Effect of nitrogen fertilization rate and regrowth interval of grass herbage on methane emission of zero-grazing lactating dairy cows

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ABSTRACT

Dairy cattle farming in temperate regions often relies on grass herbage (GH)-based diets but the effect of several grass management options on enteric CH₄ emission has not been fully investigated yet. We investigated the combined effect of N fertilization rate and length of regrowth period of GH (predominantly ryegrass) on CH₄ emission from lactating dairy cows. In a randomized block design, 28 lactating Holstein-Friesian dairy cows received a basal diet of GH and compound feed [85:15; dry matter (DM) basis]. Treatments consisted of GH cut after 3 or 5 weeks of regrowth, after receiving either a low (20 kg of N/ha) or a high (90 kg of N/ha) fertilization rate after initial cut. Feed intake, digestibility, milk production and composition, N and energy balance, and CH₄ emission were measured during a 5-d period in climate respiration chambers after an adaptation to the diet for 12 d. Cows were restricted-fed during measurements and mean DM intake was 15.0 ± 0.16 kg/d. Herbage crude protein content varied between 76 and 161 g/kg of DM, and sugar content between 186 and 303 g/kg of DM. Fat- and protein-corrected milk (FPCM) and feed digestibility increased with increased N fertilization rates and a shorter regrowth interval. Increasing the N fertilization rate increased daily CH₄ emission per cow (+10%) and per unit of DM intake (+9%), tended to increase the fraction of gross energy intake emitted as CH₄ (+7%), and (partly because of the low crude protein content for the low fertilized GH) only numerically reduced CH₄ per unit of FPCM. The longer regrowth interval increased CH₄ emission per unit of FPCM (+14%) compared with the shorter regrowth interval, but did not affect CH₄ emission expressed in any other unit. With increasing N fertilization CH₄ emission decreased per unit of digestible neutral detergent fiber intake (−13%) but not per unit of digestible organic matter intake. There was

no interaction of the effect of N fertilization rate and regrowth interval on CH₄ emission, but effects of N fertilization were generally most distinct with GH of 5 wk regrowth. The present results suggest that altering grass quality through an increase of N fertilization and a shorter regrowth interval can reduce CH₄ emission in zero-grazing dairy cows, depending on the unit in which it is expressed. The larger amount of CH₄ produced per day and cow with the more intensively managed GH is compensated by a higher feed digestibility and FPCM yield.

Key words: fresh grass, methane, grass maturity, energy balance, nitrogen balance

INTRODUCTION

Dairy systems in temperate regions largely rely on grass-based diets. Although pasture grazing is the most economical source of nutrients for dairy cows (Taweel et al., 2006; Peyraud and Delagarde, 2013), feeding grass herbage from pasture in an enclosed system (i.e., zero-grazing) has increasingly gained acceptance to allow for a greater feed intake and a better control of nutrient supply (Dohme-Meier et al., 2014). Ingested grass is fermented by ruminal microorganisms to yield energy for the host animal and CH₄ as a by-product. Because of the global warming potential (IPCC, 2006) and the potential of a loss of energy to the host animal with eructated CH₄ (Moe and Tyrrell, 1979), a socio-economic interest exists to reduce CH₄ emission from dairy cattle.

Grassland management is a valuable CH₄ mitigation strategy (Hristov et al., 2013a,b) and improving feeding value of grass to improve animal productivity will reduce CH₄ emission per unit of milk produced. Grass quality can be improved by applying more N fertilizers and by harvesting grass at an earlier (vegetative) stage. A model-simulation study (Bannink et al., 2010) predicted large effects of altered N fertilization on grass herbage composition and simulated CH₄ production. On average, CH₄ yield per unit of fat- and protein-corrected milk (FPCM) was predicted to

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be 16% smaller, and CH₄ loss as proportion of gross energy intake (**GEI**) 9% smaller when N fertilization rates increased from 150 to 450 kg/ha per year. Similar reductions in simulated CH₄ yield per unit of FPCM or GEI were predicted for early versus late cut grass silage as well as for high versus low N fertilization rate.

In vivo data on effects of grass herbage quality on CH₄ emission are limited. Hammond et al. (2009) found that DMI explains the largest part of variation in CH₄ emission, but chemical composition of grass herbage accounts for only 13% of the variation when CH₄ emission was expressed per unit of DMI. Wims et al. (2010) reported increased CH₄ emissions of up to 21% per kilogram of milk yield and 16% per kilogram of DMI for dairy cows grazing ryegrass swards of high versus low herbage mass (2,200 vs. 1,000 kg of DM/ha), although in 1 out of 2 experimental measurement periods only. A later maturity stage (reproductive vs. vegetative) increased daily CH₄ production per cow by 34% and per unit of GEI by 14% in beef cattle grazing timothy (Pinares-Patiño et al., 2003). However, with zero-grazing sheep no differences due to ryegrass maturity were observed (Molano and Clark, 2008). When CH₄ emission is expressed per unit of digestible OM intake (**DOMI**) using digestibility coefficients reported in those studies, emission was roughly 20% smaller with high- versus low-digestible grass in the respective studies. Hart et al. (2009) reported a non-significant 9%-lower CH₄ emission per unit of digestible DMI in beef cattle fed high-digestible compared with low-digestible ryegrass herbage.

To date, combined effects of N fertilization and maturity stage of grass herbage on CH₄ yield per unit of feed intake or per unit of digested feed have not been described for lactating dairy cattle. The aim of the present study was to evaluate the effect of N fertilization rate and regrowth interval of grass herbage on enteric CH₄ production, feed intake, feed digestibility, milk production, energy and N balance, and rumen fermentation in lactating cows.

