IMPROVING THE PREDICTION OF METHANE PRODUCTION DETERMINED BY *IN VITRO* GAS PRODUCTION TECHNIQUE FOR RUMINANTS*

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Abstract

Twelve feedstuffs (cereals, fibrous byproducts, protein-rich byproducts and forages) were determined for methane (CH_4) production by the *in vitro* gas production technique (IVGPT) and were correlated with their chemical compositions to predict enteric CH_4 originating from these feedstuffs in ruminants. Corn, soybean hull, soybean meal and corn silage generated the highest CH_4 production from their respective categories. The average CH_4 production of fibrous byproducts (44.6 ml/g DM incubated) was significantly higher than that of cereals (40.3 ml/g DM incubated), forages (33.3 ml/g DM incubated) and protein-rich byproducts (31.0 ml/g DM incubated) after the 48-h incubation ($P \leq 0.05$). The highest average total volatile fatty acid (VFA) concentration was determined in cereals (53.6 mM). The acetate to propionate ratio was significantly lower in cereals when compared with other categories of feedstuff ($P \leq 0.05$). The correlation analysis showed that *in vitro* true digestibility (IVTD) positively correlated with the CH_4 production in all four categories of feedstuffs ($P \leq 0.05$). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) content positively correlated with CH_4 production in every category of feedstuffs except cereals. The starch content negatively correlated with CH_4 production for fibrous and protein-rich byproducts ($P \leq 0.05$), but it positively correlated with CH_4 production for forages ($P \leq 0.05$). The CH_4 production was predicted more accurately by the equations proposed for each category ($R^2 = 0.944, 0.876, 0.942$ and 0.915 for cereals, fibrous and protein-rich byproducts and forages, respectively) than for the unclassified feedstuffs ($R^2 = 0.715$). In conclusion, the contribution of individual chemical composition to CH_4 production differed depending on the category of feedstuffs. The precision of CH_4 prediction could be substantially improved by classifying feedstuffs into categories according to their chemical composition, and selecting the appropriate predictors for each category. Information about the CH_4 output of these feedstuffs will be useful in formulating low CH_4 -producing diets for ruminants.

Key words: ruminants, feedstuffs, *in vitro* gas production technique, methane, prediction

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Ruminants depend on microorganisms to ferment plant cell wall polysaccharides into energy sources, such as volatile fatty acids (VFA). However, microbial fermentation in the rumen also produces CH_4 as an unfavorable byproduct, which represents a significant energy loss to the host animal (McAllister et al., 1996). Johnson and Johnson (1995) suggested that the energy lost as CH_4 in ruminants ranges from 2% to 12% of the gross energy intake. Therefore, decreasing CH_4 production represents a potential improvement in feed efficiency for the host animal for short-term economic and long-term environmental benefits.

Dietary manipulation is the most efficient way to reduce methanogenesis in the rumen (Waghorn and Hegarty, 2011). Ruminal CH_4 production mainly depends on the chemical compositions, the nature of carbohydrates fermented in the diet such as cellulose, hemicelluloses and soluble residues, and the intake and digestibility of nutrients (Takahashi, 2001). Fermentation of plant materials with high nonstructural carbohydrates (NSC) results in a lower CH_4 production, a decrease in the molar proportion of acetate and an increase in the molar proportion of propionate (Johnson and Johnson, 1995). On the contrary, ruminal fermentation with diets containing a high amount of plant cell wall components produces a higher molar proportion of acetate than propionate and CH_4 production. Jentsch et al. (2007) reported that digestible ADF, cellulose and hemicellulose are important fiber fractions influencing CH_4 production in the rumen, and Estermann et al. (2002) observed that the CH_4 production positively correlated with the digestible NDF intake in cows. Therefore, increasing dietary starch content has been suggested as a potential option to reduce ruminal CH_4 production (Hristov et al., 2013). However, increasing the amount of rapidly fermentable starch in the diet may raise the risk of ruminal acidosis because of the increase of VFA production beyond the buffering and absorptive capacity of the rumen, leading to negative consequences on fiber degradation and production of dairy cows (Dijkstra et al., 2012). Protein degradation also associated with lower CH_4 production than the fermentation of carbohydrates (Jentsch et al., 2007).

Since *in vivo* studies of methanogenesis in ruminants are time consuming, and require specialized facilities, *in vitro* ruminal fermentation techniques have been applied to determine CH_4 production from forages with various compositions to allow an estimation of enteric CH_4 production from ruminants. Blümmel et al. (2005) and Bhatta et al. (2007) proposed that the *in vitro* batch fermentation techniques reflect the *in vivo* conditions for identifying CH_4 production of ruminant diets. The *in vitro* gas production technique (IVGPT) has been widely used to study the extent and the rate of microbial fermentation in the gastrointestinal tracts of ruminants and nonruminants (Navarro-Villa et al., 2011; Chen et al., 2013), and it has been applied to study the fermentation kinetics and CH_4 production associated with contrasting legumes and grasses, respectively (Lovett et al., 2004; Kim et al., 2013). Relationships between chemical composition and CH_4 production of feedstuffs and diets have been reported by *in vivo* and *in vitro* studies (Santoso and Hariadi, 2009). Equations have been proposed by many researchers to predict CH_4 production and to elucidate the factors affecting the CH_4 output of feedstuffs or diets (Singh et al., 2012; Ramin and Huhtanen, 2013). However, most of the equations presented by previ-

ous studies conducted with the same type of feedstuffs with similar chemical compositions (Ramin and Huhtanen, 2013) cannot be applied to a wide range of feedstuffs varying in chemical constituents. Information on relationships between CH₄ production and chemical composition of feedstuffs classified according to their composition into cereals, fibrous and protein-rich byproducts and forages is almost absent in the literature. Therefore, the objective of this study was to estimate CH₄ production of feedstuffs using an *in vitro* gas production technique, and to improve the accuracy of CH₄ prediction by classifying feedstuffs according to their composition for subsequent use in formulating low CH₄-producing diets for ruminants.

