

## PAPER

## Methane yield from dry and lactating cows diets in the Po Plain (Italy) using an *in vitro* gas production technique

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### Abstract

The aim of the study was to measure total gas and methane (CH<sub>4</sub>) production from 30 total mixed rations (TMRs) fed to dry and lactating cows in 20 commercial dairies in the Po Plain (Italy). Samples were analysed for chemical composition, *in situ* 48 h fibre digestibility (NDFD) and *in vitro* gas production (GP) and CH<sub>4</sub> concentration at 24 h of incubation. NDFD of TMRs from dry and lactating cows was identical (52.1%; P=0.995). The TMRs fed to dry and lactating cows differed for GP (43.0 and 54.4 mL/200 mg DM, respectively; P<0.001) and CH<sub>4</sub> (7.24 and 8.85 mL/200 mg DM, respectively; P=0.001), but not for CH<sub>4</sub> as percentage of GP (24.3 and 23.7%, respectively; P=0.286). The data were analysed dividing the TMRs into quartiles depending on starch:ADF ratio; the average ratios of the groups 1, 2, 3 and 4 were 37, 77, 116 and 138, respectively. Increasing starch:ADF ratio determined a higher GP: 42.2, 51.4, 55.1 and 56.2 mL/200 mg DM for groups 1, 2, 3 and 4, respectively (P<0.001), whilst CH<sub>4</sub> (mL/200 mg DM) was lower (P<0.001) for group 1 (7.12) in comparison with the others (8.82 on average). Acetate (% on total VFA) decreased for increasing starch:ADF ratio (P=0.009), whereas butyrate tended to increase (from 8.11 to 9.23% on total VFA; P=0.069) and the acetate:propionate ratio to decrease (from 3.35 to 3.09; P=0.082). The lack of a higher CH<sub>4</sub> concentration in GP from diets richer in fibre might be attributed mainly to the relatively short time of incubation.

### Introduction

Methane (CH<sub>4</sub>) is one of the most important greenhouse gases (GHG) emitted from anthro-

pogenic sources with a contribute to climatic change and global warming 21 times more effective than carbon dioxide (CO<sub>2</sub>) (IPCC, 2007). Agriculture accounts for approximately 10 to 12% of the estimated anthropogenic greenhouse effect, producing about 45% of overall anthropogenic CH<sub>4</sub> emissions, with a wide range of uncertainty in the estimates of both the agricultural contribution and the anthropogenic total (IPCC, 2007). Domestic ruminants are the main responsible of these emissions, which derive primarily from enteric fermentations (Ellis *et al.*, 2007) but also from the fermentation of organic matter in manure. Methane emissions from gastrointestinal tract are an indirect result of ruminal fermentation processes performed by microorganisms that digest and ferment carbohydrates into energy sources such as volatile fatty acids (VFA) (Getachew *et al.*, 2005b; Hariadi and Santoso, 2010). Ruminal CH<sub>4</sub> production is energetically a wasteful process (Getachew *et al.*, 2005b), since the proportion of feed converted to CH<sub>4</sub> represents a loss of approximately 2% to 12% of the gross energy intake (Johnson and Johnson, 1995). In the European Union approximately two-thirds of annual regional methane emissions, amounting to about 6.8 million tonnes, have been attributed to enteric fermentation in ruminants (Moss *et al.*, 2000). Among European countries, Italy has a legally binding commitment under the Kyoto Protocol to reduce GHG emissions by 6.5% below base-year levels, on average, over the first commitment period, 2008-2012. There is still uncertainty about the real impact of animal husbandry on GHG: from the first very high estimates (18%) of Steinfeld *et al.* (2006) lower impacts (12 to 13%) were reported by IPCC (2007), even lower (3 to 8%) by Capper *et al.* (2009) and eventually to an impact of 3% reported by Pulina *et al.* (2011) for Italy.