MATERIALS AND METHODS

Experimental Design

Experimental procedures and surgical cannulation of cows were approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) and carried out under the Dutch Law on Animal Experimentation. The animal experiment was conducted over 6 wk in summer 2012 (June 4 to July 16) at Carus Research Facilities of Wageningen University, Wageningen, the Nether-

lands. The experiment followed a staggered approach because the 4 climate respiration chambers (**CRC**) available offered space for testing a maximum of 4 cows simultaneously. The experiment was thus designed as a completely randomized block design with 28 lactating dairy cows allocated to 7 blocks, based on parity, lactation stage, FPCM production, and whether cows were rumen cannulated or not. Within each block, 4 similar cows were randomly assigned to 1 of 4 experimental treatments (Table 1).

Grass Herbage

The grass herbage originated from a mixed sward established in 2010 and composed of diploid perennial ryegrass (*Lolium perenne*) cultivars of intermediate- and late-heading type (36% each) and timothy (*Phleum pratense*; 28%) (BG Superplus, Barenbrug, Oosterhout, the Netherlands) sown on 3 grassland fields on sandy soil. Grass swards received an initial 80 kg of N/ha of animal manure (2.86 g of N/kg of manure) and 30 kg of N/ha of artificial fertilizer (calcium ammonium nitrate; Rijnvallei, Wageningen, the Netherlands) 2 wk before the start of the initial cut (May 2012 onward). After the initial cut, fields were divided into 60 plots of varying plot size and, according to a 2 × 2 randomized design, were fertilized per plot at a low (20 kg of N/ha; **N20**) or high (90 kg of N/ha; **N90**) rate with calcium ammonium nitrate, and herbage was cut after 3 wk (**R3**) or 5 wk (**R5**) of regrowth. Each of the 3 fields comprised plots for all 4 treatments to prevent confounding between field used and treatment. Each plot size was designed such to accommodate sufficient grass DM yield for 3 consecutive feeding days. It was assumed that within these 3 feeding days differences in grass characteristics of harvested grass remained negligible. The initial cut and N application was staggered per individual plot with a 3-d interval to coincide with the scheduled harvest date of the respective plot. Thus, plots of 5 wk of regrowth were given a corresponding head start relative to plots of 3 wk of regrowth to be able to supply all treatments at the same time at the start of a feeding period. Average daily temperature and precipitation during the entire growing season (May to July 2012) were 15.3°C and 2.6 mm.

Animals, Feeding, and Housing

Twenty-eight dairy cows (4 primiparous and 24 multiparous cows) in mid to late lactation (193 ± 137 DIM; mean ± SD) with an average FPCM production of 34 ± 5.9 kg/d were used in the experiment. Twelve cows were previously fitted with a permanent rumen cannula

Table 1. Chemical composition of grass herbage harvested at 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) at 3 and 5 wk of regrowth (R3 and R5, respectively) and of compound feed

Chemical composition ¹	N20		N90		Compound feed ²
	R3	R5	R3	R5	
DM (g/kg of product)	256 ± 42.4	251 ± 10.3	206 ± 43.6	215 ± 29.1	871
OM	918 ± 9.8	935 ± 2.0	912 ± 5.6	926 ± 5.3	946
CP	100 ± 5.9	76 ± 4.3	161 ± 6.8	102 ± 6.9	114
Crude fat	24 ± 5.2	21 ± 3.2	34 ± 3.3	25 ± 4.3	41
Sugars	273 ± 36.2	303 ± 15.2	186 ± 29.2	236 ± 22.5	44
Starch	— ³	—	—	—	506
NDF	390 ± 12.2	416 ± 6.4	412 ± 17.5	454 ± 8.6	158
ADF	225 ± 11.3	241 ± 6.1	244 ± 13.3	268 ± 6.3	86
ADL	13 ± 0.5	15 ± 0.8	13 ± 1.1	17 ± 1.3	27
Gross energy (MJ/kg of DM)	17.5 ± 0.17	17.8 ± 0.20	18.4 ± 0.32	18.0 ± 0.24	17.9
Chromium ⁴ (mg/kg of DM)	—	—	—	—	2,047

¹In grams per kilogram of DM, unless specified otherwise, with means ± SD for grass herbage harvested over a 6-wk period.

²Ratio of compound feed to grass herbage in the diet: 15:85 on DM basis; ingredients (g/kg of DM): corn (692), palm kernel meal (175), sugarcane molasses (50), wheat (47), salt (18), limestone (14), mineral premix (2.5; Research Diet Services, Wijk bij Duurstede, the Netherlands), chromium oxide (3.0).

³Not analyzed.

⁴Added as chromium oxide to the compound feed.

(10 cm i.d., Type 1C, Bar Diamond Inc., Parma, ID) for rumen fluid collection.

The experimental diet consisted of 85% grass herbage and 15% compound feed (on DM basis; Table 1). Grass herbage was harvested daily at 1300 h by cutting to a height of approximately 5 cm above soil level, and 2 equal portions were offered twice daily (at 0600 and 1600 h). The portion of grass to be fed the next morning was immediately placed in a cooling unit at 4°C to limit nutrient losses through respiration during overnight storage, because respiration losses decline exponentially with decrease in temperature (Wood and Parker, 1971). The daily grass herbage portions offered were based on the DM content of representative samples collected on the previous day. The pelleted compound feed (Table 1; Research Diet Services, Wijk bij Duurstede, the Netherlands) was provided twice daily separately from the grass herbage to ensure complete compound feed uptake. Chromium oxide (Cr₂O₃; 2,047 mg of chromium/kg of DM) was added as an external marker to the compound feed for estimation of apparent total-tract feed digestibility. Feed residues during the period cows were in the CRC pertained to grass herbage alone and were minimal (an average of 80 to 760 g of fresh weight per feeding over the 4 treatments); thus, roughage-to-concentrate ratio was constant throughout the experiment.