Material and methods

Feedstuffs and chemical analysis

Twelve most common used feedstuffs in Taiwan, including barley, corn, wheat, beet pulp, soybean hull, wheat bran, dry distiller's grain with solubles (DDGS), soybean meal, rapeseed meal, alfalfa hay, Bermuda hay and corn silage, were oven dried at 55°C for 48 h, and ground through a 1 mm screen. Samples for each feedstuffs collected from five different batches were analyzed individually for their chemical composition as statistical replicates (n=5). Each sample of feedstuffs was analyzed in triplicate for dry matter (DM; 976.63), nitrogen (N; 984.13), ether extract (EE; 930.39) and ash (942.05) according to AOAC (1995), and crude protein (CP) was calculated as $6.25 \times N$. Acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) analyses were determined using the filter bag technique with a Fiber analyzer (ANKOM Technology, Fairport, NY, USA) based on the analytical method of Van Soest et al. (1991). Heat stable alpha amylase and sodium sulfite were used in NDF determination. Starch content was determined by a total starch analysis kit (Megazyme, K-TSTA). The *in vitro* true digestibility (IVTD) was determined using Daisy incubator (ANKOM Technology, Fairport, NY, USA), and the incubation buffer and procedure were conducted as described in Getachew et al. (2004). Ground feedstuffs (0.5 g per bag) were weighed into triplicate bags, heat sealed and placed in digestion jars. At the end of the 48 h incubation, jars were soaked in ice water, and bags rinsed four times with distilled water. After that, bags were placed in an ANKOM fiber analyzer and boiled in neutral detergent solution for 75 min. Bags were removed, soaked twice in acetone for 5 min and dried at 100°C for 6 h. The IVTD was calculated as the difference in the DM incubated and in the residual DM after neutral detergent treatment. Feedstuffs were preliminarily classified into three groups including cereal grains, byproducts and forages according to their sources. Since the agricultural byproducts have varying chemical constituents, they were further classified into fibrous and protein-rich byproducts according to their chemical composition. Byproducts containing NDF content of higher than 300 g/kg DM and CP contents of less than 200 g/kg DM were assigned to the fibrous byproducts; a CP content greater than 200 g/kg DM were assigned to protein-rich

byproducts. In the end, the feedstuffs assayed in this study were grouped into four categories including cereals (barley, corn and wheat), fibrous byproducts (beet pulp, soybean hull and wheat bran), protein-rich byproducts (DDGS, soybean meal and rapeseed meal) and forages (alfalfa hay, Bermuda hay and corn silage).

***In vitro* gas production technique**

The *in vitro* gas production technique (IVGPT), as described by Pellikaan et al. (2011), was used in this study. Gas production incubations were performed as described by Cone et al. (1996). Rumen content and fluid were obtained from two rumen-cannulated Holstein dairy cows (534 ± 15 body weight). Cows were fed *ad libitum* with a total mixed ration (TMR) twice daily in equal amounts at 07:00 and 18:00 h according to their requirements (NRC, 2001). The TMR consisted of 448 g/kg corn silage, 37 g/kg alfalfa hay, 20 g/kg Bermuda hay, 123 g/kg DDGS, 87 g/kg soybean hull, 25 g/kg wheat bran, and 260 g/kg concentrate. The composition of the concentrate was 542 g/kg corn, 325 g/kg soybean meal, 34 g/kg fish meal, 40 g/kg molasses, 7 g/kg salt, and 52 g/kg mineral plus vitamin premix as fed basis. The protocol for animal care was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University. The rumen content and fluid were collected via a rumen cannula from each cow at 2 h after the morning feeding of concentrate, and transported to the laboratory within 30 minutes. The content and fluid were pooled, homogenized and strained through four layers of cheesecloth into a serum bottle with an O₂-free headspace. Particle-free fluid was mixed with the prewarmed (39°C) anaerobic buffer solution, as described by Pellikaan et al. (2011) in the proportion 1:2 (v/v), and kept at 39°C in a water bath used as inoculum in the *in vitro* gas production incubations. All procedures were kept under anaerobic conditions by a constant stream of CO₂.

Inoculum (60 ml) was added into a serum bottle containing 400 mg of substrate under CO₂ flushing and was incubated at 39°C for 48 h. Blanks containing only inoculum were systematically included for each batch of *in vitro* incubation to correct values for gas production released from endogenous substrates as described by Cone et al. (1996). Bottles were attached to an automated pressure estimation system. The total gas production was measured and recorded by a pressure transducer at 2-h intervals. Overall, fifteen batches of IVGPT were performed in this study. Three different batches of IVGPT were performed as replicates for each feedstuff sample. Each batch of IVGPT was conducted in a separate single run, which contained 13 bottles, including twelve feedstuffs and a blank (one bottle for each).

Analytical procedures

During the incubation, gas samples (0.1 ml) were collected from the headspace of each bottle by an auto sampler for the determination of gas composition at 2-h intervals, and the CH₄ concentration was immediately determined by gas chromatography (Perkin Elmer, Clarus 400) equipped with a thermal conductivity detector and a column packed with Carboxen 1000 (60–80 mesh) (Supelco). Cumulative CH₄ production was calculated as the sum of the increased CH₄ in headspace and the amount of CH₄ vented from the bottle that produced in each time interval:

$$M = \sum_{i=1}^n \{V_H(C_{i-1}) + V_{Ri}C_i\}$$

where:

M – cumulative CH_4 production (ml/g);

V_H – bottle headspace volume (ml);

C_i and C_{i-1} – CH_4 concentration in the bottle headspace gas at time i and $i-1$, respectively;

V_{Ri} – the amount of gas (ml) released from the bottle between time i and $i-1$.