The amount of enteric CH<sub>4</sub> production is determined by many factors including: dry matter intake (Reynolds *et al.*, 2010; Tamburini *et al.*, 2010), diet characteristics such as supplementation with lipids (Grainger and Beauchemin, 2011) and type and level of carbohydrates fermented (Ellis *et al.*, 2007), technological processes applied to feeds, and management practices that manipulate ruminal population (number of protozoa and methanogens) (Odongo *et al.*, 2007). The adoption of these factors can reduce CH<sub>4</sub> production improving nutrient and energy utilization efficiency and decreasing environmental pollution. Hence, it is essential to be able to quantify the CH<sub>4</sub> produced from ruminal fermentation of different diets fed to dairy cows. Methane emissions are determined using both

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*in vivo* and *in vitro* assays. However, direct techniques are expensive, require complex equipment and are labour expensive and time consuming. Consequently, *in vitro* gas production (GP) method is an alternative relatively labour saving and affordable technique (Getachew *et al.*, 2005a) which allows different diets to be tested simultaneously, alone or in presence of additives and inhibitors, for their effect on methanogenesis.

Due to lack of measured data on methane enteric emission by dairy cows in the Po Plain (Lombardy, Italy), aim of this study was to measure CH<sub>4</sub> production from total mixed rations (TMRs) fed to dairy cows in commercial dairies in this area using an *in vitro* GP technique.

### Materials and methods

#### Sample collection and preparation

Thirty TMRs (22 and 8 samples for lactating and dry cows, respectively) were collected from 20 Italian Friesian commercial dairy farms in the Po plain (Italy) in the period November-December 2010. Average milk yield of cows in the selected farms was 30±4.6 kg/d. Samples were dried at 55°C in a forced air oven for 48 h and then ground to pass a 1 mm sieve using a Wiley mill (Pulverisette, Fritsch, Idar-Oberstein, Germany).

## Chemical analysis, dry matter and fibre digestibility

Dry matter (DM) was determined following the AOAC procedure (AOAC, method 945.15, 1995), and organic matter was calculated as weight lost upon ignition at 600°C (AOAC, method 942.05, 1995). The crude protein (CP) content was determined by the macro-Kjeldahl technique (AOAC, method 984.13, 1995) using a 2300 Kjeltac Analyzer Unit (FOSS, Hillerød, Denmark). Ether extract was determined following the method 920.29 of the AOAC (1995). Neutral detergent fibre (NDF) was determined according to Mertens (2002), with addition of sodium sulphite and  $\alpha$ -amylase to the neutral detergent solution. Acid detergent fibre (ADF), determined not sequentially to NDF, and acid detergent lignin (ADL) were calculated according to the method of Van Soest *et al.* (1991) using the Ankom 200 fibre apparatus (ANKOM Technology Corporation, Fairport, NY). Fibre fractions are reported on an ash-free basis. Neutral detergent insoluble crude protein and acid detergent insoluble crude protein were determined according to Licitra *et al.* (1996). Starch content was determined using Megazyme kit K-TSTA (Megazyme International Ireland Ltd., Wicklow, Ireland) for total starch assay procedure (AOAC, method 996.11, 1998). Ruminant NDF digestibility (NDFD) was determined using an *in situ* assay as reported by Spanghero *et al.* (2010) with an incubation time of 48 h. Each sample was weighed (250 mg as fed) in duplicate into Ankom F57 filter bags (ANKOM Technology Corporation, Fairport, NY, USA). Samples were incubated in 2 incubation runs repeated on different days and using for each sample 2 rumen fistulated dry Italian Friesian cows fed a ration composed of meadow hay, flaked maize and a commercial protein concentrate (forage:concentrate ratio 65:35 on DM) twice daily. After 48 h of incubation, bags were collected from the rumen, washed thoroughly using cold tap water and then analysed using the Ankom 200 fibre apparatus (ANKOM Technology Corporation, Fairport, NY) according to Mertens (2002), with addition of sodium sulphite and  $\alpha$ -amylase to the neutral detergent solution.

Animals on trial were handled as outlined by the guidelines of the 116/92 Italian law about animal welfare on experimental animals (Italian Regulation, 1992).