Prior to the onset of the experiment, animals were group housed for 7 d and fed a regular dairy ration to acclimatize to the experimental location. Drinking water was provided ad libitum during the entire experiment. In each 17-d treatment period, 4 cows of one

block were individually housed in tie-stalls for a 12-d period to adapt to the experimental diets, and animals were fed ad libitum for the first 8 d. From d 9 onward, feed intake was restricted per block to 95% of the ad libitum feed intake of the animal with the smallest DMI during the 5 preceding days as described previously by van Zijderveld et al. (2011), while ensuring that sufficient herbage was offered each day to enable at least 80% of the ad libitum feed intake of the animals with the greatest DMI within the block. From d 13 (1500 h) through 17 (0900 h), animals were individually housed in 1 of 4 identical CRC units for CH₄ measurements and measurements of energy and N balance. Cows were moved to the CRC in blocks of 4 animals each by following a staggered approach. After leaving the CRC, cows within a block were followed by another block of 4 cows. A detailed description of the CRC was recently given by van Gastelen et al. (2015). Briefly, in each CRC (volume 35 m³) relative humidity was maintained at 70% and temperature at 16°C, and cows were exposed to 16 h of light per day. The ventilation rate was 42 m³/h per compartment, and inlet and exhaust air of each compartment was sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure, temperature, and humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Staff entered each CRC compartment twice daily at 0600 and 1600 h for approximately 30 min for milking and feeding. The gas measurements during the opening of the CRC were not used for data analysis. Cows were weighed immediately after entering and just before leaving the CRC.

Sampling and Measurements

Feed samples were taken every day at the moment of feed preparation, immediately placed in a freezer at -20°C , and pooled per cow before wet-chemical analyses. On d 10 and 11, rumen liquid samples (~ 350 mL) were collected in duplicate proportionally from a cranial, middle, and caudal direction from rumen-cannulated cows before morning feeding (0 h) and 1, 2, 3, 4, 6, 8, and 10 h after morning feeding on both days using a solid and perforated plastic tube (2.5 cm i.d.). Rumen pH was measured immediately using a mobile electronic pH meter (pH electrode HI1230, Hanna Instruments, IJsselstein, the Netherlands). Subsamples of 50 mL were immediately stored at -20°C pending ammonia (NH_3)-N analyses, and subsamples of 750 μL were acidified with an equal volume of ortho-phosphoric acid and stored at -20°C pending VFA analyses.

In the CRC, Orts were collected each morning and afternoon and stored at 4°C , pooled per cow during the measurement period, and stored at -20°C pending analyses. Milk was collected twice daily at 0600 and 1600 h from cows in the CRC and recorded for individual cows. A milk sample (10 mL) of each milking was collected in a tube containing sodium azide (5 μL) for preservation and analyzed for fat, protein, and lactose content. Additional representative milk samples (5 g/kg of milk) were taken at each milking, pooled per cow, and stored at -20°C pending analyses for milk energy, MUN, and N. Milk composition reported was corrected for differences in milk yield between individual milking events. Excreta (feces plus urine) were quantitatively collected from the CRC after the measurement period and homogenized, and subsamples of ~ 500 mL were immediately placed in a freezer at -20°C . Respiration gases and condensed water from the CRC were collected and stored at 4°C pending analyses. Grab samples of feces were collected twice daily at 0600 and 1600 h, pooled per cow, and stored at -20°C for estimation of apparent total-tract feed digestibility. Rumen degradation characteristics of grass were determined with in situ nylon bag incubations, and results have been reported by Heeren et al. (2014).

Chemical Analyses

Subsamples of feed, Orts, excreta, and fecal grab samples were freeze-dried, ground to pass a 1-mm sieve (Peppink 100 AN, Peppink, Olst, the Netherlands), and analyzed by wet chemistry according to the methods described in detail by Abrahamse et al. (2008). In addition, gross energy (GE) content was determined by bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany; ISO, 1998). Grass and concentrate

samples were analyzed for DM, ash, N, crude fat, starch (concentrate only), sugars, NDF, ADF, ADL, and GE. Orts were analyzed for DM and ash. Feces and total excreta samples were analyzed for DM, ash, N, NDF, and GE. Samples of condensed water (i.e., collected from the heat exchanger) and 25% sulfuric acid solution wt/wt (i.e., through which the outflowing air was led to trap aerial ammonia) of each CRC compartment were analyzed for N. Chromium was determined in compound feed and fecal grab samples by using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) after oxidation with wet destruction as described by Pellikaan et al. (2013). The concentration of individual VFA in rumen fluid samples was determined using gas chromatography (GC type Fisons HRGC MEGA2, Milan, Italy) as described by Warner et al. (2013), and that of NH_3 -N was determined using the indophenol reaction as described by Searle (1984). Milk samples were analyzed by mid-infrared reflection spectroscopy for milk constituents (Milk Control Station VVB, Nunspeet, the Netherlands; ISO, 1999), for GE and N content as described previously, and for urea content based on the enzymatic pH difference method (ISO, 2004).

