a compromise between digestibility and blank effects. In the present study aNDFomD, TOMD and AOMD decreased linearly with increased sample size (Table 3). This is in agreement with Cone et al. (1996) who reported a decrease in total gas production after 10 h of incubation when more than 0.5 g of a highly fermentable substrate (corn cob mix) was used. The decrease in digestibility could be a result of saturation of VFA's as Beuvink and Spoelstra (1992) reported a saturation by addition of more than 4.5 mmol acetic acid in 60 ml buffered rumen fluid.

4.3. Effects of sample size on methane production

Sample size could have an important effect on methane production measurements during the incubation in a closed *in vitro* GP system, since it can influence the rate of digestion and lyses of the microbial pool in the system. The first-order methane and gas production rates were not significantly influenced by the amount of substrate as also reported by Rymer et al. (2005) for the gas production rate. The results of the first order rate in our study are in line with the report by Theodorou et al. (1994) who reported a constant rate of fermentation with increased sample size (0.2–2.0 g) substrate.

Observed methane production increased as sample size increased (data not shown). This is in line with well-recognised relationship between intake and methane production observed *in vivo* (Blaxter and Clapperton, 1965; Yan et al., 2000). In contrast, predicted methane production per g DM decreased as the sample size increased from 300 to 1200 mg. In the present study decreases in methane production with increasing sample size can be attributed to reduced digestibility, increased molar proportion of propionate and increased partitioning of carbon towards microbial cells with increased sample size. Blümmel et al. (1997) reported that producing large amounts of gas and VFA yields smaller amounts of microbial mass. Another study, Blümmel et al. (2005) reported that the production of microbial protein and efficiency of synthesis were negatively related to methane production per kg digestible OM. Reduced methane production per g DM *in vitro* with increased substrate level is consistent with *in vivo* data (Blaxter and Clapperton, 1965; Yan et al., 2000). Faster fractional passage rate with increased feed intake reduce diet digestibility and improve the efficiency of microbial protein synthesis which decreases methane production per unit of substrate. Even at a fixed level of feed intake methane production was negatively correlated with fractional passage rate and positively with cellulose digestibility (Pinares-Patino et al., 2003).

4.4. Evaluation of methane estimates

Assuming a gross energy (GE) concentration of 18.5 MJ/kg DM, the predicted proportion of methane energy varied proportionally from 0.080 (300 mg) to 0.061 (1200 mg) of GE. These values are close to observed *in vivo* values at the maintenance (300 mg) level of intake and production (1200 mg) levels of intake in dairy cows (Yan et al., 2000). The equation of Blaxter and Clapperton (1965) predicted a proportional value of 0.073 for methane energy of GE for the hay used in our trial. This value compares well with the values obtained at 300 and 600 mg sample sizes. Assuming that the sample size of 300 mg corresponds to the maintenance level of intake, the predicted proportional decrease in methane production (GE) was 0.0058 per multiple of maintenance that is similar to the value of 0.0057 derived from Blaxter and Clapperton (1965) for the hay used in the present study. In contrast to model predicted values asymptotic values clearly over-predicted methane production compared to *in vivo* data (0.097 of GE with 300 mg sample size). Using a shorter MRT (35 *versus* 50 h) decreased predicted methane production probably underestimated *in vivo* responses. The mean proportional difference in methane energy as

GE between 50 and 35 h MRT (1×maintenance *versus* approximately 3×maintenance) was 0.0091 units (0.0046 units per multiple of maintenance), *i.e.* less than 0.0078 per multiple of maintenance as reported by Yan et al. (2000). This is mainly because the mechanistic rumen model only takes into account the reduced digestibility with increasing intake, whereas improved efficiency of microbial synthesis with increasing intake that reduces the utilisation of carbon for VFA and gas production was not included in the model. In the present study, observed methane production was closely correlated ($R^2 = 0.97$) with that predicted from VFA production. This is in line with Getachew et al. (2005) who reported slightly (on average 0.09) higher values of methane calculated from VFA stoichiometry. A high correlation between measured and calculated methane production values in this study suggests that methane production was measured accurately.

5. Conclusion

An *in vitro* method based on an automated gas production system and using the estimated kinetics parameters in a dynamic mechanistic rumen model to determine methane production was developed. The results of the present study indicate that increasing the sample size up to 1200 mg (20 g/l) did not change the rate of estimated methane production, and that it is possible to use greater amounts up to 1000 mg (16.6 g/l) of substrate in the system in order to decrease the depletion of samples and the effects of the blank in the *in vitro* gas system. High correlation was achieved between measured and stoichiometrically predicted methane production indicating the accuracy of the system. Realistic values of predicted methane production suggest that the developed method can be a useful tool for evaluating the diet effects on methane production and for screening feed additives. However, the model and sample size need to be evaluated with a wider range of diets, especially with higher quality feeds, and feed additives influencing methane production as well needs to be validated with the *in vivo* data. Such work is in progress in our laboratory.

Acknowledgements

The financial support from Raisio Ltd. (Raisio, Finland) Valio (Helsinki, Finland) and the Swedish Farmers' Foundation of Agricultural Research are greatly acknowledged. The authors also wish to thank Ms. Evelina Viklund and Ms. Malin Barrlund for their assistance with rumen fluid sampling and Ms. Ann-Sofi Hahlin for her assistance in the laboratory.

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