## *In vitro* gas production and calculations of energetic value of rations

Gas production was determined using a semi-automatic system (Theodorou *et al.*, 1994), based on the measurement of the head-

space gas pressure in the incubation bottles. Samples (250 mg as fed) were weighed in duplicate into 120 mL serum bottles. Buffered mineral solution and reducing solution were prepared according to Menke and Steingass (1988), stored in a water-bath at 39°C and purged with CO<sub>2</sub>. Rumen fluid was collected before the morning feeding from two fistulated dry Italian Friesian cows fed as previously described. At the barn rumen fluid was squeezed through a cheesecloth layer and stored into a pre-warmed thermos flask, transferred to the laboratory, strained through four layers of cheesecloth and flushed with CO<sub>2</sub>. The rumen fluid was added to the buffered mineral solution (rumen fluid:buffer solution ratio=1:2) with constant stirring, while maintained at 39°C. Thirty mL of inoculum was dispensed into the bottles containing the TMR samples, for a corresponding headspace volume of 90 mL. The procedures were conducted under anaerobic conditions, flushing the bottle headspace with CO<sub>2</sub>. The serum bottles were closed hermetically with rubber tops and placed in a shaking water-bath (75 RPM) at 39°C for 24 h. Each sample was analysed in 2 incubation runs. Headspace pressure was recorded after 2, 4, 6, 8, 10 and 24 h of incubation using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA), avoiding that headspace pressure exceeded 48 kPa to preserve the normal microbial activity, as reported by Theodorou *et al.* (1994). The gas pressure data recorded at each time-point were converted to moles of gas using the ideal gas law ( $n=p*(V/R*T)$ ), where: n: gas produced (mol), p: pressure (kPa), V: headspace volume in bottles (L), R: gas constant (8.314 L\*kPa\*K<sup>-1</sup>\*mol<sup>-1</sup>, T: temperature (K)), converted to millilitres of gas and then cumulated to obtain GP at 24 h of incubation. The GP values at 24 h were corrected for blanks and standard feeds included in each run in order to calculate the rumen organic matter digestibility (OMD, %) and the energetic value of TMRs (NE<sub>L</sub>, Mcal/kg DM) according to Menke and Steingass (1988). At each reading time, after gas pressure data recording, a fixed-volume sample of gas (5 mL/bottle) was also collected for subsequent methane analysis using gas-tight syringes fitted with needles through the bottle top. After collection, remaining gas was released by needles and the pressure was brought back to atmospheric value.

## Methane measurement

The gas composition of the headspace was determined by injecting 5 mL of gas into an Agilent 3000A micro GC gas chromatograph (Agilent Technologies, Santa Clara, CA, USA)

using N as carrier. An external standard mixture of CO<sub>2</sub> and CH<sub>4</sub> prepared by SIAD SpA (Bergamo, Italy) was used for instrument calibration. Peak areas were calculated by automatic integration. The CH<sub>4</sub> volume (mL) produced between two time points and final cumulated volume were calculated as reported by Tavendale *et al.* (2005).

## Ruminal volatile fatty acids determination

At the end of the incubation period, 10 mL of inoculum was sampled and clarified by centrifugation at 3500 × g for 10 min. After centrifugation 5 mL of supernatant was sampled and added with 25% meta-phosphoric acid at a ratio of 5 parts of inoculum to 1 part of acid. The mixture was covered, held at room temperature for 30 min and centrifuged again at 3500 × g for 10 min. A 1 mL aliquot of supernatant was pipetted into a GC auto-sampler vial containing 100 µL of internal standard solution (2 mL of 2-ethylbutyric acid in 15 mL of 90% ethanol brought to a final volume of 500 mL with deionized water), sealed and placed in an auto-sampler tray. Volatile fatty acid concentrations were determined using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) using a Nukol fused silica capillary column (30 m length; 0.25 mm diameter; 0.25 µm film thickness; Supelco, Bellefonte, PA, USA). Initial column temperature was 80°C for 2 min, increased at a rate of 5°C/min to 100°C maintained for 1 min, increased at a rate of 7°C/min to 140°C and finally increased at a rate of 15°C/min to a final temperature of 200°C, with a total running time of 21 min. Injector and FID temperatures were 220°C and 250°C respectively, with a carrier gas (He) flow of 4 mL/min and a split ratio of 50:1. VFA were identified and quantified from chromatograph peaks areas using calibration with internal standard, according to a modified procedure introduced by Moore *et al.* (2002).

