Effect of maturity at harvest on *in vitro* methane production from ensiled grass

L. Holtshausen[a*], S. H.-O. Liestøl[b], S. K. Nes[b], K. A. Beauchemin[a], O. M. Harstad[b], T. A. McAllister[a]

[a]Agriculture and Agri-Food Canada Research Centre, P.O. Box 3000, Lethbridge, Alberta, Canada T1J 4B1

[b]Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

[*]Corresponding author:
phone: 403-327-4561 ext. 4143
fax: 403-317-2182
e-mail: lucia.holtshausen@agr.gc.ca

Abstract

Controlling the time of harvest to affect grass maturity for silage was evaluated as a methane (CH_4) mitigation strategy in a batch culture *in vitro* with ruminal fluid as inoculum and silage from a mixed timothy (*Phleum pretense*)-meadow fescue (*Festuca pratensis*) stand. The stand was cut in May (EM; first cutting), June (LM; first cutting) and August (MM; third cutting). Disappearance of NDF (EM: 0.58; MM: 0.50; LM: 0.45) and ADF (EM: 0.57; MM: 0.49; LM: 0.45) after 48 h were greater for EM compared to MM and LM, with no difference between the latter two. With advancing maturity, total gas (EM: 166.6; MM: 149.7; LM: 119.3 mL), CH_4 production (EM: 21.4; MM: 17.6; LM: 14.8 mL) and methane production per g NDF digested decreased at 48 h (EM: 120; MM: 92; LM: 74 mL/g NDF digested). Ensiling less mature grass resulted in more CH_4 per unit of NDF digested.

Keywords: grass silage; maturity; methane production

1. Introduction

Grass silage forms an important part of ruminant diets in Western Europe and many other regions of the world (Wilkinson et al., 1996). It is well known that its nutritive value can greatly affect animal performance and the need for supplemental concentrates (Randby et al., 2012). The nutritive value of grass silage is influenced by the stage of maturity of the grass at the time of harvest. Advanced maturity in grass is associated with a decrease in crude protein (CP) and an increase in neutral detergent fibre (NDF) and acid detergent fibre (ADF) content (Rinne et al., 1997a; Cone et al., 1999).

Changes in nutrient composition of grass will have an effect on digestibility and the proportions and amount of fermentation end-products produced in the rumen. Grass harvested for ensiling at an advanced maturity has decreased organic matter (OM), CP, NDF and ADF digestibility (Rinne et al., 1997a,b; Cone et al., 1999; Rinne et al., 2002). Fermentation of silage from more mature grass yielded less total ruminal volatile fatty acids (VFA) compared to silage from less mature grass (Rinne et al., 2002). The effect of stage of maturity of grass at ensiling on ruminal VFA proportions is not consistent, but the majority of experiments show higher proportions of acetate and lower proportions of butyrate and propionate in late as compared to early maturity grass at ensiling (Bosch et al., 1992; Rinne et al., 1997a; Rinne et al., 2002).

Acetate and butyrate production promotes hydrogen gas (H_2) and methane (CH_4) formation, with propionate acting as a net sink of H_2 (Hegarty & Gerdes, 1998; McAllister & Newbold, 2008). Furthermore, because fermentation of CP gives rise to less VFA compared to carbohydrates (Sveinbjörnsson et al., 2006), the higher CP content of silage from grass harvested at an earlier stage of maturity may result in less CH_4 per unit dry matter (DM) digested.

Early maturity grass silage promotes high milk production of dairy cows (Randby et al., 2012) and therefore decreased stage of maturity at harvest is often promoted as a CH_4 mitigation strategy for dairy cows to decrease enteric CH_4 production when expressed on the basis of milk yield (*i.e.*, g CH_4/kg milk; Beauchemin et al., 2009). However, it is uncertain whether CH_4 production in the rumen is also affected.

Therefore, the objective of this study was to determine the impact of grass ensiled at an early, mid or late stage of maturity on CH_4 production and VFA profiles. We hypothesized that harvesting grass silage at a vegetative stage (early maturity) would decrease NDF content of silage, increase *in vitro* DM (DMD) and NDF disappearance (NDFD) and CH_4 production, and decrease CH_4 production per g DMD and per g NDFD.

