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 (CH4) 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 CH4 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 CH4 (7.24 and 8.85 mL/200 mg DM, respectively; P=0.001), but not for CH4 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 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 CH4 (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 CH4 concentration in GP from diets richer in fibre might be attributed mainly to the relatively short time of incubation.

Introduction

Methane (CH4) is one of the most important greenhouse gases (GHG) emitted from anthropogenic sources with a contribute to climatic change and global warming 21 times more effective than carbon dioxide (CO2) (IPCC, 2007). Agriculture accounts for approximately 10 to 12% of the estimated anthropogenic greenhouse effect, producing about 45% of all anthropogenic CH4 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 CH4 production is energetically a wasteful process (Getachew et al., 2005b), since the proportion of feed converted to CH4 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. (2006) for Italy. Hence, it is essential to be able to quantify the CH4 produced from ruminal fermentation of different diets fed to dairy cows. Methane emissions are determined using both 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 CH4 production from total mixed rations (TMRs) fed to dairy cows in commercial dairies in this area using an in vitro GP technique.
Chemical analysis, dry matter and fibre digestibility

Dry matter (DM) was determined following the AOAC procedure (AOAC, method 915.15, 1955), 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 Kjeltec 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 α-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 Ltd., Wicklow, Ireland) for total starch assay procedure (AOAC, method 996.11, 1998). Ruminal 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 of 5 parts of inoculum to 1 part of acid). The inoculum was sampled and clarified by 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 N as carrier. An external standard mixture of CO2 and CH4 prepared by SIAD SpA (Bergamo, Italy) was used for instrument calibration. Peak areas were calculated by automatic integration. The CH4 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 N as carrier. An external standard mixture of CO2 and CH4 prepared by SIAD SpA (Bergamo, Italy) was used for instrument calibration. Peak areas were calculated by automatic integration. The CH4 volume (mL) produced between two time points and final cumulated volume were calculated as reported by Tavendale et al. (2005).

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 headspace 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 CO2. Rumien fluid was collected before the morning feeding from two fistulated dry Italian Friesian cows fed as previously described. At the barn rumien fluid was squeezed through a cheesecloth layer and stored into a pre-warmed thermosts flask, transferred to the laboratory, strained through four layers of cheesecloth and flushed with CO2. The rumien fluid was added to the buffered mineral solution (rumen fluid:buffer solution ratio=12) 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 CO2. The serum bottles were sealed 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 of 90 mL, R: gas constant (8.314 L*kPa*(mol), T: 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 N as carrier. An external standard mixture of CO2 and CH4 prepared by SIAD SpA (Bergamo, Italy) was used for instrument calibration. Peak areas were calculated by automatic integration. The CH4 volume (mL) produced between two time points and final cumulated volume were calculated as reported by Tavendale et al. (2005).

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 CH4 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

Diet composition and chemical analysis

The feeds used to formulate dry cows TMRs were primarily meadow hay, corn silage, wheat straw and protein concentrate (39±14.8, 32±2.32, 26±12.5 and 14±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±7.60% of total DM), corn (both ground and high moisture, 15±5.25 and 14±4.74% of total DM, respectively), protein concentrate (29±8.63% of total DM) and soybean meal (12±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±3.07 and 13.6±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±5.27 in dry TMRs, whereas in lactating TMRs ranged from 25.5 and 41.3, with a mean value of 32.1±4.17% DM basis. Starch content of lactating TMRs (23.5±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±2.93% on DM) is consistent with that found in previous experiments (Getachew et al., 2005a; 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 24 h of incubation (P<0.001) and in CH₄ production, expressed as mL/200 mg DM (P<0.001).