## Statistical analysis

Statistical analysis was carried out using one-way variance analysis procedure of IBM SPSS Statistics 18 (IBM Corporation, Armonk, NY, USA). Data deriving from biological analyses were analysed considering the type of TMR (dry vs lactating) as factor. The Pearson correlation between the starch:ADF ratio of the all 30 diets and the GP and the CH<sub>4</sub> production was first studied. Then the entire population (30 TMRs) was divided into quartiles considering starch:ADF ratio as parameter to take into account the effects of both rapidly and slowly

fermentable carbohydrates. Data were analysed considering the quartile as main factor (fixed effect), the period (incubation run) as random effect, and their interaction. Differences among means with  $P < 0.05$  were declared significant. Subsequent *post-hoc* multiple comparisons were performed using Bonferroni test (equal variances assumed) or Dunnett (T3) test in case of heteroskedasticity of variances.

## Results and discussion

### Diets composition and chemical analysis

The feeds used to formulate dry cows TMRs were primarily meadow hay, corn silage, wheat straw and protein concentrate ( $39 \pm 14.8$ ,  $32 \pm 2.32$ ,  $26 \pm 12.5$  and  $14 \pm 9.68\%$  of total DM, respectively). For lactating TMRs there was a wide range in terms of feeds used and levels of inclusion. Corn silage ( $29 \pm 7.60\%$  of total DM), corn (both ground and high moisture,  $15 \pm 5.25$  and  $14 \pm 4.74\%$  of total DM, respectively), protein concentrate ( $20 \pm 8.63\%$  of total DM) and soybean meal ( $12 \pm 5.99\%$  of total DM) were the main ingredients included in the majority of the TMRs fed to lactating cows. The average chemical analysis of the TMRs fed to dry and lactating cows is reported in Table 1. The CP content was  $11.5 \pm 3.07$  and  $15.6 \pm 1.73\%$  DM in dry and lactating TMRs, respectively and it seems to be fairly low as required by the need to meet the European Union Nitrate Directive constraints (European Commission, 1991). The average NDF content was  $49.2 \pm 5.27$  in dry TMRs, whereas in lactating TMRs ranged from 25.5 and 41.3, with a mean value of  $32.1 \pm 4.17\%$  DM basis. Starch content of lactating TMRs ( $23.5 \pm 4.41\%$  on DM) is slightly lower than the level normally registered (28% on DM) in the Po plain area for high producing cows (Crovetto and Colombini, 2010), but non-fibrous carbohydrate (NFC) content ( $42.4 \pm 2.93\%$  on DM) is consistent with that found in previous experiments (Getachew *et al.*, 2005b; Masoero *et al.*, 2006).

The diets for both groups appear well balanced to meet the nutritional requirements of the animals in the first phase of the dry period and adequate for lactating cows producing 30 kg milk daily, on average (Fox *et al.*, 2004).

### Nutritive value and methane production of the total mixed rations for dry and lactating cows

The different composition and analysis of

the two groups of TMRs (dry and lactating) significantly influence GP, OMD and consequently the energetic value (Table 2). There were differences between dry and lactating TMRs in total GP at 24h of incubation ( $P < 0.001$ ) and in  $\text{CH}_4$  production, expressed as mL/200 mg DM ( $P \leq 0.001$ ).

The higher  $\text{CH}_4$  emission of TMRs for lactation is explained by the higher starch and NFC contents in comparison with the TMRs for dry animals. Higher  $\text{CH}_4$  production is normally associated with fibre fermentation, however, for highly digestible feeds, such as TMRs for lactation, a higher quantity of  $\text{CH}_4$  is produced

in early hours of fermentation. In the present experiment GP and  $\text{CH}_4$  productions were registered at 24 h, a time sufficient for a complete fermentation of the readily fermentable carbohydrates, but not for the fibrous fractions. These results are consistent with those reported by Getachew *et al.* (2005a) who found a positive correlation between  $\text{CH}_4$  production and organic matter, NFC, dNDF, and DM digestibility in the first 24 h of *in vitro* incubation. Similarly Lee *et al.* (2003) registered a comparative higher  $\text{CH}_4$  production for grains among other feed ingredients rich in fibre or protein or oil, that might be attributed to the high con-