2. Materials and Methods

2.1. Substrates

Three grass silages grown near Ås, Norway (longitude, 11.3°W; latitude 61.3°N) were used as substrates. The grass was from a single sward consisting of 0.66 timothy (*Phelum pratense*), 0.20 meadow fescue grass (*Festuca pratensis*), 0.05 red clover (*Trifolium pratense*), 0.04 smooth meadow grass (*Poa pratensis*) and 0.05 weeds. Grass was harvested on May 15 (first cutting, early maturity; EM), June 11 (first cutting, late maturity; LM) and August 6 (third cutting, mid-maturity; MM) of 2007 and made into three silages differing in grass maturity at time of harvest. Grass NDF was measured frequently and NDF content served as the criteria for harvesting. Grass harvested on June 11 was considered a late maturity grass because this grass had not been cut before that date and therefore contained growth from the start of the season through June 11. Grass harvested on August 6, on the other hand, was considered a mid-maturity grass because this grass had been cut twice before and therefore contained younger plant material compared to the June 11 cutting.

The grass was wilted before baling, but due to unfavorable weather the target DM content of 27% was not achieve for all three silages. A preservative (740 g/kg formic acid plus sodium formate containing 20 g lactose/kg) was added at a rate of 4.5 L/t during baling of the grass (baler: Orkel GP 1260, Fannrem, Norway; plastic bale wrap: Trio wrap, Trioplast, Smålandsstenar, Sweden) to inhibit undesirable bacterial and mold growth. Bales were ensiled for at least 7 months before it was processed through a feed mixer (Kuhn Euromix I, Saverne, France) to ensure consistent chop length. Immediately thereafter quantities of 20 kg of silage from each individual bale were packed in plastic bags and stored at -20°C. A representative sample was taken from several bags for each of the three silages, combined per silage and then freeze-dried and ground through a 1 mm screen Wiley mill (standard model 4, Arthur H. Thomas, Philadelphia, PA, USA).

2.1. In vitro incubation

The freeze-dried samples were sent to the Agriculture and Agri-Food Canada's Research Center in Lethbridge, Alberta, Canada where three replicate 24-h and 48-h *in vitro* batch cultures (fermentation runs) were conducted. Approximately 0.7 g (± 0.01 g) DM of dried ground silage was weighed into 5 replicate filter bags (F57, ANKOM Technology, Macedon, NY, USA) for each of the three silages by incubation time (24 and 48 h) combination. Five blanks (buffered medium and inoculum plus bags with no substrate) were also included for each of the 24 and 48 h incubations.

The filter bags were heat-sealed and placed in 120 mL glass vials (one bag per vial). Sixty mL of buffered medium (Goering & Van Soest, 1970) was added to each glass vial and closed with a rubber stopper (2048-11800, Bellco Glass Inc., Vineland, NJ, USA). The vials were placed in an incubator at 39°C to pre-warm while inoculum was being collected. For the inoculum, ruminal contents (2 L per cow) were obtained approximately 3 h after the morning feeding from 2 non-lactating cannulated Holstein cows that were fed a diet at maintenance level of consumption consisting of (g/kg DM basis): 720 barley silage, 240 steam-rolled barley and 40 mineral-vitamin supplement. The ruminal fluid was strained through a PECAP polyester screen (pore size 355 µm; B & S H Thompson, Ville Mont-Royal, QC, Canada) into an insulated flask, pooled across the 2 cows and immediately transported to the laboratory. After adding 15 mL of the inoculum to the pre-warmed buffered medium while gassing the headspace with CO_2, the vials were crimp-sealed with rubber stoppers to avoid gas leakage, and placed on a rotary shaker platform at 120 rpm in an incubator at 39°C.

