Agraarteadus
1 * XXVII * 2016 42–47

Journal of Agricultural Science
1 * XXVII * 2016 42–47

COMPARISON OF METHANE PRODUCTION FROM INDIVIDUAL FEEDS AND TOTAL DIETS – AN *IN VITRO* EVALUATION

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Saabunud: 13.04.16
Received:
Aktsepteeritud: 01.06.16
Accepted:

Avaldatud veebis: 16.06.16
Published online:

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Keywords: feed interaction, greenhouse gases, gas production

Link: http://agrt.emu.ee/pdf/2016_1_ramin.pdf

ABSTRACT. The objective of the current study was to compare methane (CH_4) production from the *in vitro* gas production system by incubating feeds either individually or as mixed total diet. Eleven diets varying in the forage to concentrate ratio were tested. The forages were tropical grass or corn silages and the concentrate mixtures consisted of soybean grain, soybean meal, corn grain, wheat bran, urea and minerals in different proportions. There were three replicates for each diet. Methane production was reported as weighted mean for individual feeds and total diet separately. The mean of CH_4 production from total diet was 30.1 mL g^{-1} dry matter (DM) and 30.8 mL g^{-1} DM from the weighted mean of individual feeds. There was a weak correlation between weighted CH_4 production from individual feeds and complete diet (r = 0.15). It can be concluded that individual feeds cannot be used as a proxy to estimate CH_4 production from total mixed diets.

Introduction

Methane (CH_4) production is a major problem in ruminant production system as it represents a significant energy loss from the diet *i.e.*, 6–6.5% of gross energy intake on average for dairy cows fed on grass silage based diets in Scandinavian countries (Huhtanen *et al.*, 2013). Many factors influence CH_4 production in ruminants such as digestibility, fat, and dry matter intake (Beauchemin *et al.*, 2009; Ramin, Huhtanen, 2013). Measuring CH_4 production from animals by respiration chambers is laborious and costly, but at the same time it is considered the most accurate method (Hellwing *et al.*, 2012).

In this context, the *in vitro* technique is an alternative method to estimate CH_4 production from ruminants (Cone *et al.*, 1996; Ramin, Huhtanen, 2012). One main disadvantage of the *in vitro* technique is that it does not take into account the dynamic of rumen, including the interaction between degradation and passage (Huhtanen *et al.*, 2008). Huhtanen *et al.* (1991) reported possible interaction between dietary components on diet digestibility, on the other hand, interaction among feeds can influence the stoichiometry of rumen fermentation which could modify CH_4 production as well. However, the interaction between feeds has not been considered in the *in vitro* evaluation of diets.

In this way, we hypothesized that CH_4 production from individual feeds evaluation cannot be used as a predictor of CH_4 production from total diets. The objective of the current study was to compare CH_4 production from the *in vitro* gas production system by incubating feeds either individually or as total mixed diets.

Materials and methods

The *in vitro* trial was carried out at the Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, Umeå, Sweden. The study was conducted with the permission of the Swedish Ethical Committee on Animal Research.

Eleven diets were evaluated, with seven different forage-to-concentrate ratios ranging from 90:10 to 55:45 (Table 1).

The forages (n = 9) were tropical grass (*Brachiaria decumbens*, n = 5) and corn silages (*Zea mays*, n = 4). The concentrate mixtures (n = 7) were obtained by mixing whole soybean grain, soybean meal, corn grain, wheat bran, urea and minerals in different proportions (Table 2). Seven diets were taken from two grazing trials using Zebu heifers supplemented with concentrates

containing different soybean meal and whole soybean grains contents (Silva, 2012). Three forages were taken from the first grazing trial and two forages were taken from the second grazing trial from the study conducted by Silva (2012). Moreover, four diets were evaluated from a feedlot trial using Nellore bulls fed corn silage (n = 4) and concentrates (n = 4) (Costa e Silva *et al*., 2013) (Table 1 and 2). The ingredient proportion of concentrates in feedlot trail were the same (the ratio of different feeds) but differed in their chemical composition as the evaluations were performed along the feedlot period using the animals at different maturities (Costa e Silva *et al*., 2013).