**Table 1. Chemical composition of total mixed rations fed to dry and lactating cows.**

	Dry				Lactating			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Dry matter, %	62.4	10.9	47.8	77.5	49.9	5.01	35.3	56.4
Ash, % DM	9.19	1.12	7.70	11.5	7.00	0.69	6.13	8.32
Crude protein, % DM	11.5	3.07	8.12	15.7	15.6	1.73	11.8	18.6
Ether extract, % DM	2.39	0.50	1.89	3.32	3.76	0.83	2.75	5.49
NDF, % DM	49.2	5.27	45.9	61.8	32.1	4.17	25.5	41.3
NDFIP, % DM	2.63	0.68	1.75	3.73	2.01	0.73	1.21	4.60
NDFIP, % CP	23.6	6.46	14.7	33.4	13.0	4.91	7.21	30.4
ADF, % DM	31.7	3.82	27.6	40.2	21.1	2.83	15.0	26.5
ADL, % DM	4.83	0.75	3.68	5.88	3.57	0.76	2.40	5.89
Starch, % DM	12.5	4.29	5.89	19.4	23.5	4.41	16.1	33.4
NFC, % DM	28.4	4.76	18.4	35.3	42.4	2.93	35.6	47.4
Starch:NDF, %	26.0	9.88	9.53	42.3	75.4	21.6	38.9	128
Starch:ADF, %	40.4	15.2	14.7	66.0	115	35.7	60.5	223

SD, standard deviation; DM, dry matter; NDF, neutral detergent fibre; NDFIP, Protein bound to the NDF fraction; ADF, acid detergent fibre; ADL, acid detergent lignin; NFC, non-fibrous carbohydrate.

**Table 2. Total gas and methane production, organic matter and neutral detergent fibre digestibility, and volatile fatty acids production of total mixed rations fed to dry and lactating cows.**

	Dry	Lactating	SE	P
GP, mL/200 mg DM	43.0	54.4	1.11	<0.001
Methane <sup>o</sup> , mL/200 mg DM	7.24	8.85	0.19	0.001
Methane <sup>o</sup> , % total GP	24.3	23.7	0.22	0.286
OMD, %	64.3	74.8	1.03	<0.001
NEL, Mcal/kg DM	1.25	1.62	0.04	<0.001
NDFD, %	52.1	52.1	1.04	0.995
dNDF, % DM	25.6	16.8	0.95	<0.001
VFA, mmol/L	49.2	47.7	0.99	0.516
Acetate, mmol/L	34.9	33.2	0.77	0.340
Acetate, % VFA	70.6	69.3	0.26	0.025
Propionate, mmol/L	10.4	10.3	0.21	0.750
Propionate, % VFA	21.3	21.6	0.20	0.448
Butyrate, mmol/L	3.89	4.25	0.09	0.066
Butyrate, % VFA	8.14	9.08	0.17	0.011
Acetate:Propionate	3.34	3.24	0.04	0.314

GP, gas production (at 24 h of incubation corrected for blank and standard feeds gas production); DM, dry matter. <sup>o</sup>Cumulated methane production at 24 h of incubation; the percentage on total GP is referred to the raw GP, not corrected for standard feeds gas production. OMD, organic matter digestibility (calculated according to Menke and Steingass GP technique, 1988); NEL, net energy of lactation (stimated using equation no. 171 for compounds and roughage feeds, Menke and Steingass, 1988); NDFD, neutral detergent fibre digestibility; dNDF, digestible neutral detergent fibre; VFA, volatile fatty acids.

tent of easily fermentable sugars, starch and pectins of grains. Similar results were obtained by Navarro-Villa *et al.* (2011) who found an increase in CH<sub>4</sub> output with feeds rich in rapidly fermentable carbohydrates.