Gas pressure was measured at 4, 8 12, 18, 24, 30, 42 and 48 h using a manual pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada) fitted with a 1.5 inch 22 gauge needle at one end of a three-way stopcock, and connected to a visual display (Data Track, Christchurch, UK). Gas samples (approximately 10 mL) for determination of CH_4 concentration were taken at 4, 8, 12, 24 and 48 h from another outlet of the three-way stopcock with a gas-tight syringe and transferred to pre-evacuated 5.9 mL glass vials (Catalog code: Exetainer 718W, Labco Ltd., Buckinghamshire, UK) while ensuring positive pressure to prevent contamination of the gas sample with atmospheric gas. Positive pressure was ensured by applying pressure to the plunger of the syringe before, during and after transferring the gas sample from the syringe into the glass vial. Gas was vented after each gas sampling and pressure measurement. At the end of 24 and 48 h of incubation the fermentation was terminated by placing the glass vials in ice water and removing the rubber stoppers to expose the samples to air. A 1 mL aliquot of the supernatant was transferred to microcentrifuge vials containing 200 µL of 25% metaphosphoric acid solution, and stored at –10°C until analysis for VFA concentrations. The filter bags were then carefully removed from the vials using tweezers and rinsed under a gentle stream of cold water until the water ran clear and then transferred to an oven for determination of DM disappearance (DMD).

2.2. Analytical procedures and calculations

Representative samples of the three silages were oven-dried at 55°C for 48 h for DM determination (Table 1). For analytical DM, OM, CP, NDF and ADF content silage samples were freeze-dried and ground through a 1 mm screen. Analytical DM was determined by drying samples at 135°C for 2 h, followed by hot weighing (AOAC, 1995; method 930.05). The OM content was calculated as the difference between 100 and the percentage ash (AOAC, 1995; method 942). Crude protein (N × 6.25) was determined by the Kjeldahl method (AOAC, 1995; method 984.13) on a Foss Kjeltec^{TM} 2400 (Tecator^{TM} Technology, Foss, Höganäs, Sweden) using a Cu catalyst. The ANKOM^{200} Fiber Analyzer (Ankom Technology, Macedon, NY, USA) was used to determine NDF, with heat stable α-amylase and sodium sulfite, and ADF; both expressed inclusive of residual ash. Following *in vitro* incubation, filter bags were placed in a 55°C oven for 48 h to determine DMD. Thereafter, sequential NDF and ADF analyses, using the ANKOM^{200} Fiber Analyzer (Ankom Technology, Macedon, NY, USA), were performed to determine NDF and ADF disappearance (NDFD and ADFD, respectively).

A sub-sample of gas (3 mL) was removed from each glass vial and CH_4 as a percentage of total gas was analyzed using a dual channel gas chromatograph (model 4900, Varian Canada Inc., Mississauga, ON, Canada) equipped with two micro-thermal conductivity detectors. The second channel had a 10 meter PPU H column and resolved CH_4 at 0.77 min. The carrier gas was helium at 80 kPa. The column temperature was

36°C and the injector was at 70°C. The run was isothermal (75 s) and the injection time was 40 ms with no back flush.

Gas pressure measurements were converted to volume (mL) produced using the equation developed by Mauricio et al. (1999) and then corrected for average gas production from blank fermentation vials. Volume (mL) of CH_4 produced was calculated by multiplying the volume of gas at each specific sampling time by the percentage of CH_4 (of total gas) at the midpoint between the applicable sampling time and the preceding sampling time. Cumulative total gas and cumulative CH_4 production at 24 and 48 h were calculated by adding the respective gas volumes for all applicable sampling hours.

The VFA were quantified using a gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA, USA) with a capillary column (30 m × 0.32 mm i.d., 1-µm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA), and flame-ionization detection. The oven temperature was 170°C held for 4 min, which was then increased by 5°C/min to 185°C, and then by 3°C/min to 220°C, and held at this temperature for 1 min. The injector temperature was 225°C, the detector temperature was 250°C, with helium as the carrier gas.

2.3. Statistical analysis

Data were analyzed as a completely randomized design, using the MIXED procedure of SAS (2001). A BY statement was used to analyze sampling time points (24 and 48 h) separately. Substrate (n = 3) was considered a fixed effect and fermentation run (n = 3) a random effect. Multiple LSM comparison was performed using the PDIFF option. Significance was declared at $P < 0.05$ and a tendency at $0.05 \leq P < 0.10$.

3. Results

Silage CP content decreased and NDF and ADF content increased with increasing maturity (Table 1). Dry matter disappearance decreased with an increase in maturity of grass after 24 and 48 h of fermentation. Disappearance of NDF after 24 h tended to decrease with increasing grass maturity, whereas ADFD after 24 h did not differ among silages. After 48 h of fermentation, NDFD and ADFD were higher for EM compared to MM and LM, with no difference between the latter two silages.