Statistical Analyses

Measurements related to CH_4 were restricted to the last 3 d of measurements in the CRC. Data on DMI, milk production, and milk composition were pooled per animal. One animal (treatment N90-R3) was removed from the experiment because of general health issues not related to the dietary treatments or experimental conditions, and 2 more animals (treatments N20-R3 and N20-R5) were removed from the statistical analyses on measurements taken in the CRC due to outlying values ($3 \times \text{SEM}$) observed for feed efficiency, FPCM, and CH_4 production.

Data were subjected to ANOVA in a randomized block design with a 2×2 factorial arrangement of treatments (2 N fertilization rates $\times$ 2 regrowth intervals) by mixed model procedures of SAS (version 9.3, SAS Institute Inc., Cary, NC). Treatments and their interactions were considered fixed effects; blocks were considered random effects. Each block of 4 animals was completed within one experimental period; thus, period effect was completely confounded with block effect. Covariance parameters were estimated using the REML method with variance components structure as the covariance structure, and denominator degrees of freedom were estimated using the Kenward-Roger approximation.

For DMI, distribution of residuals was nonnormal, and because no transformation met the assumptions

of normality, the generalized linear mixed model procedure of SAS (GLIMMIX procedure; version 9.3) was applied. Rumen fluid measurements from 2 sampling days were pooled, and data were subjected to analyses of variance as described above with the addition of sampling time as the repeated variable ($t = 0, 1, 2, 3, 4, 6, 8$, and 10 h after morning feeding). Because of the inherent unbalanced sampling-time interval, spatial power variance components were used as the covariance structure of choice to account for within-cow variation. All results are reported as least squares means, and significance of treatment effects was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

RESULTS

Intake and Milk Production

Dry matter intake (on average, 15.0 ± 0.16 kg/d) was significantly greater with N90 relative to N20, but the numerical difference of less than 2% remained small (Table 2). The FPCM yield increased by 16% with N fertilization rate and decreased by 13% with a longer regrowth interval. Similar differences were observed for milk fat and milk protein yields. No significant treatment interaction effect on milk yield and composition was observed, except for MUN, where the significant interaction indicates a more pronounced increase in MUN upon increased N fertilization with R3 than with R5 herbage.

Methane Production

Daily CH_4 production per cow was 10% larger with N90 relative to N20 but did not change with regrowth interval (Table 3). Similar differences were observed for CH_4 per unit of DMI (+9%) and as a trend for

proportion of GEI (+7%; $P = 0.070$). When enteric CH_4 production was scaled to unit of digestible nutrient intake, CH_4 production was reduced by 13% per unit of digestible NDF intake (**DNDFI**) with increasing N fertilization rate but was unaffected by regrowth interval, and no effects were observed per unit of DOMI. When scaled to kilograms of FPCM produced, CH_4 emission increased by 14% with a longer regrowth interval but was not significantly affected by N fertilization rate (numerical decrease of 5% for N90 vs. N20). The largest difference in CH_4 per unit of FPCM occurred between the most extensively managed (N20–R5) and the most intensively managed grassland (N90–R3). Despite a numerically 9% smaller daily CH_4 production per cow with the N20–R5 diet compared with the N90–R3 diet, cows restricted to the same DMI produced 19% more CH_4 per kilogram of FPCM with the N20–R5 diet because of the lower feed digestibility and FPCM production.

Apparent Digestibility, Energy, and Nitrogen Balance

Apparent total-tract digestibility increased with N fertilization rate for all nutrients (8, 31, 17, and 9 percent units for OM, N, NDF, and GE, respectively) and decreased with length of regrowth interval (–4, –18, –8, and –5 percentage units, respectively; Table 4).

No difference in GEI among treatments was found, but ME intake increased by 10% with increasing N fertilization rate and decreased by 5% with a longer regrowth interval (Table 5). The amount of energy lost to CH_4 increased with N fertilization rate for R5 herbage but did not change with regrowth interval. Cows partitioned more energy to milk production with N fertilization rate for R5 herbage and with a shorter regrowth interval for N20 herbage. Cows excreted more N in feces plus urine with a higher N fertilization rate

Table 2. Dry matter intake, milk production, and milk composition of dairy cows fed rations containing grass herbage with 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) and harvested at 3 or 5 wk of regrowth (R; R3 and R5, respectively)

Item	N20		N90		SEM	P-value		
	R3	R5	R3	R5		N	R	N × R
DMI (kg/d)	14.9	14.9	15.1	15.0	0.16	0.027	0.32	0.73
Milk yield								
Milk (kg/d)	19.6	16.6	20.1	19.8	1.51	0.13	0.15	0.25
FPCM ¹ (kg/d)	21.1	16.8	22.6	21.2	1.38	0.006	0.006	0.13
Fat (g/d)	913	710	995	917	67.0	0.010	0.010	0.21
Protein (g/d)	651	538	701	661	35.9	0.005	0.009	0.17
Milk composition (g/kg)								
Fat	46.2	42.2	48.7	48.0	3.79	0.25	0.54	0.66
Protein	33.8	32.2	35.2	34.4	1.65	0.27	0.43	0.80
Lactose	45.2	44.3	45.5	44.3	1.14	0.94	0.34	0.88
MUN (mg/dL)	7.3 ^{a,x}	4.9 ^{a,x}	13.6 ^{a,y}	7.0 ^{b,x}	0.87	<0.001	<0.001	0.016

^{a,b; x,y}Significant ($P < 0.05$) differences within N (^{a,b}) and within R (^{x,y}) for a significant treatment interaction (N × R).

¹Fat- and protein-corrected milk.

