Frequency of diet delivery to dairy cows: Effect on nutrient digestion, rumen fermentation, methane production, nitrogen utilization, and milk production

C. Benchaar* and F. Hassanat

Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, Sherbrooke, QC, Canada J1M 0C8

ABSTRACT

The objective of this study was to examine the effect of frequency of diet delivery to dairy cows on nutrient digestion, rumen fermentation, milk production, nitrogen utilization, enteric methane emission, and manure methane production potential. Twelve lactating cows were used in a replicated 3×3 Latin square design (35-d period) and offered a TMR ad libitum [56:44 ratio of forage to concentrate, dry matter (DM) basis] once (0930 h), twice (0930 and 2130 h), or 4 times daily (0930, 1300, 1630, and 2130 h). Frequency of diet delivery did not affect intake or apparent total-tract digestibility of DM and nutrients. Likewise, milk production, milk composition (fat, protein, and lactose), and milk production efficiency (kg of milk/kg of DM intake or g of milk N/g of N intake) were not changed by frequency of diet delivery. Although diurnal variation of ruminal pH, total VFA, and acetate molar proportion were influenced by frequency of diet delivery, daily average ruminal pH, total VFA, and acetate and propionate molar proportions were not affected by frequency of diet delivery. Daily enteric CH₄ emission averaged 534 g/d and was not changed by frequency of diet delivery. Methane energy losses (on gross energy intake basis) were lower when cows received the diet once daily (5.8%) versus twice or 4 times daily (6.1%). Urinary N excretion was higher for cows receiving the diet 4 times daily compared with cows receiving the diet once or twice daily (36 vs. 34% of N intake). Frequency of diet delivery had no influence on manure volatile solids excretion or maximal CH₄ production potential. Results from this study show that delivering the diet once daily reduces enteric CH₄ energy losses compared with twice or 4 times daily, whereas urinary N losses increased by delivering the diet 4 times daily compared with once or twice daily. However, milk production and maximal

manure CH₄ emission potential were not affected by frequency of diet delivery.

Key words: frequency of diet delivery, methane emissions, dairy cow

INTRODUCTION

Methane emissions from enteric fermentation and manure management contribute 30% of Canada's national CH₄ emissions (Environment Canada, 2019). Diet manipulation has been shown to influence CH₄ production in dairy cows (Brask et al., 2013; Benchaar et al., 2015; Hassanat et al., 2017). When dietary interventions affected DMI or ruminal OM fermentation, the amount and the composition of manure volatile solids (VS) were changed, affecting CH₄ emissions from stored manure (Møller et al., 2014; Benchaar and Hassanat, 2019; Hassanat and Benchaar, 2019). However, studies investigating the effect of non-nutritional feeding management practices (e.g., frequency of diet delivery) on CH₄ emissions from enteric fermentation and manure storage are scarce.

Under conventional feeding practices, dairy producers offer TMR to dairy cows once or twice daily. Feeding rate (kg of DMI/h) and time spent eating (min/h) are highest immediately after diet delivery (DeVries and von Keyserlingk, 2005; King et al., 2016). Moreover, DeVries et al. (2005) reported that cows spend more time during the day eating when the diet is delivered more frequently. Also, according to Shabi et al. (1998, 1999) and Cabrita et al. (2006), increasing frequency of diet delivery prevents extreme fluctuation of ruminal pH and metabolite concentrations (VFA, NH₃) in ruminal fluid, resulting into a more stable rumen environment. As a result, DMI, fiber digestion, and microbial protein synthesis increase by increasing frequency of diet delivery (Shabi et al., 1998, 1999; Cabrita et al., 2006). These findings indicate that increasing frequency of diet delivery may increase the amount of CH₄ emitted daily by dairy cows.

Studies investigating the effect of frequency of diet delivery on enteric CH₄ production are very scarce, and no information is available on the effects of frequency of

Received January 14, 2020.

Accepted April 4, 2020.