Differences among treatments for total gas and CH_4 production, and CH_4 production per gram DMD followed the same pattern for the 24 and 48 h incubations. Total gas and CH_4 production decreased with increasing silage NDF (Table 2). Methane production per g DMD did not differ among silages, whereas CH_4/g NDFD was higher for EM compared to MM (24 h: $P = 0.02$; 48 h: $P = 0.03$) and LM (24 and 48 h: $P < 0.01$), and did not differ between the latter two silages (24 h: $P = 0.16$; 48 h: $P = 0.10$).

Total VFA concentration after 48 h fermentation was higher for EM and MM compared with LM; Table 2), whereas after 24 h there was a higher concentration for EM compared with MM ($P = 0.02$) and LM ($P < 0.01$), but no difference in concentrations for the latter two silages ($P = 0.23$). The proportion of acetate after 24 h tended to increase with grass maturity, but did not differ after 48 h of fermentation among silages. The proportion of propionate did not differ among silages after 24 h, whereas after 48 h there was a lower propionate proportion for EM compared with MM ($P = 0.02$) and LM ($P < 0.01$), and no difference in concentrations for the latter two silages ($P = 0.31$). Butyrate proportion after 24 h of fermentation did not differ among silages. After 48 h of fermentation, however, butyrate proportion tended to differ, with a lower proportion for LM compared to EM ($P = 0.05$), a tendency for a lower butyrate proportion for LM compared to MM ($P = 0.06$) and no difference in proportions for MM and EM ($P = 0.82$). The ratio of acetate+butyrate to propionate after 48 h of fermentation for EM was higher compared with MM ($P < 0.01$) and LM ($P < 0.01$), and tended to be higher for MM compared with LM ($P = 0.09$).

4. Discussion

There is general acceptance that grass harvested in an early stage of maturity is a valuable forage for dairy cows because of its relatively low production cost and high nutritive value (Randby et al., 2012). However, effects of grass maturity on enteric CH_4 production are not well known. Thus, we examined the effects of maturity of ensiled grass on *in vitro* fermentation and CH_4 production. While the study design permitted us to explore the forage maturity effects on *in vitro* fermentation, it should be noted that one limitation to the design was that the early and late forages were from the same cutting, whereas the mid-maturity forage was from the third cutting. Nevertheless, the forages obtained provided the desired range in NDF and ADF content, this limitation was considered minor.

The general decrease in CP content and the increase in cell wall content (NDF and ADF) with increased maturity was expected and in agreement with other reports (Rinne et al., 1997; Cone et al., 1999). Of interest though is the fact that the CP content was similar for the silages from grass harvested in June and August. The grass harvested in June (LM) was from the first cutting, whereas that from August (MM) was from the third cutting. Therefore, the re-growth of the grass harvested in August could have contributed to the similar CP content compared to grass harvested in June (Kuoppala et al., 2008). Also, the fibre component of this re-growth (younger plant material) might have been less lignified, which could explain why despite the higher NDF and ADF content for LM compared to MM, fibre disappearance was similar after 48 h of fermentation for these two silages. The apparent difference in cell wall composition and degradation characteristics between the first cutting in June and that from the third cutting in August could also have been influenced by the difference in growing conditions (*e.g.*, light intensity, temperature).

Total gas and CH_4 production decreased as grass was ensiled with increasing maturity in accord with the decrease in DM disappearance. Reduction in CH_4 production can also result from a shift in the VFA pattern. The difference in the ratio of acetate+butyrate to propionate in the current study support the effect that increased maturity of ensiled grass had on CH_4 production as acetate and butyrate production promotes H_2 and CH_4 formation and propionate is a net sink of H_2 (McAllister & Newbold, 2008). In agreement with other reports (Rinne et al., 2002), the proportion of acetate tended to increase with increasing forage maturity after 24 h of incubation. However, the increase in the proportion of propionate with increased maturity of ensiled grass after 48 h of incubation is contrary to other studies which reported no change (Bosch et al., 1992; Rinne et al., 1997a, Rinne et al., 2002).