At the end of the incubation, the bottles were uncapped and soaked in ice to stop the microbial fermentation. A sub-sample (1.0 ml) of the culture solution from each bottle was transferred to 15 ml centrifuge tubes containing 4.0 ml 30% (w/v) metaphosphoric acid and centrifuged at $14,000 \times g$ for 10 min to precipitate particles. The supernatant was transferred into microcentrifuge tubes and stored at -20°C for later analysis of volatile fatty acid (VFA) concentrations, which were determined by gas chromatography (Clarus 580, Perkin Elmer) equipped with a flame ionization detector and capillary column (Nukol 15 m $\times$ 0.25 mm $\times$ 0.25 μm , Supelco). Acetic, propionic, butyric and valeric acids were expressed as molar proportions relative to the total VFA (mmol/mol).

Following the collection of the supernatants samples for VFA analyses, the contents of the serum bottles were transferred and rinsed with distilled water into pre-weighed 50 ml centrifuge tubes, and centrifuged twice at $500 \times g$ for 10 min at 4°C . The supernatant was discarded, and the residue was dried at 55°C for 48 h and weighed to estimate DM and OM disappearance as described in Meale et al. (2012).

Statistical analyses

The following model, with the feedstuff as fixed factor and the batch as random factor, was used for chemical composition, IVTD, gas production, and VFA concentration analysis:

$$Y = \mu + M_i + B_j + \varepsilon_{ij}$$

where:

Y – the parameter to be tested,

μ – the overall mean,

M_i – the effect of feedstuff ($i = 1, \dots, 12$),

B_j – the random effect of the batch ($j = 1, \dots, 5$),

ε_{ij} – the error term.

Differences between the main effects were analyzed using the Tukey multiple comparison method in the general linear model (GLM) procedure (SAS, 2006), with effects considered significant at a probability value of $P \leq 0.05$. In addition, to test the effect of classes of feedstuffs (cereal, fibrous byproduct, protein-rich byproduct and forage), pre-determined single degrees of freedom orthogonal contrasts were generated using the CONTRAST option (SAS, 2006). In order to quantify the relationship between chemical constituents, IVTD and CH_4 production, correlation analysis was

conducted using the PROC CORR statement in SAS. The forward stepwise linear regression analysis was used to develop predictive equations for CH₄ production using PROC REG of SAS (2006).

Results

Chemical composition and IVTD of feedstuffs

Table 1 summarizes the chemical composition and *in vitro* true digestibility (IVTD) of cereals, fibrous and protein-rich byproducts and forages. As expected, there was a large variation in the chemical composition among the categories of feedstuffs. Cereal feedstuffs had the lowest average fiber content (147, 36.6 and 9.96 g/kg DM in NDF, ADF and ADL, respectively) ($P \leq 0.05$). The highest average starch content was determined in cereal feedstuffs ($P \leq 0.05$), and the lowest average content in starch was observed in protein-rich byproducts, which varied from 4.21 g/kg DM in SBM to 49.6 g/kg DM in DDGS. The average CP content in protein-rich byproducts was the highest (370 g/kg DM). The average IVTD determined in cereal feedstuffs was the highest (898 g/kg), followed by that in protein-rich and fibrous byproducts (837 g/kg and 814 g/kg, respectively). The lowest average IVTD was determined in forages ($P \leq 0.05$).

Volatile fatty acid production of feedstuffs

The VFA concentration and proportion of feedstuffs incubated with diluted rumen fluid for 48 h are presented in Table 2. The highest average total VFA concentration was determined in cereals (53.6 mM) ($P \leq 0.05$); the lowest was observed in protein-rich byproducts (26.5 mM). The acetic acid proportions and acetic to propionic acid ratio (C2/C3) were significantly lower, but the proportion of propionic acid was significantly higher in cereals when compared with fibrous and protein byproducts and forages ($P \leq 0.05$). The proportion of isovaleric and valeric acid in protein-rich byproducts was significantly higher than for cereal, fibrous byproducts and forages ($P \leq 0.05$).

Total gas production of feedstuffs

The total gas and CH₄ productions of feedstuffs incubated with diluted rumen fluid for 48 h are shown in Table 3. The total gas production of feedstuffs varied from 234 ml/g DM incubated for alfalfa hay to 398 ml/g DM incubated for corn after 48 h of incubation. Cereal feedstuffs produced the highest average total gas production, followed by fibrous byproducts, forages and protein-rich byproducts after 48 h of incubation (390, 328, 258 and 245 ml/g DM incubated, respectively). The average total gas production either expressed in ml per gram DM or OM of incubated feedstuffs was consistently higher in cereals and fibrous byproducts than in protein-rich byproducts and forages ($P \leq 0.05$) whereas the total gas production of forages expressed in ml per gram DM or OM disappeared was similar to the amount produced by cereal feedstuffs ($P \geq 0.05$).

Table 1. Chemical composition (g/kg DM) and *in vitro* dry matter digestibility (IVTD, g/kg) of feedstuffs (n = 5)

Item ³	Cereal			Fibrous byproduct			Protein-rich byproduct			Forage			Mean	SEM ²			
	barley	corn	wheat	Mean	beet pulp	soybean hull	wheat bran	Mean	DDGS ¹	SBM	rapeseed meal	Mean			Forage		
															alfalfa hay	Bermuda hay	corn silage
DM (g/kg)	933	891	889	904 b	917	896	893	902 b	928	889	913	910 b	948	968	925	947 a	10.2
OM	983	989	985	986 a	933	959	950	947 b	960	940	925	942 b	913	924	942	926 c	6.53
EE	24.8	47.2	23.9	31.9 b	8.46	40.8	75.0	41.4 ab	139	19.3	35.0	64.4 a	9.83	11.3	23.6	14.9 b	19.0
CP	124	81.8	140	115 b	72.8	148	182	134 b	300	465	346	370 a	161	99.7	87.9	116 b	28.6
NDF	200	84.9	156	147 c	382	650	416	483 a	298	171	253	241 b	463	647	569	560 a	49.1
ADF	36.2	25.8	47.9	36.6 c	262	468	139	290 a	127	160	158	148 b	359	330	338	342 a	42.4
ADL	9.07	8.81	12.0	9.96 c	53.1	22.6	36.0	37.2 b	22.7	23.0	59.8	35.2 b	94.6	69.1	50.8	71.5 a	8.86
Starch	578	680	621	626 a	10.1	16.4	236	87.6 bc	49.6	4.21	6.42	20.1 c	49.0	71.5	360	160.2 b	60.0
IVTD	898	893	904	898 a	866	864	713	814 b	736	955	821	837 b	622	601	635	619 c	24.3

¹DDGS – dry distiller's grain with solubles; SBM – soybean meal.²SEM – standard error of the mean.³DM – dry matter; OM – organic matter; EE – ether extract; CP – crude protein; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; IVTD – *in vitro* true digestibility.a, b, c – means in the same row among the categories of feedstuffs with different letters are significantly different ($P \leq 0.05$).