*Corresponding author: chaouki.benchaar@canada.ca

diet delivery on CH₄ emissions from stored manure. We hypothesized that the increasing frequency of diet delivery would (1) induce a more stable rumen environment, thereby increasing OM degradation in the rumen; (2) increase DMI, nutrient digestion, and milk production; and (3) increase enteric CH₄ production (g/d) but reduce CH₄ production potential during manure storage. Therefore, the objective of this study is to investigate the influence of frequency of diet delivery (once, twice, or 4 times per day) on digestion, ruminal fermentation characteristics, N excretion, milk production, and CH₄ emissions from enteric fermentation and stored manure.

MATERIALS AND METHODS

The study was conducted at the Sherbrooke Research and Development Centre (Sherbrooke, Quebec, Canada). Animal procedures were conducted under the approval of the local Institutional Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009).

Cows, Experimental Design, and Diet

Twelve multiparous (3.0 ± 1.0 parity) lactating Holstein cows fitted with rumen cannulas (10 cm, Bar Diamond Inc., Parma, ID) were used in a replicated 3×3 Latin square design (35-d period). The cows averaged (mean $\pm$ SD) 85 ± 23.7 DIM at the beginning of the experiment with an average BW of 681 ± 76 kg and milk yield of 50 ± 3.7 kg/d. During the experiment, cows were housed in individual tiestalls and had free access to water. Cows were fed ad libitum (5% orts, on an as-fed basis) a TMR consisting of 56% forage and 44% concentrate (DM basis; Table 1) formulated to meet the nutrient requirements of cows (NRC, 2001). The experimental treatments were as follows: (1) TMR delivered once daily; (2) TMR delivered twice daily; and (3) TMR delivered 4 times daily. The TMR was delivered at 0930h; 0930 and 2130 h; and 0930, 1300, 1630, and 2130 h for cows fed once, twice, and 4 times, respectively. The diet was pushed up at 1300, 1630, and 2130 h. Separators were installed between cows to minimize the disruption caused by the different frequencies of diet delivery.

After 14 d of adaptation to dietary treatments, feed intake, apparent total-tract digestibility, N excretion, milk production and milk composition (7 d); in sacco ruminal degradability (5 d); rumen fermentation characteristics (2 d); BW (2 d); and CH₄ production (5 d) were measured. Because only 2 respiration chambers were available, cows were staggered by 5 d to facilitate

measurements. In addition, measurements (digestibility, N excretion, rumen fermentation, in sacco ruminal degradability, and CH₄) were spread over the 21 d of sampling and data collection. Manure CH₄ emissions were measured from manure collected over 2 consecutive days during the last period of the experiment.

Intake, Apparent Total-Tract Digestibility, N Excretion, and BW

Apparent total-tract digestibility and N excretion were measured as described in Benchaar et al. (2013). Feed consumption was recorded daily by weighing feeds delivered to and refused by the cows. Total collection of feces and urine was performed as described in Benchaar et al. (2013) by fitting cows with harnesses and tubes allowing the collection of feces and urine separately. Samples of the diet, feed ingredients, refusals, and feces were collected daily and stored at -20°C . These samples were later thawed, composited by cow within period, freeze-dried, ground to pass a 1-mm screen using a Wiley mill (standard model 4; Arthur M. Thomas,

Table 1. Ingredient and chemical composition of the TMR (% of DM unless otherwise noted)

Item	Amount
Ingredient	
Corn silage	24.9
Alfalfa silage	24.9
Corn grain, ground	23.1
Protein supplement ¹	6.20
Timothy hay, chopped	6.06
Soybean hulls	6.02
Soybean meal, 48% solvent extracted	5.27
Mineral and vitamin supplement ²	1.57
Rumen inert fat supplement ³	1.19
Calcium carbonate	0.78
Chemical composition	
OM	92.9
CP	17.5
RDP ⁴ (% of CP)	60.9
RUP ⁴ (% of CP)	39.1
NDF	30.8
ADF	19.9
Starch	25.4
Gross energy (Mcal/kg of DM)	4.45
NE _L ⁴ (Mcal/kg of DM)	1.60

¹Top supplement, composed of 30% corn gluten meal, 20% heat-treated soybean, 20% canola meal, and 30% dried corn distillers grains (Bélisle Solution Nutrition Inc., St-Mathias, QC, Canada).