Our hypothesis was that *in vitro* DM disappearance and CH_4 production would decrease as grass with increasing maturity was ensiled, but that CH_4 production per g DMD would increase. However, instead of an increase in CH_4 per g DMD for silages from more mature grass, there was no difference and CH_4/g NDFD actually decreased. The higher CH_4/g NDFD observed for silage from less mature grass could have been due to CH_4 production resulting from the highly fermentable non-NDF fraction combined with more potentially digestible NDF. Less mature grass usually has high water soluble carbohydrate concentration and the NDF fraction is less lignified compared with mature grass (Randby et al., 2012).

The increase in DM and NDF disappearance of grass that is less mature at harvest would be expected to improve animal performance (Randby et al., 2012). Methane intensity (*i.e.*, emission per unit of meat or milk produced) typically declines in a curvilinear manner with improved animal productivity, because the maintenance energy requirement of the animal is proportionally larger at low levels of productivity (Beauchemin et al., 2009). Thus, an earlier stage of maturity of grass at harvest may decrease CH_4 intensity, but our study suggests that the scale of reduced CH_4 intensity is curtailed in part by increased CH_4 emissions per unit of forage fibre digested. The extent to which this offset occurs needs further study in vivo.

5. Conclusions

Total *in vitro* gas production and CH_4 production decreased, in accord with the decrease in DM and NDF disappearance with increasing maturity of ensiled grass. The *in vitro* CH_4/g NDFD for silages also decreased with advancing maturity. Therefore, when recommending harvesting grass at an early stage of maturity as a CH_4 mitigation practice, the expected decreases in CH_4 per unit of animal product due to improvements in energy partitioning need to offset increased CH_4 emissions per unit of forage fibre consumed and digested.

References

AOAC. 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Arlington, VA.

Beauchemin, K.A., McAllister, T.A. & McGinn, S.M. (2009). Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 4 (No. 035), 18 pp.

Bosch, M.W., Tamminga, S., Post, G., Lefferinger, C.P. & Muylaert, J.M. (1992). Influence of stage of maturity of grass silage on digestion processes in dairy cows. 1. Composition, nylon bag degradation rates, fermentation characteristics, digestibility and intake. Livest. Prod. Sci. 32, 245-264.

Cone, J.W., Van Gelder, A.H., Soliman, I.A., De Visser, H. & Van Vuuren, A.M. (1999). Different techniques to study rumen fermentation characteristics of maturing grass and grass silage. J. Dairy Sci. 82, 957-966.

Goering, H.K. & Van Soest, P.J. (1970). Forage fiber analysis (apparatus, reagents, procedures and some applications). Agric. Handb. No. 379. ARS-USDA, Washington, DC.

Hegarty, R. & Gerdes, R. (1998). Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. 1, 37–44.

Kuoppala, K., Rinne, M., Nousiainen, J. & Huhtanen, P. (2008). The effect of cutting time of grass silage in primary growth and regrowth and the interactions between silage quality and concentrate level on milk production of dairy cows. Livest. Sci. 116, 171–182.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S. & Theodorou, M.K. (1999). A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. Anim. Feed Sci. Technol. 79, 321-330.

McAllister, T.A. & Newbold, C.J. (2008). Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7-13.

Randby, Å.T., Weisbjerg, M.R., Nørgaard, P. & Heringstad B. (2012). Early lactation feed intake and milk yield responses of dairy cows offered grass silages harvested at early maturity stages. J. Dairy Sci. 95, 304–317.

Rinne, M., Jaakkola, S. & Huhtanen, P. (1997a). Grass maturity effects on cattle fed silage-based diets. 1. Organic matter digestion, rumen fermentation and nitrogen utilization. Anim. Feed Sci. Technol. 67, 1-17.

Rinne, M., Huhtanen, P. & Jaakkalo, S. (1997b). Grass maturity effects on cattle fed silage-based diets. 2. Cell wall digestibility, digestion and passage kinetics. Anim. Feed Sci. Technol. 67, 19-35.

Rinne, M., Huhtanen, P. & Jaakkola, S. (2002). Digestive processes of dairy cows fed silages harvested at four stages of grass maturity. J. Anim. Sci. 80, 1986-1998.

SAS Institute. (2001). SAS System for Windows Release 9.1. SAS Inst. Inc., Cary, NC.

Sveinbjörnsson, J., Huhtanen, P. & Udén, P. (2006). The Nordic dairy cow model Karoline – development of VFA sub-model. In: Kebreab, E., Dijkstra, J., Bannink, A., Gerrits, W.J.J., France, J. (Eds.), Nutrient digestion and utilisation in farm animals: modelling approaches. CAB International, Wallingford, UK, pp. 1-14.