Table 2. The VFA production (mM) and molar proportion of feedstuffs incubated with diluted rumen fluid for 48 h (n = 5)

Item	Cereal			Mean		Fibrous byproduct			Mean		Protein-rich byproduct			Mean		Forage			Mean		SEM ²
	barley	corn	wheat			beet pulp	soybean hull	wheat bran			DDGS ¹	SBM	rapeseed meal			alfalfa hay	Bermuda hay	corn silage			
Total VFA	58.5	59.6	42.7	53.6 a	52.6	41.2	41.2	30.1	41.3 b	26.9	32.6	20.1	26.5 c	39.8	30.3	34.4	34.8 bc	1.74			
Molar proportion																					
C2 ³	0.648	0.601	0.652	0.634 c	0.690	0.675	0.661	0.675 a	0.640	0.640	0.634	0.645	0.640 bc	0.681	0.678	0.634	0.664 ab	0.006			
C3	0.229	0.259	0.218	0.235 a	0.197	0.202	0.206	0.202 b	0.223	0.196	0.196	0.198	0.206 b	0.186	0.191	0.231	0.203 b	0.003			
Iso-C4	0.010	0.019	0.016	0.015	0.012	0.018	0.018	0.016	0.017	0.018	0.018	0.017	0.017	0.016	0.016	0.019	0.017	0.001			
C4	0.079	0.081	0.080	0.080 a	0.070	0.074	0.078	0.074 b	0.072	0.089	0.089	0.088	0.083 a	0.071	0.078	0.076	0.075 b	0.003			
Iso-C5	0.024	0.028	0.026	0.026 bc	0.018	0.019	0.026	0.021 c	0.031	0.044	0.044	0.034	0.036 a	0.031	0.026	0.030	0.029 b	0.001			
C5	0.010	0.012	0.008	0.010 b	0.013	0.012	0.011	0.012 b	0.017	0.019	0.019	0.018	0.018 a	0.015	0.011	0.010	0.012 b	0.001			
C2/C3	2.83	2.32	2.99	2.71 b	3.48	3.34	3.21	3.34 a	2.87	3.23	3.26	3.12 a	3.66	3.55	2.74	3.30 a	0.094				

¹DDGS – dry distiller’s grain with solubles; SBM – soybean meal.

²SEM – standard error of the mean.

³C2 – acetic acid; C3 – propionic acid; Iso-C4 – isobutyric acid; C4 – butyric acid; Iso-C5 – isovaleric acid; C5 – valeric acid; C2/C3 – acetic acid to propionic acid ratio.
a, b, c – means in the same row among the categories of feedstuffs with different letters are significantly different (P≤0.05).

Table 3. Total gas and methane production of feedstuffs incubated with diluted rumen fluid for 48 h (n = 5)

Item	Cereal			Mean	Fibrous byproduct			Mean	Protein-rich byproduct			Mean	Forage			Mean	SEM ²
	barley	corn	wheat		beet pulp	soybean hull	wheat bran		DDGS ¹	SBM	rapeseed meal		alfalfa hay	Bermuda hay	corn silage		
Total gas																	
DMI ³	386 ab	398 a	389 ab	390 A	379 ab	366 b	250 de	328 B	255 de	263 d	219 f	245 C	234 ef	237 ef	300 c	258 C	9.54
DMD	429 b	446 ab	431 b	434 A	437 b	424 b	351 de	402 B	346 e	275 f	266 f	296 C	377 cd	394 c	472 a	415 AB	10.3
OMi	392 a	402 a	395 a	396 A	406 a	382 a	264 c	347 B	265 c	280 c	236 d	260 C	257 cd	256 cd	318 b	278 C	9.98
OMD	433 bc	449 ab	432 bc	437 A	460 ab	434 bc	353 e	413 A	348 e	291 f	280 f	306 B	395 d	413 cd	475 a	428 A	10.1
Methane																	
DMI	40.0 d	42.1 c	39.1 de	40.3 B	46.4 b	52.0 a	36.7 f	44.6 A	25.9 i	38.1 def	29.0 h	31.0 C	32.3 g	30.0 h	37.3 ef	33.3 C	1.16
DMD	44.5 e	47.2 d	43.3 e	44.9 B	53.6 b	60.2 a	51.5 bc	54.9 A	35.3 g	39.9 f	35.3 g	36.8 C	51.9 bc	49.9 c	58.8 a	53.7 A	0.91
OMi	40.7 d	43.0 c	39.7 d	41.0 B	49.7 b	54.3 a	38.6 d	47.1 A	27.0 g	40.6 d	31.3 f	33.0 C	35.4 e	32.4 f	39.6 d	36.0 C	1.22
OMD	45.0 de	47.5 d	43.4 ef	45.2 B	56.3 b	61.6 a	51.8 c	56.3 A	35.5 g	42.2 f	37.1 g	38.3 C	54.4 bc	52.3 c	59.2 a	55.4 A	0.92

¹DDGS – dry distiller's grain with solubles; SBM – soybean meal.²SEM – standard error of the mean.³DMi – ml per g DM incubated; DMd – ml per g DM disappeared; OMi – ml per g OM incubated; OMd – ml per g OM disappeared.

a, b, c, d, e, f, g, h, i – values in the same row with different letters are significantly different (P<0.05).