²Contained 9.27% Ca, 4.80% P, 4.80% Mg, 1.57% S, 12.68% Na, 1.23% K, 23 mg/kg I, 2,014 mg/kg Fe, 440 mg/kg Cu, 1,797 mg/kg Mn, 2,657 mg/kg Zn, 7 mg/kg Co, 467,556 IU/kg vitamin A, 56,669 IU/kg vitamin D, 2,739 IU/kg vitamin E, and 20 mg/kg Se.

³Energy Booster 100 (Milk Specialties Global Animal Nutrition, Carpentersville, IL).

⁴Calculated using values from NRC (2001) tables.

Philadelphia, PA), and analyzed for DM, OM, total N, NDF, ADF, starch, and gross energy (**GE**). Urine (acidified with 50% H₂SO₄) was analyzed for total N content. Cows were weighed at the beginning and end of each experimental period on 2 consecutive days before the a.m. feeding and after the a.m. milking.

In Sacco Ruminal Degradability

Ruminal degradability of the TMR was determined (6 cows; Latin square design) using the nylon bag procedure as described in Benchaar et al. (2013). Samples were ground to pass a 2-mm screen using a Wiley mill, and 5 g (DM) were weighed in quadruplicates in polyester bags (17 cm × 9 cm; 53-μm pore size) made of monofilament PeCAP Polyester (Sefar Nitex, Sefar AG, Heiden, Switzerland). Bags were incubated in the ventral sac of the rumen for 2, 4, 8, 16, 24, 48, 72, 96, and 120 h. Bags used for time 0 disappearance were soaked in water at 37°C for 5 min and then treated similarly to the other bags. Residues recovered after incubation were dried at 55°C for 48 h and ground to pass through a 1-mm screen for subsequent determination of DM.

Kinetics of ruminal degradation of DM of the TMR were calculated using a nonlinear model of McDonald (1981). The NLIN procedure of SAS (SAS Institute Inc., Cary, NC) was used to fit the following model:

$$P(t) = a + b(1 - e^{-c(t-L)}) \text{ for } t > L;$$

$$P(t) = a \text{ for } t \leq L,$$

where $P(t)$ = percentage of DM disappearance from the nylon bag at time t , a = soluble and rapidly degradable fraction (%), b = slowly degradable fraction (%), c = fractional rate of disappearance of the fraction b (per h), L = lag time (h), and t = time of incubation (h). The model was fitted using $(a + b) \leq 100$, $b \geq 0$, $c \geq 0$, and $L \geq 0$ constraints.

The effective ruminal degradability of DM was calculated using the following equation:

$$\text{Effective ruminal degradability} = a + \left[bc / (c + k_p) \right] \times e^{(-k_p L)},$$

where k_p is the ruminal fractional passage rate, calculated from the equations suggested by the NRC (2001).

Ruminal Fermentation Characteristics and Protozoa Enumeration

Ruminal fluid was collected from each cow before (0 h) and 1, 2, 3, 4, 6, and 8 h after the a.m. meal, as

described in Benchaar et al. (2013). A total of 250 mL was collected from several locations within the rumen using a 50-mL syringe screwed to a stainless steel tube ending with a probe covered by a fine metal mesh (RT Rumen Fluid Collection Tube, Bar Diamond Inc.). Ruminal fluid was subsampled (15 mL) and frozen at −20°C immediately for later determination of VFA concentrations. Another subsample (15 mL) was acidified to pH 2 with 50% sulfuric acid and frozen at −20°C for later determination of NH₃ concentration.

Protozoa were counted from ruminal content collected before the a.m. meal as described in Benchaar et al. (2013). Ruminal content (approximately 1 L) was strained through 4 layers of cheesecloth, and a 5-mL portion of the strained ruminal fluid was preserved using 5 mL of methyl green formalin-saline solution for protozoa enumeration (Ogimoto and Imai, 1981). Protozoa samples were stored at room temperature in darkness until counting. Protozoa were microscopically enumerated using a counting chamber (Neubauer Improved Bright-Line counting cell, 0.1-mm depth; Hausser Scientific, Horsham, PA), and genera were identified as outlined by Dehority (1993).