The higher CH₄ emission of TMRs for lactation is explained by the higher starch and NFC contents in comparison with the TMRs for dry animals. Higher CH₄ production is normally associated with fibre fermentation, however, for highly digestible feeds, such as TMRs for lactation, a higher quantity of CH₄ is produced in early hours of fermentation. In the present experiment GP and CH₄ 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 CH₄ 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 CH₄ 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. |
|--------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                                     | 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 |
| NFCIP, % DM                         | 2.63   | 0.68  | 1.75 | 3.73 | 2.01     | 0.73  | 1.21 | 4.60 |
| NFCIP, % CP                         | 23.6   | 6.46  | 14.7 | 33.4 | 13.0     | 4.91  | 7.21 | 30.4 |
| NFC, % 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  | 2.49 | 5.89 |
| Starch, % DM                        | 12.5   | 4.29  | 5.89 | 19.4 | 23.5     | 4.41  | 16.1 | 33.4 |
| NDF, % DM                           | 28.4   | 4.76  | 18.4 | 35.3 | 42.4     | 2.93  | 35.8 | 47.4 |
| Starch/NDF, %                       | 26.0   | 9.88  | 9.53 | 42.3 | 75.4     | 21.6  | 38.9 | 128 |
| Starch/NDF, %                       | 40.4   | 15.2  | 14.7 | 66.0 | 115      | 35.7  | 60.5 | 223 |

SD, standard deviation; DM, dry matter; NFC, neutral detergent fibre; NFCIP, Protein bound to the NFC fraction; 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*, mL/200 mg DM              | 7.24         | 8.85          | 0.19 | 0.001       |
| Methane*, % total GP                | 24.3         | 23.7          | 0.22 | 0.286       |
| OMD, % DM                           | 64.3         | 74.8          | 1.03 | <0.001      |
| NFCIP, % DM                         | 1.25         | 1.62          | 0.04 | <0.001      |
| NDFD, %                             | 52.1         | 52.1          | 1.04 | 0.959       |
| 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. "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 Steinhaas GP technique, 1988); NEL, net energy of lactation (estimated using equation no. 17f for compounds and roughage feeds, Menke and Steinhaus, 1988); NDFL, 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$_4$ output with feeds rich in rapidly fermentable carbohydrates.

Unexpectedly, no difference was observed in the percentage of CH$_4$ 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$_4$ 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$_4$ 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$_4$ production, expressed as mL/200 mg DM (P<0.001) were observed (Table 3). The volume of CH$_4$ 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=0.97; P<0.001) and the volume of CH$_4$ 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$_4$ than roughages with higher levels of fibre. Overall, the average volume of CH$_4$ 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$_4$ 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$_4$. 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$_4$ production in *in vitro* 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 ruminant 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$_4$ 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.

| Starch:ADF, % | Group 1 | Group 2 | Group 3 | Group 4 | SE | P |
|---------------|---------|---------|---------|---------|----|---|
| GP, mL/200 mg DM | 42.2$^a$ | 51.4$^b$ | 55.1$^{ab}$ | 56.2$^a$ | 1.51 | <0.001 |
| Methane$^c$, mL/200 mg DM | 7.12$^a$ | 8.54$^b$ | 9.04$^a$ | 8.87$^a$ | 0.19 | <0.001 |
| Methane$^c$, % total GP | 24.2 | 24.2 | 23.8 | 23.2 | 0.22 | 0.382 |
| OMD, % | 63.9$^a$ | 71.5$^ab$ | 75.6$^a$ | 76.5$^a$ | 1.51 | <0.001 |
| NEL, Mca/kg DM | 1.23 | 1.51$^a$ | 1.63$^a$ | 1.69$^a$ | 0.06 | <0.001 |
| NDFD, % | 52.1 | 51.5 | 52.7 | 52.0 | 3.21 | 0.985 |
| dNDF, % DM | 25.9$^a$ | 19.1$^b$ | 16.5$^b$ | 15.3$^b$ | 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$^a$ | 70.3$^{ab}$ | 69.9$^{ab}$ | 68.5$^b$ | 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. °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 (stimulated 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).

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Conclusions

In the present experiment methane production of different TMRs resulted positively related to the starch and the NE\textsubscript{L} concentrations. Unexpectedly, CH\textsubscript{4} 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 vitro technique (e.g., with respiration chambers) is the most accurate in determining the CH\textsubscript{4} 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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