A, B, C, D – means in the same row among the categories of feedstuffs with different letters are significantly different (P<0.05).

Methane production of feedstuffs

The highest CH₄ production was generated by soybean hull (52.0 ml/g DM incubated), and the lowest was produced by DDGS (25.9 ml/g DM incubated) after 48 h of incubation. Among the categories of feedstuffs, fibrous byproducts produced the highest average CH₄ production, and protein-rich byproducts produced the lowest average CH₄ production despite the unit expressed. There were variations in average CH₄ production expressed in different units especially for forages. The average CH₄ production per unit of DM or OM incubated for forages was similar to the amount produced by protein-rich byproducts, although there was no significant difference in the average CH₄ production between forages and fibrous byproducts when it was expressed in the DM or OM disappeared after 48 h of incubation.

Table 4. Correlation (r) between chemical composition and methane production (ml/g DM incubated) for different categories of feedstuffs incubated with diluted rumen fluid for 48 h

Composition	Overall (n = 60)	Cereal (n = 15)	Fibrous byproduct (n = 15)	Protein-rich byproduct (n = 15)	Forage (n = 15)
OM ¹	0.217	0.126	0.124	-0.273	0.248
EE	-0.156	0.340	-0.657 +	-0.682 x	-0.849 xx
CP	-0.388 x	-0.499 +	-0.450 +	0.429	-0.185
NDF	0.101	-0.254 +	0.656 x	0.910 xxx	0.270 +
ADF	0.230	-0.580 +	0.904 xxx	0.841 xx	0.116 +
ADL	-0.339 x	-0.701 x	-0.239 +	-0.197+	-0.381 +
Starch	-0.141	0.245	-0.907 xx	-0.778 x	0.705 x
IVTD	0.511 xxx	0.498 x	0.835 xxx	0.927 xxx	0.463 x

¹OM – organic matter; EE – ether extract; CP – crude protein; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; IVTD – *in vitro* true digestibility.

+ – P≤0.10.

x – P≤0.05.

xx – P≤0.01.

xxx – P≤0.001.

Correlation between chemical composition and methane production

Table 4 summarizes the relationship between chemical composition and CH₄ production of feedstuffs in 48 h *in vitro* fermentation. The CH₄ production positively correlated with IVTD (P≤0.01), but it negatively correlated with CP and ADL content (P≤0.05) for overall feedstuffs. The relationships between CH₄ production and other chemical compositions including EE, NDF, ADF and starch were not significant for overall feedstuffs.

Table 5. Linear regression equations to predict CH₄ (ml/g DM incubated) from chemical compositions and *in vitro* true digestibility of feedstuffs

	R ²	P-value
Overall (n = 60)		
CH ₄ = 39.96 - 0.017 NDF + 0.056 ADF - 0.214 ADL	0.440	≤0.001
CH ₄ = 60.79 - 0.027 NDF + 0.047 ADF - 0.266 ADL - 0.039 CP - 0.078 EE - 0.013 Starch	0.668	≤0.001
CH ₄ = 9.848 + 0.017 NDF + 0.034 ADF - 0.063 ADL - 0.028 CP - 0.011 EE + 0.001 Starch + 0.048 IVTD	0.715	≤0.001
Cereal (n = 15)		
CH ₄ = 28.88 + 0.018 Starch	0.105	0.183
CH ₄ = 46.57 - 0.010 NDF - 0.095 ADF - 0.130 ADL	0.823	0.036
CH ₄ = 86.98 - 0.011 NDF - 0.051 ADF - 0.215 ADL + 0.046 IVTD	0.870	0.029
CH ₄ = 76.70 - 0.033 NDF - 0.212 ADF - 0.460 ADL + 0.034 IVTD - 0.101 CP	0.944	≤0.001
Fibrous byproduct (n = 15)		
CH ₄ = 40.56 + 0.033 NDF + 0.070 ADF - 0.001 ADL	0.816	≤0.001
CH ₄ = 39.15 + 0.023 NDF + 0.055 ADF - 0.011 ADL - 0.009 Starch + 0.003 IVTD	0.841	≤0.001
CH ₄ = 34.21 + 0.039 NDF + 0.144 ADF - 0.029 ADL - 0.144 Starch + 0.005 IVTD - 0.101 CP - 0.191 EE	0.876	≤0.001
Protein-rich byproduct (n = 15)		
CH ₄ = 6.159 + 0.066 CP	0.566	≤0.001
CH ₄ = 7.017 + 0.019 NDF + 0.216 ADF - 0.123 ADL	0.892	≤0.001
CH ₄ = - 46.80 + 0.163 NDF + 0.419 ADF - 0.478 ADL - 0.052 EE - 0.225 Starch	0.939	≤0.001
CH ₄ = - 50.51 + 0.150 NDF + 0.387 ADF - 0.419 ADL - 0.065 EE - 0.131 Starch + 0.010 IVTD	0.942	≤0.001
Forage (n = 15)		
CH ₄ = - 26.26 + 0.015 NDF + 0.242 ADF - 0.251 ADL	0.698	≤0.001
CH ₄ = 25.18 + 0.008 NDF + 0.001 ADF - 0.064 ADL - 0.469 EE + 0.007 Starch	0.836	≤0.001
CH ₄ = 108.7 + 0.027 NDF + 0.019 ADF - 0.047 ADL - 0.381 EE + 0.018 Starch + 0.106 IVTD	0.915	≤0.001

NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; CP – crude protein; EE – ether extract; IVTD – *in vitro* true digestibility.