Table 3. Methane production from dairy cows fed rations containing grass herbage with 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) and harvested at 3 or 5 wk of regrowth (R; R3 and R5, respectively)

CH ₄ ¹	N20		N90		SEM	P-value		
	R3	R5	R3	R5		N	R	N × R
CH ₄ (g/d)	306	297	326	337	16.1	0.016	0.97	0.36
CH ₄ (g/kg of FPCM)	14.9	17.4	14.6	16.2	0.88	0.33	0.015	0.53
CH ₄ (g/kg of DMI)	20.4	19.9	21.5	22.4	0.80	0.017	0.83	0.32
CH ₄ (g/kg of DOMI)	29.3	29.2	28.7	30.9	1.06	0.55	0.22	0.19
CH ₄ (g/kg of DNDFI)	78.8	78.8	67.9	68.9	3.19	<0.001	0.82	0.80
CH ₄ (% of GEI)	6.5	6.4	6.7	7.1	0.27	0.070	0.62	0.16

¹FPCM = fat- and protein-corrected milk; DOMI = digestible OM intake; DNDFI = digestible NDF intake; GEI = gross energy intake.

and a shorter regrowth interval and retained more N in milk with a higher N fertilization rate for R5 herbage and with a shorter regrowth interval for N20 herbage, resulting in a negative N balance for N20 herbage. Accompanied by the level of N intake, N excretion in feces plus urine was more strongly affected by reducing the regrowth interval (+28% with R3 compared with R5) than by increasing N fertilization rate (+14% with N90 compared with N20).

Rumen Fermentation

Rumen fluid pH was not affected by dietary treatments (Table 6). Concentrations of rumen fluid NH₃ markedly increased with a higher N fertilization rate for R3 herbage and with a shorter regrowth interval for N90 herbage. Total VFA concentrations in the rumen and branched-chain VFA molar proportions (R3 only) increased with N fertilization rate and there was a trend for increased acetate molar proportion, but butyrate molar proportion and the ratio of nonglucogenic-to-glucogenic VFA (R5 only) decreased. Regrowth interval did not affect total concentrations and molar proportions of VFA, except for a trend for an increased propionate (N90 only) and branched-chain VFA molar proportion. Sampling time after morning feeding had clear effects on rumen liquid characteristics, with only a trend for NH₃ concentrations and valerate molar proportions. Acetate molar proportions initially decreased, whereas propionate molar proportions initially increased until approximately 4 to 6 h after morning feeding to return

to baseline levels with incremental sampling time (Figure 1). A similar time effect was observed for rumen pH, branched-chain VFA, and nonglucogenic-to-glucogenic VFA with an initial decrease and for butyrate with an initial increase in molar proportions and concentrations of total VFA (data not shown). Acetate, butyrate, and valerate molar proportions were generally correlated with CH₄ per unit of DMI (*r* of 0.65, −0.64, and −0.58, respectively; *P* < 0.05) and GEI (*r* of 0.66, −0.63, and −0.66, respectively; *P* < 0.05) but not with CH₄ per unit of DOMI. Propionate and branch-chained VFA were not correlated (*P* > 0.05) with CH₄ emission.

DISCUSSION

This study reports the effect of grass herbage quality on enteric CH₄ emission measured by indirect calorimetry. To our knowledge no *in vivo* data exist for combined effects of N fertilization and maturity stage of grass herbage on CH₄ production in lactating dairy cows. The results indicate that altering grass quality by a shorter regrowth interval and increased N fertilization reduce CH₄ emission of dairy cattle, although the size of effects depends on the unit in which CH₄ emission is expressed. An increased N fertilization rate resulted in some 10% larger CH₄ production per day and cow or per unit of DMI because of a higher grass digestibility. This higher CH₄ emission was more than offset by an increased milk yield, resulting in numerically less CH₄ produced per unit of FPCM. In contrast

Table 4. Total-tract apparent nutrient digestibility in dairy cows fed rations containing grass herbage with 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) and harvested at 3 or 5 wk of regrowth (R; R3 and R5, respectively)

Digestibility (%)	N20		N90		SEM	P-value		
	R3	R5	R3	R5		N	R	N × R
OM	77.0	74.0	83.6	79.6	1.38	<0.001	0.002	0.62
N	56.5	47.1	74.9	60.6	2.29	<0.001	<0.001	0.16
NDF	74.1	67.5	85.9	80.3	2.39	<0.001	0.003	0.78
Gross energy	72.9	70.3	80.2	75.7	1.42	<0.001	0.002	0.33

Table 5. Energy and N balance for dairy cows fed rations containing grass herbage with 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) and harvested at 3 or 5 wk of regrowth (R; R3 and R5, respectively)