Ruminal pH was measured continuously for 48 h using eCow bolus system (eBolus, eCow Ltd., Exeter, UK). The system consisted of eCow bolus probes, handset (Moto G Model XT1032, Motorola, Chicago, IL) equipped with Hathor V4.8 software, and an adapter (dongle) fitted with antenna as described by Falk et al. (2016). The probes were calibrated to pH 4.00 and 7.00 at 39°C prior to use. The probes were attached to a 0.5-kg metal weight, to ensure that they would always be immersed in the ruminal fluid, and then placed in the ventral sac of the rumen. Ruminal pH was recorded every 15 min. After 48 h, the probes were removed and data downloaded. The probes were then immersed in pH 4.00 and pH 7.00 buffers kept at 39°C, and the probe drift did not exceed ±0.05. The pH data were summarized as mean pH, minimum pH, maximum pH, time spent below pH 6.0, time spent below pH 5.6, and time spent below pH 5.2 (Plaizier et al., 2001; Keunen et al., 2002).

Milk Production and Milk Composition

Cows were milked twice daily at 0700 and 1930 h in their stalls. Milk production was recorded at each milking, and milk data recorded during the 7 d of apparent total-tract digestibility measurement were used for the statistical analysis. Milk samples were taken from each cow at each milking, stored at 4°C with a preservative (2-bromo-2-nitropropan-1,3-diol), and sent to a commercial laboratory (Lactanet, Ste-Anne-de-Bellevue,

QC, Canada) for analysis of fat, protein, lactose, MUN, and SCC.

Enteric Methane Production

Respiration chambers measured 4.09 m long $\times$ 2.95 m wide $\times$ 2.84 m high. When doors were closed, air entered the chamber through a ventilation duct and exited through an exhaust. Air temperature within the chamber was maintained at $17 \pm 0.5^\circ\text{C}$. Airflow into and out of the chamber was measured using inline mass flowmeters (FT2; Fox Thermal Instruments Inc., Marina, CA) and maintained at $180 \pm 10 \text{ m}^3/\text{h}$. Methane concentration was continuously measured at the air entrance and exhaust ducts using CH_4 analyzers (SERVOPRO 4100; Servomex, Brighton, UK). The amount of CH_4 entering and leaving the chamber was calculated by multiplying the concentration of CH_4 by the airflow at entrance and at exhaust. The difference between the incoming and outgoing mass of CH_4 corresponded to the amount of enteric CH_4 emitted in each chamber by the animal. Chambers were calibrated at the beginning of each experimental period by releasing known amounts of CH_4 in each empty chamber. Recovered amounts of CH_4 were calculated from the difference between incoming and outgoing CH_4 . The recovered amounts averaged 96% (± 1.0) and 105% (± 2.4) for the first and second chamber, respectively. The calibration factors (i.e., to adjust each chamber to 100% recovery) were used to correct CH_4 emissions data. A small positive pressure was generated inside each chamber to prevent inflow of gases to the chambers. Methane was recorded every minute over 5 consecutive days, and fluxes were averaged to derive 24-h CH_4 emissions. Cows were preconditioned to the respiration chambers before the beginning of the experiment. To reduce the effect of confinement on animal behavior, the chambers were equipped with windows and speakers so the cow in the chamber could see and hear her peers. Cows entered the chambers 18 h before starting CH_4 measurements. Within each chamber, the cow was kept in a tiestall that measured 1.82 m long $\times$ 1.60 m wide, elevated from the floor by 25 cm. Manure was collected in a stainless steel box placed below and to the rear of each stall. Farm personnel accessed the chambers to feed and milk the cows and remove the manure. The doors were kept open for 1 to 1.5 min to allow exchange of material in and out of the chambers. This resulted in interruptions of flux measurements for 15 to 30 min, which is the time required for gas concentrations to reach steady state. These interruptions had little effect on daily emissions, because fluxes were calculated every minute and used to derive the 24-h emissions values.