After the feedstuffs were classified by their chemical composition, IVTD remained consistently in a positive relationship with CH_4 production in all categories ($P \leq 0.05$). The negative relationship between CH_4 production and ADL content remained constant in every category of feedstuffs. However, other chemical compositions including NDF, ADF and starch content exhibited an inconsistent effect on CH_4 production among the feedstuff categories. The NDF and ADF content of feedstuffs positively correlated with CH_4 production for fibrous and protein-rich byproducts ($P \leq 0.05$), and the positive correlation also tended to occur for forages ($P \leq 0.1$). Starch content negatively correlated with CH_4 production for fibrous and protein-rich byproducts ($P \leq 0.05$), but it positively correlated with CH_4 production for forages ($P \leq 0.05$).

Linear regression equations for methane production

Linear regression equations are derived to predict CH_4 production from the chemical composition and IVTD of feedstuffs as seen in Table 5. Cell wall content, including NDF, ADF and ADL, were poor predictors of CH_4 production ($R^2 = 0.440$) in linear regression for overall feedstuffs. The inclusion of IVTD and chemical composition such as EE, CP and starch content as predictors could improve the accuracy of an equation, although this still remained at a low level for the prediction of CH_4 production ($R^2 = 0.715$).

The precision of equations for CH_4 production was greatly improved by classifying the feedstuffs into four categories according to their chemical composition. For the cereal feedstuffs, linear regression indicated that starch content alone was a poor predictor for CH_4 production ($R^2 = 0.105$). The accuracy of CH_4 prediction was sharply improved by the inclusion of NDF, ADF and ADL content of cereal feedstuffs ($R^2 = 0.823$). A further increase in the R^2 was achieved by the inclusion of IVTD and CP content as predictors of CH_4 production ($R^2 = 0.944$). The precision of CH_4 prediction for the protein-rich byproducts could be improved from 0.566 to 0.892 by replacing CP with the NDF, ADF and ADL content as predictors. The accuracy of CH_4 prediction for protein-rich byproducts and forages was largely improved by the cell wall content (NDF, ADF and ADL) combined with EE and starch content and IVTD ($R^2 = 0.915$). The cell wall content (NDF, ADF and ADL) was a good predictor for CH_4 production of fibrous byproducts ($R^2 = 0.816$), and the inclusion of starch, CP and EE content and IVTD resulted in a small improvement in the accuracy of CH_4 prediction for fibrous byproducts ($R^2 = 0.876$).

Discussion

Chemical composition and IVTD of feedstuffs

In this study, chemical compositions of feedstuffs and forages are within the range of values reported earlier (Getachew *et al.*, 2004; Pellikaan *et al.*, 2011; Kim *et al.*, 2013). Variations in chemical compositions and IVTD among the classes of feedstuffs were consistent with previous studies (Lee *et al.*, 2003; Getachew *et*

al., 2004). Higher starch content and lower fiber (NDF, ADF and ADL) content in cereals than in fibrous and protein-rich byproducts and forages were expected. Such variations in chemical composition of feedstuffs and forages are caused by factors such as crop type, variety, fertilizer, stage of harvest and environment (Lovett et al., 2004). It is also affected by the method of processing and components extraction (DePeters et al., 1997). The CP content of protein-rich byproducts (DDGS, SBM and rapeseed meal) was higher than that of cereals, fibrous byproducts and forages due to the concentrated effect of oil extraction or starch fermentation.

Volatile fatty acid production of feedstuffs

An *in vitro* gas production technique primarily evaluates the digestive rate through the amount of gas produced from feedstuffs or diets during microbial fermentation. It reflects the production of volatile fatty acids (VFA), which supply energy to ruminants (Dijkstra et al., 2005). The VFA production is influenced by the amount of substrate that can be utilized by microorganisms which is associated with the chemical composition of feedstuffs or diets (Getachew et al., 2004). In the present study, the highest total VFA production was determined in cereals (barley, corn and wheat) after *in vitro* fermentation (Table 2). This suggested that the highest amount of substrate could be provided by cereal feedstuffs as evidenced by the highest average IVTD for cereals (Table 1). Conversely, forages had the lowest total VFA production as a result of the lowest IVTD and higher undegradable cell wall content (ADL) in forages compared with other feedstuffs.

Previous studies have reported that VFA are either lipogenic (acetate and butyrate) or glucogenic (propionate and valerate) in nature. A shift in balance between lipogenic and glucogenic VFA production determines the partition of energy within the host animal (Dijkstra et al., 2005) and the availability of H_2 for methanogenesis (Bannink et al., 2006). Higher propionic acid and lower acetic and butyric acid molar proportions are associated with lower H_2 production and thus, lower CH_4 production (Janssen, 2010). Therefore, the differences in VFA profiles among the categories were determined in this study (Table 3). The acetate to propionate ratio of feedstuffs in this study is within the range of 2.0 to 4.1 as reported by Brown et al. (2002). The current study showed that the molar proportion of propionate was significantly increased, but acetate was decreased when cereal feedstuffs were used as a substrate in the *in vitro* fermentation; this finally led to lower acetate to propionate ratio than other categories, which is consistent with previous studies (McAllister et al., 1996; Getachew et al., 2004). The molar proportion of individual VFAs is affected by a number of factors, including nutrient composition and availability of the substrate, microbial species occurring and physiochemical characteristics of the rumen environment (Dijkstra et al., 2005). Getachew et al. (2004) showed that high acetate to propionate ratio is an indication of proportionally higher digestible NDF in the feeds. This is evidenced by the higher acetate to propionate ratio in alfalfa hay and beet pulp (Pellikaan et al., 2011). The high starch content of corn grain and corn hominy leads to proportionally higher propionate production, thereby decreasing the ratio of acetate to propionate (Christophersen et al., 2008).

In addition to carbohydrate fermentation, protein degradation also leads to a small proportion of VFA. The amount and proportion of VFA production from protein depends on the amino acid composition of the substrate and the extent of microbial deamination of these amino acids such as the fermentation of leucine and valine leads to isovaleric acid and isobutyric acids, respectively (Allison, 1970). This is in accordance with the higher molar proportion of isovaleric acid for protein-rich byproducts compared with other feedstuffs in the current study.