Item	N20		N90		SEM	P-value		
	R3	R5	R3	R5		N	R	N × R
Metabolic BW (kg ^{0.75})	118	127	124	120	3.3	0.83	0.40	0.032
Energy balance (kJ/kg of BW ^{0.75} per d)								
Gross energy (GE) intake	2,190	2,046	2,199	2,213	76	0.13	0.24	0.16
ME intake ¹	1,428	1,302	1,514	1,499	50	<0.001	0.031	0.080
ME:GE intake ratio (%)	65.5	64.0	68.6	67.8	0.78	<0.001	0.13	0.68
Energy in methane	143 ^{a,x}	130 ^{a,x}	148 ^{a,x}	157 ^{a,y}	7.2	0.004	0.68	0.020
Heat production	798	772	800	790	25.8	0.63	0.38	0.69
Energy in milk	546 ^{a,x}	414 ^{b,x}	570 ^{a,x}	544 ^{a,y}	33.2	0.006	0.004	0.036
N balance (mg/kg of BW ^{0.75} per d)								
N intake	2,021 ^{a,x}	1,461 ^{b,x}	3,003 ^{a,y}	2,038 ^{b,y}	98.9	<0.001	<0.001	0.004
N in excreta	1,326	1,083	1,573	1,184	78.1	0.003	<0.001	0.14
N in milk	853 ^{a,x}	643 ^{b,x}	895 ^{a,x}	855 ^{a,y}	46.0	<0.001	<0.001	0.015
Balance ²	-157 ^{a,x}	-265 ^{a,x}	535 ^{a,y}	-1 ^{b,y}	64.9	<0.001	<0.001	<0.001
Energy retention (kJ/kg of BW ^{0.75} per d)								
Total ³	89	121	143	165	33.1	0.13	0.37	0.88
Protein ⁴	-23 ^{a,x}	-39 ^{a,x}	79 ^{a,y}	0 ^{b,y}	9.6	<0.001	<0.001	<0.001
Fat ⁵	114	161	63	166	28.3	0.39	0.009	0.30

^{a,b; x,y}Superscript letters indicate significant ($P < 0.05$) differences within N (^{a,b}) and within R (^{x,y}) for a significant treatment interaction (N × R).

¹ME intake = GE intake – energy in excreta – energy in methane.

²N retained = N intake – N in excreta – N in milk – N in condensate collected from heat exchanger – N trapped from the outflowing air.

³Energy retention total = ME intake – heat production – energy in milk.

⁴Energy retention protein = (N balance/1,000) × 6.25 × 23.6 kJ/g.

⁵Energy retention fat = energy retention total – energy retention protein.

to N fertilization, regrowth interval did not influence daily CH₄ production per cow or per unit of DMI, but because FPCM yield was greater, CH₄ production per unit of FPCM was smaller with the shorter regrowth interval. Interactions among dietary treatments did not occur, but numerically, effects of N fertilization rate on CH₄ production, either expressed as absolute or relative yields (see following paragraphs), were larger for the 5- than 3-wk regrowth interval. In line with observed

total-tract OM, N, and NDF digestibility, in situ rumen degradability of the same grass herbage was reduced with increased stage of regrowth or reduced N fertilization rate (Heeren et al., 2014). In situ, significant effects of N fertilization on effective degradability of NDF and OM occurred at 5 wk of regrowth but not at 3 wk of regrowth, in line with the smaller numerical differences observed for in vivo total-tract digestibility at 3 wk of regrowth.

Table 6. Rumen liquid characteristics of dairy cows fed rations containing grass herbage with 2 N fertilization rates (20 and 90 kg/ha; N20 and N90, respectively) and harvested at 3 or 5 wk of regrowth (R; R3 and R5, respectively)

Item	N20		N90		SEM	P-value ¹			
	R3	R5	R3	R5		N	R	N × R	Time
pH	6.5	6.6	6.4	6.4	0.07	0.11	0.35	0.89	<0.001
NH ₃ (mmol/L)	1.7 ^{a,x}	0.7 ^{a,x}	10.2 ^{a,y}	2.8 ^{b,x}	0.67	<0.001	<0.001	<0.001	0.092
Total VFA (mmol/L)	77.2	76.6	99.2	89.6	3.97	<0.001	0.23	0.26	<0.001
VFA (mol/100 mol)									
Acetate	65.0	62.9	65.9	65.7	1.3	0.056	0.22	0.29	<0.001
Propionate	16.8 ^{a,x}	16.6 ^{a,x}	16.3 ^{a,x}	17.8 ^{b,x}	0.40	0.29	0.061	0.020	<0.001
Butyrate	15.8	18.0	14.4	13.8	1.17	0.008	0.33	0.095	<0.001
Valerate	1.0	1.0	1.0	0.9	0.10	0.28	0.77	0.71	0.059
Branched-chain VFA ²	1.4 ^{a,x}	1.5 ^{a,x}	2.5 ^{a,y}	1.7 ^{a,x}	0.22	0.005	0.082	0.044	<0.001
NGR ³	5.4 ^{a,x}	5.6 ^{a,x}	5.3 ^{a,x}	4.9 ^{a,y}	0.13	0.015	0.43	0.029	<0.001

^{a,b; x,y}Superscript letters indicate significant ($P < 0.05$) differences within N (^{a,b}) and within R (^{x,y}) for a significant treatment interaction (N × R).

¹Analyses of variance with sampling times 0, 1, 2, 3, 4, 6, 8, and 10 h after morning feeding as repeated measures; interactions time × N and time × R not significant ($P > 0.05$).

²Branched-chain VFA = isobutyrate + isovalerate.

³Nonglucogenic-to-glucogenic VFA [= (acetate + 2 × butyrate + 2 × isobutyrate + valerate + isovalerate)/(propionate + valerate + isovalerate)].

Figure 1. Rumen molar proportions of acetate (continuous line) and propionate (dashed line) with respective upper standard error bars from time = 0 h (before morning feeding) through 10 h after morning feeding for dairy cows fed grass herbage at 2 N fertilization rates and 3 or 5 wk of regrowth.