Unexpectedly, no difference was observed in the percentage of CH<sub>4</sub> of total GP (24.3±1.32 and 23.7±1.18 for dry and lactation TMRs, respectively; P=0.286). Consistently with the present results, a study of Getachew *et al.* (2005b) did not show any difference in CH<sub>4</sub> percentage between 7 diets for lactation in the first 24 hours of *in vitro* incubation, although significant differences among diets and an overall increase in CH<sub>4</sub> proportion were registered at 48 and 72 hs. No differences were detected among groups for NDFD (P=0.995) (Table 2), however, due to the higher NDF content in dry cows TMRs, digestible NDF (dNDF, % on DM) in this group resulted significantly higher (P<0.001). This resulted in a significant higher acetic acid concentration, expressed as percentage of total VFA produced, for dry cows TMRs (P=0.025) although the difference between the two groups was numerically quite small. On the contrary, butyric acid concentration, as percentage of total VFA, resulted significantly higher for lactating cows TMRs. No significant difference between groups was observed for the acetate:propionate ratio (P=0.314) (Table 2).

### Methane production and dietary starch:ADF ratio

The period (random effect) and the interaction period x starch:ADF ratio were never significant and hence removed from the statistical model. Considering the entire population of TMR tested, divided into quartiles using the starch:ADF ratio as parameter, significant differences among groups in total GP (P<0.001) and in CH<sub>4</sub> production, expressed as mL/200 mg DM (P<0.001) were observed (Table 3). The volume of CH<sub>4</sub> produced increased significantly for increasing starch:ADF ratio, confirming the positive effect of starch on methanogenesis within the first 24 h of *in vitro* incubation. This is confirmed by the highly significant correlation between the starch:ADF ratio and the GP (r=0.87; P<0.001) and the volume of CH<sub>4</sub> produced (r=0.66; P<0.001). Similarly, Singh *et al.* (2011), in an *in vitro* study, showed that dry roughages with a higher content of non-structural and soluble carbohydrates produced more CH<sub>4</sub> than roughages with higher levels of fibre. Overall, the average volume of CH<sub>4</sub> produced after 24 h of incubation was consistent with the values reported by Lee *et al.* (2003) for cereal grains

(6.9 to 11.6 mL/200 mg DM).

As previously observed, no differences were detected for CH<sub>4</sub> as percentage of total GP. This is confirmed by the low correlation coefficient (r=-0.28; P=0.138) between the starch:ADF ratio and the percentage of CH<sub>4</sub>. The lack of difference can be partially due to the short incubation time. Furthermore *in vitro* fermentation is essentially a type of enrichment culture, in which the particular set of environmental conditions used (pH, substrate type and concentration, presence of growth stimulants or inhibitors, *etc.*) are likely to set the stage for preferential rate or extent of growth of some microbial species over other species present in the original *inoculum* (Madigan *et al.*, 2000). The strongly buffered *in vitro* system also did not determine the low pH that is associated with inhibition of fibrolytic bacteria (Argyle and Baldwin, 1988) and methanogens (Van Kessel and Russell, 1996). Russel (1998) showed that CH<sub>4</sub> production *in vivo* decreased dramatically at pH below 6.3. In our experiment, as in most of *in vitro* studies, the use of a buffer solution avoided such a drop in pH which was maintained in the range of 6.5-7.0, optimal for the fermentation of the cellulolytic microbes (McGeough *et al.*, 2011). In the present experiment all diets in every incubation run were treated with the same *inoculum*, therefore the comparison between treatments

is valid. However, a possible interaction between *inoculum* characteristics and the diet fed to the donor animals can be expected. Demeyer and Fievez (2000) reported that a greater proportion of cellulolytic bacteria and methanogenic *Archea* is expected in rumen fluid obtained from ruminants fed high forage diets. Consequently, the *in vitro* fermentation pattern can be indirectly influenced by the diet of the donor animals. Martinez *et al.* (2010), in a study on 24 h *in vitro* methanogenesis, found that the use of an *inoculum* obtained from animals fed diets with a 30:70 forage:concentrate ratio on DM determined a higher CH<sub>4</sub> production in comparison with an *inoculum* obtained from animals fed diets with a 70:30 forage:concentrate ratio. The NDFD did not differ among groups but the proportion of dNDF significantly decreased (P<0.001) as the starch:ADF ratio increased, due to a subsequent lower NDF content. As a consequence acetic acid as percentage of total VFA slightly decreased from 70.7±1.67 (group 1, lowest starch:ADF ratio) to 68.5±0.82% (group 4, highest starch:ADF ratio) (P=0.009), whilst butyric acid percentage numerically increased with increasing starch:ADF ratio. The proportion of propionic acid did not differ among groups; consequently only a tendency (P=0.082) in acetate:propionate ratio decrease was registered as starch:ADF ratio increased (Table 3).