Total gas production of feedstuffs

Gas production has been suggested as a good indication of the fermentation extent of feedstuffs and diets in ruminants (Cone et al., 1996; Getachew et al., 2005). Variations in total gas and CH₄ production among the feedstuffs might be due to variations in their chemical composition. Most of the gas was generated from carbohydrates, including sugars, starch, hemicelluloses and cellulose, fermented by ruminal microorganisms. Cereals applied in this study had the highest total gas production after 48 h of *in vitro* fermentation (Table 4). On the contrary, protein-rich byproducts had the lowest average total gas production after 48 h of *in vitro* fermentation. These results were comparable with Singer et al. (2008) and suggest there were more available carbohydrates and a greater extent of ruminal fermentation for cereals compared to fibrous and protein-rich byproducts and forages. This extensive fermentation is related to higher starch and lower undegradable carbohydrate content, and is reflected in the higher IVTD and total VFA production of cereals than in other categories of feedstuffs. Both fibrous byproducts and forages contain a high level of cell wall content, but the total gas production of forages was significantly lower than fibrous byproducts as expressed in ml per gram of DM or OM incubated in the current study. These results can be explained by the difference in the nature of the carbohydrates between fibrous byproducts and forages. Singh et al. (2012) suggested that sugars, starch and NDF can be fermented more rapidly than ADF, but the lignin cannot be degraded by cellulolytic microorganisms in the rumen. Therefore, forages applied in this study comprised more lignin, resulting in less total gas produced from each gram of substrate incubated and lower IVTD than from fibrous byproducts. This difference in degradability between fibrous byproducts and forages resulted in a similar total gas production on the basis of substrate disappeared.

In spite of values in IVTD indicating that the fermentation extent was similar for fibrous and protein-rich byproducts (Table 1), the total gas production for fibrous byproducts was significantly higher than for protein-rich byproducts. A similar trend was reported by Getachew et al. (2004), who suggested lower gas production was associated with high CP content in feedstuffs. Proteins in diet were mostly released by microbial degradation as amino acids, peptides and ammonia, and eventually converted into microbial protein. Although soluble protein components are also degraded rapidly, like NSC in a diet, protein fermentation produces less gas than carbohydrate fermentation (Singh et al., 2012). Consequently, protein-rich byproducts produced less total gas than cereals and fibrous byproducts in the current study.

Methane production of feedstuffs

Methane is a proportion of the total gas produced by methanogens, the major archaeal members in the rumen that utilize H_2 as the major energy source to reduce CO_2 to CH_4 (Janssen, 2010). Hence, the production of H_2 and CO_2 positively correlated with CH_4 production during microbial fermentation of feed in rumen. The CH_4 production of feedstuffs determined in this study is within the range of values previously reported (Navarro-Villa et al., 2011; Pellikaan et al., 2011; Kim et al., 2013). The proportions of CH_4 in total gas for cereals, fibrous and protein-rich byproducts and forages in the current study were 10.3%, 13.6%, 12.7% and 13.0%, respectively. These relative productions of CH_4 are comparable to the results presented by Pellikaan et al. (2011), who reported that the relative CH_4 production of feedstuffs generally ranges from 10% to 20%.

Earlier studies suggest the quality of the diet has a major effect on CH_4 production as the relative proportions of VFA are affected by the nature and fermentation of carbohydrate as described above (Johnson and Johnson, 1995). A large proportion of H_2 and CO_2 is produced along with the acetate and butyrate, which are the principal products of cell wall content fermentation. Thus, substrates that produce a higher acetic to propionic acid ratio, result in a higher proportion of H_2 produced by cellulolytic microorganisms and a higher CH_4 production (Blümmel et al., 2005). Such variations in CH_4 production for feedstuffs during the *in vitro* fermentation period are in accordance with the findings of Santoso and Hariadi (2009), who reported that the slowly fermentable carbohydrates of feedstuffs (hemicelluloses and cellulose) are associated with higher *in vitro* CH_4 production. In contrast to the fibrous byproducts and forages, cereals contained higher rapidly fermentable carbohydrates (starch) that resulted in a lower level of CH_4 production during the incubation period, which was associated with a lower acetic to propionic acid ratio (Table 2). The relatively higher protein content in feedstuffs and diets also contributed to lower CH_4 output due to the lower H_2 and CO_2 production of protein than carbohydrates that suggested a relatively limited methanogenic potential of protein compared to carbohydrates (Singh et al., 2012). This is consistent with the result observed in the current study that protein-rich byproducts produced the lowest CH_4 production during the *in vitro* fermentation. The microbial degradability also affects the *in vitro* CH_4 production of feedstuffs (Hindrichsen et al., 2004; Navarro-Villa et al., 2011). The lower average CH_4 production relative to DM or OM incubated (CH_4 /DMi and CH_4 /OMi) associated with forages compared to fibrous byproducts was related to the less extensive *in vitro* rumen fermentation as evidenced by the lower IVTD, total VFA and total gas production, which is in agreement with Lee et al. (2003). Therefore, when the DM or OM disappeared (CH_4 /DMd and CH_4 /OMd) during incubation was accounted for, the contrasting fermentation between the fibrous byproducts and forages was insufficient to create a significant difference in CH_4 production.

The differences in the nature of carbohydrate fraction also explained the distinct pattern of CH_4 production within the categories of feedstuffs (Hindrichsen et al., 2004). Soybean hull producing the highest CH_4 production within the fibrous byproducts was related to the higher structural carbohydrates (NDF and ADF) and lower unfermentable cell wall content (ADL) compared to other fibrous byproducts

(Hindrichsen et al., 2004). Corn silage yielded the highest CH_4 production within the forages due to the extensive fermentation, which sheltered the suppression effect of NSC on CH_4 production. This is also the explanation for higher CH_4 production from alfalfa hay compared to Bermuda hay as evidenced by the relatively higher IVTD. This agrees with Singh et al. (2012) who reported legumes produced more CH_4 than grasses due to their extensive fermentation, although they contained fewer structural carbohydrates. Kim et al. (2013) suggested that higher *in vitro* CH_4 production determined from soybean meal compared to other feedstuffs in the same category was also caused by the extensive fermentation, which is consistent with the result observed in the current study. Other than the carbohydrate and protein fractions, the suppression effect of lipids on CH_4 production has been studied (Dohme et al., 2001). In this study, the lowest CH_4 production determined from DDGS partially resulted from the highest EE content, which interfered with the fiber fermentation (Dohme et al., 2001) as evidenced by lower IVTD than in other feedstuffs.