All forage samples were oven-dried (55 ºC, 48 h) and were ground using a knife-mill to pass through a 1-mm screen sieve. The same procedure was performed with the concentrate feeds and carefully mixed according to the correct proportion given in Table 2 to make the concentrate mixture.

Table 1. Chemical composition and digestibility of diets taken from *in vivo* trials (n = 11)

Item	Grazing trial 1[A]			Grazing trial 2[B]				Feedlot trial[C]			
	SM	SM:SG	SG	SM1	SM2	SG1	SG2	MS1	MS2	MS3	MS4
Actual chemical composition, g kg^{-1} DM											
Organic matter	917	918	918	912	921	922	922	945	946	943	942
Crude protein	112	114	112	74	80	77	75	125	132	125	120
Ether extract	15	23	30	14	13	33	28	33	29	21	24
Neutral detergent fiber	565	555	549	616	598	615	588	326	342	352	364
Diet characteristics[D]											
F:C	90:10	86:14	88:12	85:15	84:16	82:18	86:14	55:45	55:45	55:45	55:45
Organic matter digestibility (g kg^{-1})	587	614	616	507	493	494	497	679	696	714	732

[A]SM – diet including forage (forage 1) and concentrate mixture with soybean meal as the main protein source; SM:SG – diet including forage (forage 2) and concentrate mixture with soybean meal and soybean grain as the main protein source; SG – diet including forage (forage 3) and concentrate mixture with soybean grain as the main protein source. Adapted from Silva (2012)
[B]SM1 and SM2 – diet including forage (forages 1 and 2) and concentrate mixture with soybean meal as the main protein source; SG1 and SG2 – diet including forage (forages 1 and 2) and concentrate mixture with soybean grain as the main protein source. Adapted from Silva (2012).
[C]MS1-4 – diets that include corn (4) silage with concentrate mixtures (4). Adapted from Costa e Silva *et al.* (2013)
[D]F:C – forage to concentrate ratio (DM basis). For chemical composition of forages and concentrate mixtures see Table 2; The OM digestibility was measured *in vivo* (Silva, 2012; Costa e Silva *et al.*, 2013).

Table 2. Average chemical composition of concentrate mixture and forages separately and feed composition of concentrate mixtures

Item	Feeds from grazing trials[A]					Feeds from feedlot trial	
	SM	SM:SG	SG	Forage trial 1	Forage trial 2	Concentrate	Corn silage
	n = 1	n = 1	n = 1	n = 3	n = 2	n = 4	n = 4
Chemical composition, g kg^{-1} DM							
Organic matter	910	919	918	918	920	947	943
Crude protein	339	295	296	86	31	195	65
Ether extract	7	65	131	16	13	30	23
NDF	208	239	141	606	686	134	521
Feed composition of concentrate mixtures, g kg^{-1} DM							
Corn grain	0	0	0	–	–	816	–
Soybean meal	500	250	0	–	–	138	–
Soybean grain	0	250	500	–	–	0	–
Wheat bran	430	425	415	–	–	0	–
Minerals	60	60	60	–	–	26	–
Urea	10	15	25	–	–	20	–

[A]SM – concentrate mixture with soybean meal as the main protein source; SM:SG – concentrate mixture with a combination of soybean meal and soybean grain as the main protein source; SG – concentrate mixture with soybean grain as the main protein source; Forage 1 and 2 – average composition of forages from trails 1 and 2; n – represents number of forage:corn silages or concentrate mixture used for that specific trial

Thus, the proportions needed for each concentrate mixture and forage for each diet (n=11) were weighted separately (depending on the ratio between forage and concentrate) in the *in vitro* bottles using an AG204DR (Mettler Toledo, Switzerland) analytical balance. Forage:corn silages and concentrate mixtures of all 11 diets were also incubated individually to later calculate CH_4 production based on the weighted mean of forage to concentrate (F:C) ratios of each diet.