**Table 3. Total gas and methane production, organic matter and neutral detergent fibre digestibility, and volatile fatty acids production of total mixed rations divided into quartiles using starch:ADF as parameter.**

	Group 1	Group 2	Group 3	Group 4	SE	P
Starch:ADF, %	37	77	116	138		
GP, mL/200 mg DM	42.2 <sup>c</sup>	51.4 <sup>b</sup>	55.1 <sup>ab</sup>	56.2 <sup>a</sup>	1.51	<0.001
Methane <sup>o</sup> , mL/200 mg DM	7.12 <sup>b</sup>	8.54 <sup>a</sup>	9.04 <sup>a</sup>	8.87 <sup>a</sup>	0.19	<0.001
Methane <sup>o</sup> , % total GP	24.2	24.2	23.8	23.2	0.22	0.382
OMD, %	63.9 <sup>c</sup>	71.5 <sup>b</sup>	75.6 <sup>a</sup>	76.5 <sup>a</sup>	1.51	<0.001
NEL, Mcal/kg DM	1.23 <sup>c</sup>	1.51 <sup>b</sup>	1.63 <sup>a</sup>	1.69 <sup>a</sup>	0.06	<0.001
NDFD, %	52.1	51.5	52.7	52.0	3.21	0.985
dNDF, % DM	25.9 <sup>a</sup>	19.1 <sup>b</sup>	16.5 <sup>b</sup>	15.3 <sup>b</sup>	1.82	<0.001
VFA, mmol/L	49.4	48.6	47.6	46.7	3.00	0.820
Acetate, mmol/L	35.1	34.2	33.1	32.2	2.29	0.596
Acetate, % VFA	70.7 <sup>a</sup>	70.2 <sup>ab</sup>	69.3 <sup>ab</sup>	68.5 <sup>b</sup>	0.64	0.009
Propionate, mmol/L	10.4	10.2	10.2	10.3	0.64	0.976
Propionate, % VFA	21.2	21.1	21.5	22.3	0.55	0.136
Butyrate, mmol/L	3.89	4.17	4.31	4.21	0.25	0.382
Butyrate, % VFA	8.11	8.75	9.19	9.23	0.45	0.069
Acetate:Propionate	3.35	3.36	3.26	3.09	0.12	0.082

GP, gas production (at 24 h of incubation corrected for blank and standard feeds gas production); DM, dry matter. <sup>o</sup>Cumulated methane production at 24 h of incubation; the percentage on total GP is referred to the raw GP, not corrected for standard feeds gas production. OMD, organic matter digestibility (calculated according to Menke and Steingass GP technique, 1988); NEL, net energy of lactation (stimated using equation no. 17f for compounds and roughage feeds, Menke and Steingass, 1988); NDFL, neutral detergent fibre digestibility; dNDF, digestible neutral detergent fibre; VFA, volatile fatty acids. a,b,cValues on the same row with different superscript differ significantly (P<0.05).

## Conclusions

In the present experiment methane production of different TMRs resulted positively related to the starch and the NE<sub>L</sub> concentrations. Unexpectedly, CH<sub>4</sub> production as a percentage of total GP was not influenced by diet characteristics. This might be attributed to the short incubation time, to the characteristics of the *inoculum* and to the methodology applied, which hampered an effective simulation of rumen fermentation.

The long-term *in vivo* technique (e.g., with respiration chambers) is the most accurate in determining the CH<sub>4</sub> production, but it is very cumbersome, expensive and time consuming. On the contrary, the *in vitro* technique, despite some limitations, permits a precise prediction of the fermentation pattern.

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