Relationship between chemical composition and methane production

Instead of the CH_4 production relative to DM disappeared, the CH_4 production relative to DM incubated was used in the correlation and regression analysis in this study to provide direct information for CH_4 prediction of feedstuffs as per gram DM ingested by ruminants (Santoso and Hariadi, 2009; Navarro-Villa et al., 2011). It will be helpful to directly compare with the result of the *in vivo* experiment, and for the subsequent use in formulating low CH_4 -producing diets for ruminants in the future.

Previous studies have proposed correlations between chemical composition and CH_4 production by *in vivo* and *in vitro* studies (Santoso and Hariadi, 2009; Navarro-Villa et al., 2011). However, information on relationships between CH_4 production and chemical composition after classifying the feedstuffs according to their composition is almost absent. In the current study, correlations analyzed by the results determined from all feedstuffs showed that the increases in CP and ADL content have a depressing effect on *in vitro* CH_4 production per unit of incubated DM (Table 4). This agrees with Singh et al. (2012), suggesting the increment of protein and undegradable cell wall fractions will reduce the *in vitro* CH_4 production. In contrast, an increase in IVTD was related to higher CH_4 production because of the higher fermentable substrates presented in the feedstuffs (Navarro-Villa et al., 2011). However, the insignificant relationship between CH_4 production and NDF, ADF or starch content for overall feedstuffs contradicted previous studies, which suggested the high CH_4 production relates to the increase in the content of degradable cell wall fractions since the cellulolytic bacteria, which favors acetate production and consequently produces more H_2 , is available for methanogenesis (Christophersen et al., 2008; Janssen, 2010). The wide range of feedstuffs used in the current study may explain the absence of a significant relationship between CH_4 production and EE, NDF, ADF or starch content.

Once the feedstuffs were classified into four groups, the effect of chemical compositions on CH_4 production was inconsistent among the categories of feedstuffs except for the IVTD, which maintained a significantly positive relationship with CH_4 production. The inconsistent effect of chemical compositions on CH_4 production was

also observed by Lee et al. (2003). Hatew et al. (2015) suggested that depressed CH_4 production may not always be linked in a high starch diet in ruminants. The CH_4 production was encouraged by NDF and ADF content in fibrous and protein-rich byproducts and forages, but it was depressed by NDF and ADF content in cereals. Compared with starch, cell wall fractions (NDF and ADF) are relatively hard to degrade by rumen microbes, and they cause the negative relationship to the CH_4 production for cereal feedstuffs (Lee et al., 2003). Although research has shown that the CH_4 production negatively correlated with lipid content of feedstuffs (Dohme et al., 2001; Soliva et al., 2003), the suppressing effect of EE on CH_4 production was probably sheltered by the large amount of fermentable carbohydrates in cereals after the classification. On the contrary, there was a more-pronounced suppressing effect of EE on CH_4 production observed in protein-rich byproducts and forages than in cereal and fibrous byproducts. This was probably due to the lack of fermentable carbohydrates in the former categories. These results suggest that the *in vitro* CH_4 production of feedstuffs is not affected by a single chemical composition. They are affected by the interactions existing among the chemical compositions, and the contribution of individual chemical composition to *in vitro* CH_4 production was different depending on the category of feedstuffs.

Linear regression equations for methane production

Equations have been proposed by many researchers to predict *in vivo* and *in vitro* CH_4 production of feedstuffs or diets for ruminants (Hindrichsen et al., 2004; Singh et al., 2012; Ramin and Huhtanen, 2013). Most of these proposed equations were developed within the same type of feedstuffs with high precision in CH_4 prediction (Hindrichsen et al., 2004; Singh et al., 2012). However, the current study, using a broad range of feedstuffs, developed the equations based on the results determined from all feedstuffs and showed that the coefficient of CH_4 prediction ($R^2=0.715$) was lower than in previous studies (Singh et al., 2012; Ramin and Huhtanen, 2013) due to the large variations in chemical composition among the categories of feedstuffs.

After the classification, the regression coefficient was dramatically improved by selecting the suitable predictors for each category according to the results of correlation analysis since different effects of each chemical composition on CH_4 production were observed among the categories of feedstuffs as described above. The differences between the unclassified and classified feedstuffs in CH_4 predictions suggest that interactions existed between the chemical composition and the category of feedstuffs. The regression coefficient of the equations proposed in this study showed that CH_4 production of each category of feedstuffs could be accurately predicted by their chemical compositions and IVTD. Cell wall fractions (NDF, ADF and ADL) and IVTD of feedstuffs were the major predictors for CH_4 production.

In conclusion, the contribution of individual chemical composition to *in vitro* CH_4 production differed depending on the category of feedstuffs. Classifying the feedstuffs according to their chemical composition and choosing the optimal chemical compositions as the predictors for each category of feedstuffs substantially improved the accuracy of CH_4 predictions. The developed equations for each category of feedstuffs could accurately and rapidly predict the CH_4 production. The current

study provides preliminary information on CH₄ production of feedstuffs that could be used to optimize diet formulation for ruminants by selecting feedstuffs with low CH₄ output from different categories of feedstuffs in the future. Further *in vitro* and *in vivo* studies with a broader range of feedstuffs differing in constituents for each category are necessary to improve the accuracy and representation of the predictive equations before their practical application.

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