Methane per Unit of Milk

Rate of N fertilization did not significantly affect CH_4 production per unit of FPCM, although FPCM yield significantly increased by 16%. The numerical reduction of 0.8 g of CH_4 per kilogram of FPCM (−5%) with increased N fertilization is considerably smaller than the prediction made in the model-simulation study of Bannink et al. (2010). They predicted a 3.0 g/kg of FPCM smaller CH_4 production (−17%) at a comparable forage-to-concentrate ratio and feed intake level to that in the present study. However, the level as well as the difference between simulated N fertilization rates (150 kg of N/ha per year vs. 450 kg of N/ha per year) were higher compared with those in the present study. Different assumptions on the extent of N fertilization effects on grass chemical composition, in situ degradation characteristics of grass, and FPCM yield in the model study of Bannink et al. (2010) hence explained at least partly the differences in outcome of the model-simulation study and actual observed in vivo results in the present study. Bannink et al. (2010) assumed a lower NDF content with the high-fertilized grass, whereas in the present study NDF content with N90 was higher than with N20. Furthermore, grass herbage

tested in the present study had a comparably lower CP and higher sugar content and lower rumen degradability (see Heeren et al., 2014) than assumed by Bannink et al. (2010). Cows fed the N20 herbage had a negative N balance and a low rumen NH_3 content, especially at 5 wk of regrowth, which may have limited rumen N availability and microbial growth. The low content of MUN is a further indication of inadequate NH_3 levels in the rumen for optimal rumen microbial growth.

Before the actual start of the experiment, we expected the N content of the herbage to be considerably higher than the actual N content measured after finishing the experiment and chose a concentrate composition suitable for grass herbage with a higher N content. The low N availability in the rumen with N20 may have negatively affected rumen fermentation processes related to a shortage of N and may have thus reduced CH_4 emission compared with a situation of sufficient N supply. This may explain the smaller amount of CH_4 per unit of FPCM for the low N fertilization rate compared with the simulation study of Bannink et al. (2010). It can be thus assumed that CH_4 per unit of FPCM was somewhat underestimated with N20 herbage under the conditions tested here. As high-yielding dairy cows cannot cope with a prolonged N imbalance, under regular

on-farm conditions with sufficient N supply, differences in CH₄ emissions between N20 and N90 herbage are expected to be larger than the ones described here. The CH₄ emission was 2.0 g/kg of FPCM (+14%) higher with the 5-wk compared with the 3-wk regrowth interval. To date, few data are available from literature with regard to grass herbage of varying maturity on CH₄ emission of dairy cattle. Wims et al. (2010) reported a 21% greater CH₄ per kilogram of milk emission of dairy cows grazing ryegrass swards of high versus low herbage mass (2,200 vs. 1,000 kg of DM/ha) in 1 of 2 experimental periods but no significant effect in the other experimental period. Comparable differences of 15% in CH₄ per unit of FPCM were obtained by Brask et al. (2013) with late- versus early-cut herbage for making grass silages (delaying harvest time 3 wk compared with early cut). Effects of a similar order were simulated for grass silage by Bannink et al. (2010).

Methane Loss per Unit of GEI

Greenhouse gas inventories often estimate CH₄ emission from dairy cows as 6.5% of GEI (Tier 2 approach; IPCC, 2006). In the present study, the GEI levels were comparable among dietary treatments. The trend for a larger CH₄ production per unit of GEI (+7%; $P = 0.070$) with N90 compared with N20 herbage resulted from an increased grass digestibility and daily CH₄ production per cow. This trend is opposite to findings of Bannink et al. (2010), who predicted a 10% reduction with high- compared with low-N-fertilized grass herbage. As previously argued, the contrasting observations in the present study are most likely due to the different chemical composition and grass digestibility than assumed by Bannink et al. (2010).

We found no effect of regrowth interval on CH₄ per unit of GEI which corresponds with observations on effects of phenological stage on CH₄ emissions from sheep fed ryegrass herbage (Molano and Clark, 2008) and beef cattle grazing timothy pastures (Pinares-Patiño et al., 2003). Similarly, in a zero-grazing experiment with ryegrass herbage of high and low digestibility fed to beef heifers, CH₄ yield per unit of GEI did not differ (Hart et al., 2009). Taken together, forage quality, as affected by N fertilization and regrowth interval, appears to have a relatively small effect on energy loss with CH₄ as proportion of GEI.

The average yield of CH₄ as a percentage of GEI in the present study was 6.7% (range 6.4 to 7.1%) and slightly greater than IPCC (2006) Tier 2 guidelines (6.5%). The large value can be explained by the large energetic value of the grass herbage fed. The ME:GE intake ratio (or metabolizability) of 0.67 was high, and

calculated net energy for lactation content of the grass herbage was approximately 7.0 MJ/kg of DM, which is well above that in average grass herbage (6.4 MJ/kg of DM; Bannink et al., 2011). Predictions with a mechanistic model, which is the basis of the national greenhouse gas inventory of the Netherlands, for grass herbage diets indicated 6.3 to 7.0% of GEI being emitted as CH₄ (on average 6.7%; Bannink et al., 2010), which matches the 6.7% average of the observed values in the present study. The amount of CH₄ (% of GEI) measured in the present experiment for grass-based diets was larger than that predicted for an average diet fed to Dutch cattle (6.0%; Bannink et al., 2011). The reason is that the average Dutch diet comprises some 25% corn silage, 25% concentrate and by-products, 30% grass silage, and less than 20% grass herbage. Despite the large CH₄ yield as a percentage of GEI, CH₄ yield per unit of FPCM was similar to that found by Bannink et al. (2011) for similar milk-production levels. These results imply that CH₄ yield expressed as a percentage of GEI has only limited value, because digestibility and intake may differ widely. It seems more informative to express CH₄ per unit of digested feed or per unit of FPCM because this better takes into account value and characteristics of GEI by cows.