Rumen fluid for all three *in vitro* incubation runs was obtained from the same two ruminally cannulated lactating Swedish Red cows about two hours after morning feeding. Cows were fed on a diet containing grass silage and commercial concentrate (60:40 on a dry matter [DM] basis). The crude protein (CP) of silage was 17.3% with a neutral detergent fibre (NDF) content of 55.1%. The commercial concentrate was Solid 220 (Lantmännen, Malmö, Sweden) mainly consisting of wheat, rapeseed meal, oat, dried sugar beet pulp and minerals. The rumen fluid was collected into pre-warmed thermos flasks previously flushed with carbon dioxide (CO_2) and afterwards filtered through four layers of cheesecloth into a buffered mineral solution (Menke, Steingass, 1988), with the ratio of rumen fluid to buffer of 20:80 (vol:vol). A fully automated *in vitro* gas production system was used as described by Cone *et al.* (1996) with recordings of gas production (GP) every 12 minutes. The recorded GP was corrected to normal air pressure (1013.5 h Pa). Samples of 1 g were weighed (total diet and individual feeds) directly into

250-mL serum bottles and incubated in 60 mL of buffered rumen fluid for 48 hours. The bottles were placed in water bath at 39 ºC and gently agitated continuously during the incubations.

All 27 samples (forage:corn silages, concentrate mixtures and total mixed diets) were incubated in three *in vitro* series (runs) and were randomly distributed within the runs, resulting in three *in vitro* observations per sample. In each run, a blank (buffered rumen fluid without a sample) was incubated in duplicates. Gas samples were drawn from each serum bottle by a gas tight syringe (Hamilton, Bonaduz, Switzerland) at 2, 4, 8, 24, 32 and 48 h of incubation through a gas tight rubber suba seal (Z124567-100EA, 13, Sigma-Aldrich, Germany) that was previously installed on the pipes leading out from the *in vitro* serum bottles. Methane concentrations were 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. Calibration gas was completed using a standard mixture of CH_4 and CO_2 (100 mmol mol^{-1}) prepared by AGA Gas (AGA Gas AB, Sundbyberg, Sweden). Peaks were identified by comparison with the standard gas. Total gas production values from the fully automated *in vitro* gas production system were recorded. Methane production was measured as described by Ramin and Huhtanen (2012) and was reported as weighted means for individual feeds and total diet separately.

The statistical comparison was performed by a simple linear regression of values obtained from total diet incubations (Y) on values obtained from the weighted sum of the individual feeds (X) of respective diet, according to the model:

$$Y_{ij} = \beta_0 + \beta_1 \times X_{ij} + R_j + \varepsilon_{ij} \quad [1],$$

where Y_{ij} is the CH_4 production obtained by incubation of diet i in the incubation run j, β_0 is the intercept, β_1 is the slope, X_{ij} is the weighted CH_4 production obtained by individual feeds incubation in the incubation run j, R_j is the effect of the j incubation run (random effect), and ε_{ij} is the random error.

The following null hypotheses were tested:

$$H_0 : \beta_0 = 0 \quad [2],$$

$$H_0 : \beta_1 = 1 \quad [3],$$

where β_0 is the intercept, and β_1 is the slope.

The CH_4 production estimates obtained by diet or individual feed incubations should be considered similar if both of the null hypotheses are not rejected.

The model adjustment was performed by using the MIXED procedure of the SAS 9.4 ($\alpha = 0.05$). As shown in Equation [1], the model adjustment took into account for the random variation among different runs.

Results and discussion

The feeds used in the current study showed to have a wide range in terms of chemical composition (Table 2). Crude protein ranging from 31 up to 339 g kg^{-1} DM and neutral detergent fiber (NDF) varied from 208 to 686 g kg^{-1} DM (Table 2). Similarly, the 11 diets used in the present study had wide ranges in chemical composition and *in vivo* digestibility (Table 1).

Descriptive statistics of the *in vitro* CH_4 production is given in Table 3. The mean of CH_4 production from the total diets was 30.1 mL g^{-1} DM and 30.8 mL g^{-1} DM from the weighted mean of individual feeds, respectively (Table 3).