Wilkinson, J.M., Wadephul, F. & Hill, J. (1996). Silage in Europe. A survey of 33 countries. Chalcombe publications, Painshall, Church Lane, Welton, Lincoln LN 3LT, United Kingdom.

Table 1.

Substrate chemical composition before incubation and *in vitro* DM, NDF and ADF disappearance after a 24-h and 48-h rumen batch culture fermentation of three grass silages from a mixed timothy (*Phleum pretense*)-meadow fescue (*Festuca pratensis*) stand cut in May (EM), June (LM) and August (MM)

	EM	MM	LM	SEM	$P_{\text{treatment}}$
Chemical composition					
DM, g/kg	310	349	242	--	--
OM, g/kg DM	928	923	934	--	--
CP, g/kg DM	215	127	125	--	--
NDF, g/kg DM	452	554	655	--	--
ADF, g/kg DM	265	319	382	--	--
24-h disappearance					
DM	0.60^{a}	0.51^{b}	0.43^{c}	0.011	< 0.01
NDF	0.37	0.31	0.29	0.048	0.06
ADF	0.31	0.29	0.26	0.041	0.13
48-h disappearance					
DM	0.73^{a}	0.64^{b}	0.56^{c}	0.015	< 0.01
NDF	0.58^{a}	0.50^{b}	0.45^{b}	0.051	0.02
ADF	0.57^{a}	0.49^{b}	0.45^{b}	0.054	0.03

ADF, acid detergent fibre; NDF, neutral detergent fibre; CP, crude protein; DM, dry matter; OM, organic matter.

^{a,b,c}LSM with different superscript within a row differ, $P < 0.05$.

Table 2.

In vitro gas production and VFA concentrations after 24-h and 48-h rumen batch culture fermentation of three grass silages from a mixed timothy (*Phleum pretense*)-meadow fescue (*Festuca pratensis*) stand cut in May (EM), June (LM) and August (MM)

	EM	MM	LM	SEM	$P_{\text{treatment}}$
24-h incubation					
Total gas production, mL	119.4^{a}	99.8^{b}	76.3^{c}	4.07	< 0.01
CH$_4$ production, mL	13.5^{a}	10.4^{b}	8.4^{c}	0.64	< 0.01
CH$_4$ production, mL/g DMD	33.5	30.1	29.6	2.34	0.11
CH$_4$ production, mL/g NDFD	127.1^{a}	90.1^{b}	73.2^{b}	9.70	0.01
Total volatile fatty acids, m*M*	60.2^{a}	51.8^{b}	48.6^{b}	7.18	0.02
Acetate (C2), mol/100 mol	54.9	55.3	56.2	0.004	0.10
Propionate (C3), mol/100 mol	18.6	21.0	20.6	0.01	0.09
Butyrate (C4), mol/100 mol	11.4	11.0	9.99	0.004	0.12
(C2 + C4) : C3	3.58	3.17	3.26	0.22	0.07
48-h incubation					
Total gas production, mL	166.6^{a}	149.7^{b}	119.3^{c}	3.50	< 0.01
CH$_4$ production, mL	21.4^{a}	17.6^{b}	14.8^{c}	1.16	< 0.01
CH$_4$ production, mL/g DMD	43.5	39.9	38.2	2.26	0.16
CH$_4$ production, mL/g NDFD	120.4^{a}	92.0^{b}	74.3^{b}	8.25	0.01
Total volatile fatty acids, m*M*	76.5^{a}	71.8^{a}	63.6^{b}	7.48	< 0.01
Acetate (C2), mol/100 mol	54.4	54.4	53.8	0.02	0.50
Propionate (C3), mol/100 mol	19.9^{b}	22.0^{a}	22.6^{a}	0.01	0.01

Butyrate (C4), mol/100 mol	11.4	11.3	10.3	0.004	0.08
(C2 + C4) : C3	3.33^{a}	3.01^{b}	2.87^{b}	0.22	< 0.01

CH_4, methane; DMD, dry matter disappearance; NDFD, neutral detergent fibre disappearance.

[a,b,c]LSM with different superscript within a row differ, $P < 0.05$.