Methane Production per Unit of Feed and Digestible Nutrient Intake

A recent meta-analysis (Ellis et al., 2007) indicated DMI as one of the main explanatory variables of CH₄ production and was also confirmed for fresh ryegrass pasture based on a large data set by Hammond et al. (2009). In our study, DMI levels were little affected because of the restricted feeding regimen employed. Thus, differences in CH₄ production among dietary treatments must be explained by differences in nutrient composition and digestibility of the grass herbage.

The reduced grass digestibility of the N20 herbage at early maturity is assumed to be partly caused by the inherent N shortage in the rumen with this treatment. This was confirmed by the *in situ* degradation study of Heeren et al. (2014), which tested the same grass herbage incubated *in situ* in *ad libitum*-fed dairy cows with a sufficient N supply, but showed no effect of N fertilization on NDF degradation at early maturity. This finding was in contrast to the *in vivo* NDF digestibility reported in the present study and implies that the N limitation inherent to the N20 herbage partly explains the observed smaller amount of CH₄ per unit of DMI compared with the N90 herbage.

In line with Pinares-Patiño et al. (2003), Molano and Clark (2008), and Hart et al. (2009), the yield

of CH₄ per unit of DMI in the present study was not affected by regrowth stage. Moreover, CH₄ yield per unit of DOMI or DNDFI was not affected by regrowth stage and numerical differences were small, whereas literature reported a higher amount of CH₄ per digestible DMI or DOMI with lower digestible grass herbage (Pinares-Patiño et al., 2003; Molano and Clark, 2008; Hart et al., 2009). As discussed previously, in the present experiment, the N availability in the rumen for microbes was limited, and this may have resulted in less pronounced differences in CH₄ production.

In contrast to an increased CH₄ per unit of DMI with N fertilization, CH₄ per unit of DOMI did not differ due to the higher OM digestibility with N90 than N20 herbage. Significantly higher VFA concentrations in rumen fluid of cows fed N90 herbage were in line with the higher OM digestibility. It should be noted that the particular high sugar and low CP content of the N20 herbage tested in the present study has not been reported in the available literature. In accordance with Ellis et al. (2012), the high sugar content of the N20 herbage in the present study was in line with the observed increase in rumen butyrate molar proportion and a trend for a decrease of acetate molar proportions relative to the N90 herbage.

Methane and Rumen Fermentation Parameters

Significant correlations were established between VFA molar proportions and CH₄ formation (for various units of expression). However, variation in CH₄ emission per unit of DOMI appeared unrelated to VFA molar proportions, probably because of the overall small differences in VFA molar proportions. Thus, differences in VFA molar proportion did not seem to explain the observed variation in CH₄ emission due to N fertilization rate and weeks of regrowth. Also no correlation was found with CH₄ per unit of DNDFI (except for butyrate), evidently because digestible NDF reflects only part of the digested OM and therefore cannot by itself explain changes in CH₄ and VFA for the whole diet. The observed correlations between molar VFA proportions and CH₄ production were in agreement with data reviewed by Moss et al. (2000), except for propionate. Although propionate was found to predict most variation in CH₄ production in the latter study, reflecting the reduced hydrogen surplus in the rumen and therefore the amount of CH₄ produced, no such relationship was established in the present study.

Implications

In the present study grass herbage was tested with a low CP content but relatively high OM and NDF

digestibility. Improving the herbage quality by increasing the N fertilization rate and reducing regrowth interval increased diet digestibility and FPCM yield and reduced CH₄ per unit of FPCM. Moreover, the increased NDF and OM digestibility with the higher N fertilization rate resulted in a reduced excretion of NDF and OM. This will reduce OM fermentation and related CH₄ emission during manure storage, whereas the increased N excretion may increase N losses on farm and lead to (direct and indirect) emissions of N₂O (Montes et al., 2013). Hence, on a whole-farm scale, the benefit of reducing relative CH₄ emission by improving the herbage quality needs to be weighed against current mitigation measures to reduce N losses into the environment (Dijkstra et al., 2011). It should be noted that the milk N efficiency (milk N output/diet N input) of the N20 and R5 diets was very high and partly related to the negative N balance of the cows. Upon expressing milk N output per unit of N in excreta, differences between diets were much smaller, indicating that potential losses of N₂O per unit of milk protein N are not very pronounced.

Given the low CP and high sugar content of the grass herbage with low N fertilization rate and longer regrowth interval in the present study and the paucity of data on effects of grass management on CH₄ yield, further work is needed to elucidate how changes in grass herbage characteristics affect chemical composition, rumen fermentation, diet digestibility, FPCM yield, and CH₄ emission. As grass herbage quality is particularly conditioned by the weather in studies in which grass is harvested and fed as herbage on a daily basis, if resources permit, researchers are invited to consider including a moderate N fertilization rate as an intermediate between 2 extreme N applications to ensure a more balanced grass herbage quality in future CH₄-emission studies.

CONCLUSIONS

This study showed that adapting grassland management (N fertilization rate and length of regrowth) affects CH₄ emission from enteric fermentation in dairy cows fed grass herbage. The CH₄ emission per unit of FPCM decreased with shortening of regrowth interval (numerically with increased N fertilization). Methane emission did not differ per unit of DOMI, but increasing the N fertilization rate did increase CH₄ emission per unit of DMI. The more intensively managed grass herbage yielded more CH₄ per unit of DMI because of its higher digestibility, but this was more than compensated by the greater FPCM yield leading to less CH₄ per unit of FPCM.

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