Table 3. Descriptive statistics for the methane production (mL g^{-1} DM) obtained from incubation of total diet and from weighted information of individual feeds

Statistic	Total diet	Weighted value
Mean	30.1	30.8
Minimum	21.3	22.9
Maximum	40.4	38.9
Standard deviation	5.64	4.31
n^A	32	

n^A: diet 11 had 2 replicates

In spite of presenting close average values, both null hypotheses were rejected ($P < 0.01$). This indicates a total lack of association between CH_4 production obtained by incubating total diets and individual feeds in the *in vitro* gas production system as shown in Figure 1.

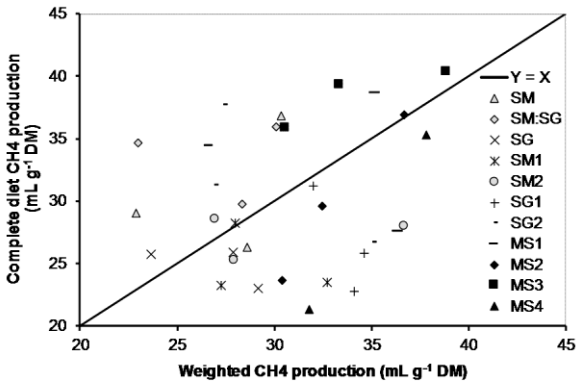

Figure 1. Descriptive relationship between the methane productions obtained from incubation of total diet (n = 11) and from weighted information of individual feeds. For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of the article. For more details about the diets see Table 1. [$\hat{Y} = 22.1 \pm 7.28 + 0.26 \pm 0.235 \times X$, r = 0.15, P value ($\beta_0 = 0$) – 0.005, P value ($\beta_1 = 1$) – 0.003, P value ($\rho = 0$) – 0.431, n = 32]

This pattern is corroborate by a weak (r = 0.15) and non-significant ($P > 0.43$) correlation between those values and plot of residuals for CH_4 production from total diets (observed) versus predicted values from the weighted individual means (Figure 2). It is important to note that no specific effect of different diets was detected in this study. This can be stated because no clusters for different diets were observed and the scatter of the paired points from different diets was found to be homogenously and randomly distributed around the equality line (Figure 1). Therefore, it can be infer that the lack of association between CH_4 production obtained from total diets and weighted individual feeds was an overall pattern that did not depend upon the evaluated diet.

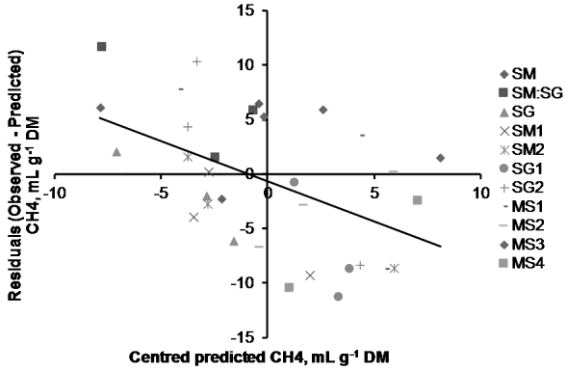

Figure 2. Plot of residuals (observed − predicted) for methane production from total diets (n = 11) versus values predicted from weighted individual feeds. The regression line in the graph represents the adjusted linear model for residual pattern. Predicted values were centred by subtracting the overall mean predicted value from each predicted value. For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of the article. For more details about the diets see Table 1. [$\hat{Y} = -0.65 \pm 0.993 - 0.74 \pm 0.234 \times X$, P value ($\beta_0 = 0$) – 0.520, P value ($\beta_1 = 0$) – 0.003, n = 32]

The purpose of this study was to use a wide range of diets varying in F:C ratios and chemical composition taken from two grazing and one feedlot trial for evaluating CH_4 production *in vitro* from total diets and calculated based on weighted mean of individual feeds. The *in vitro* method used in the current study has been used for measuring CH_4 production in the literature for other purposes, *e.g.* the effect of CH_4 inhibitors and CH_4 production (Danielsson *et al.*, 2014). Ramin and Huhtanen (2012) found a high correlation between actual CH_4 production and predicted CH_4 production based on volatile fatty acids stoichiometric equations from their *in vitro* gas production system (r = 0.97).

Assuming a gross energy (GE) concentration of 18.5 MJ kg^{-1} DM, the average proportion of CH_4 energy as a proportion of GE used in the present study was 6.5%. This value is close to observed *in vivo* values at the production level of intake in dairy cows (Yan *et al.*, 2000). The calculated average CH_4 as a proportion of GE used in the current study were lower than the values reported by Getachew *et al*. (2005), in which they were between 8–9% of GE intake. One reason could be due to the lower *in vivo* digestibility of diets used in the current study for our *in vitro* evaluation that will influence CH_4 as a proportion of GE intake.

Digestibility of feeds either individual or in a mixed form (complete diet) does not only depend on the physical constraints and chemical composition but also depends on their interaction when feeds are mixed. According to Detmann *et al.* (2005, 2008), the ruminal degradation of structural carbohydrates from forages should be seen as a second order process, as the microbial activity on fiber depends on both feed and medium characteristics. This is of great relevance for this study because all diets evaluated in the present study were obtained from tropical conditions (Silva, 2012; Costa e Silva *et al.*, 2013) in which fiber represents the main energy source for cattle production. This could be one reason for the differences in CH_4 production between observed and predicted values from individual feeds observed in the current study. Other factors such as differences in digestibility of diets and feed quality could also be a reason making this discrepancy. The associative effect of feeds on diet digestibility was also reported by Huhtanen (1991), Moss *et al*. (1992) and Detmann *et al*. (2005).

It is often assumed that energy values of feeds are additive and that there are no interactions when they are mixed. For instance, calculation of energy for dairy cattle adopted by NRC (2001) takes into account only chemical composition of feeds and that the only adjustment for energy content is based on intake level and therefore no interactions are considered. However, that might not always be true (Huhtanen, 1991). In this case, different feed ingredients of a diet can influence the proportion of fermentation end products such as volatile fatty acids and gases. Moss *et al*. (1995) reported a significant increase on CH_4 as a proportion of GE when the proportion of barley concentrate was increased from 0 to 75% in sheep fed grass silage. The main change in rumen fermentation pattern was an increase in butyrate, whereas both acetate and propionate decreased with increased concentrate. In feed lot type diets in which extreme levels of concentrate are fed (around 90%) the amount of CH_4 as a proportion of GE ranges from about 2–4% and the proportion of propionate also increases to the extent of decreased acetate and butyrate (Johnson and Johnson 1995). Other factor that could alter rumen fermentation pattern is the inclusion of diets with high fat content (Beauchemin *et al.*, 2009).

Rumen retention time could also affect CH_4 production, as increased intake declines CH_4 as a proportion of GE due to a faster passage and smaller retention time of feed particles (Ramin and Huhtanen, 2013). At the same time of increased passage, microbial cell yield will increase per unit of energy fermented by diluting maintenance expenditure (Russell *et al*., 1992). Interaction of different feeds in the rumen can change the degradation rate of fiber and modify the overall stoichiometry of the ruminal fermentation. Associative effects have occurred when the apparent digestibility of a mixture does not equal the sum of the separately determined digestibilities of its components (Mould, 1988). The same trend was found in the present study for CH_4 production, as the sum of weighted mean of CH_4 production from individual feeds was not the same as the values obtained from total diets.

When the number of feeds in a diet is increased there will be a direct effect on the equilibrium and on the relative participation of the different microbial populations in the rumen (Russell, 2002). Accordingly, the metabolic pathways of energy production in the rumen would be intensely changed, including the dynamics and amount of hydrogen production (e.g., acetate to propionate production rate) as well as the metabolic pathways for hydrogen sinks (e.g., acrylate to succinate pathways for propionate production). From this, it

could be understood that all the dynamics and the equilibrium of hydrogen incorporation in either NADH or CH_4 molecules shall be affected by diet composition and interactions caused by the presence of different feeds in the diet.

Conclusion

From the results obtained here, it can be concluded that the weighted sum of individual feeds cannot be used as a proxy for the estimation of CH_4 production from total mixed diets in *in vitro* conditions.

Conflict of interests

The authors declares that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

To Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, INCT Ciência Animal, CNPq and FAPEMIG, for financial support. To Dr. Luiz Costa e Silva and Dr. Aline Silva, for providing the feed samples.

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