Cows in the chambers were milked twice daily, had free access to water, and were fed for ad libitum intake (5% orts on an as-fed basis). Offered feed and orts were weighed daily to determine feed consumption. Samples of feed offered and orts were collected, pooled across days, and kept frozen for later determination of DM and GE concentrations.

The relationship between frequency of diet delivery and rate of CH_4 emission, by treatment, was examined using the exponential equation described by Thornley and France (2007):

$$\text{Methane production rate (g/h)} = a \exp[bc^{-1}(1 - e^{-ct}) - d],$$

where a = initial CH_4 production rate (g/h) before diet delivery; t = time of day (h); and b , c , and d = kinetic parameters characterizing diurnal variation in CH_4 emission (g/h). Kinetic parameters obtained from the previous equation were used to calculate time of CH_4 production peak (h), peak CH_4 production rate (g/h), and relative rate of decline (per h) of CH_4 using the following equations:

$$\text{Time of } \text{CH}_4 \text{ production peak (h)} = c^{-1} \ln(b/d),$$

$$\text{Peak } \text{CH}_4 \text{ production rate (g/h)} = a(b/d)^{b/c} \exp[c^{-1}(b - d)],$$

$$\text{Relative rate of decline (per h)} = b \exp[-c(t + t_f)/2] - d,$$

where t_f is final measurement time (h).

Manure Methane Production

To determine CH_4 emissions of stored manure, feces and urine and were collected from each cow as described previously. Feces and nonacidified urine were weighed daily, sampled (4% of the amount excreted), mixed based on the daily excreted feces-to-urine ratio (on a fresh-weight basis), and then pooled over the 2 collection days for each cow. Subsamples were taken and stored at -20°C and subsequently freeze-dried and ground to pass a 1-mm screen using a Wiley mill for later determination of DM, VS, and NDF. The mixture of feces and urine of each cow was mixed manually on a fresh-weight basis with psychrophilic residual sludge (1:1) obtained from a manure storage facility at the dairy operation of the Sherbrooke Research and De-

velopment Centre. In this study, the mixture of feces, urine, and residual sludge was identified as manure.

The manure was transferred under anaerobic conditions (O_2 -free N_2) into 500-mL glass bottles (3 replicates per cow; 250 g in each bottle), sealed with butyl rubber stoppers and aluminum seals (Wheaton Industries Inc., Millville, NJ), and incubated at 20°C for 17 wk in a controlled-environment chamber. Blank bottles ($n = 3$) containing residual sludge (250 g) were treated similarly for measurement of gas production. Thus, in total, 39 bottles were used in this study. Concentration of DM in residual sludge was 3.9% and was composed (on DM basis) of 71% VS and 28% NDF. Methane emissions from the residual sludge after 17 wk of incubation were negligible and averaged 8.7 L/kg of VS.

Gas pressure in the bottle headspace was measured daily using a digital pressure gauge (Ashcroft 2089, Ashcroft Inc., Stratford, CT), and the volume of gas produced was calculated according to the following equation:

$$P_1V_1 = P_2V_2,$$

where P_1 = atmospheric pressure, V_1 = volume of gas produced at atmospheric pressure, P_2 = pressure of gas measured at specific incubation day, and V_2 = volume of headspace of the bottle.

Headspace gas was sampled using a syringe connected to the pressure gauge by a sampling valve. The collected gas sample was then forced into 6-mL screw-capped vacuum vials (Labco Ltd., Lampeter, UK) covered with a rubber septum (National Scientific, Rockwood, TN) for measurement of CH_4 concentration. Gas pressure in the bottle headspace was then reduced to room pressure by leaving the sampling syringe open for 2 min. Gas production from blanks was not subtracted from gas produced from the experimental bottles.

Chemical Analyses

Dry matter content was determined by drying samples in a vacuum oven at 100°C overnight (AOAC International, 2005; method 934.01). Ash content was determined by sample incineration at 550°C overnight in a muffle furnace (AOAC International, 2005; method 942.05), and the OM content was calculated as the difference between 100 and the percentage of ash. Crude protein ($N \times 6.25$) was determined using the macro-Kjeldahl procedure (AOAC International, 2005; method 954.01). The concentration of NDF was determined as described by Van Soest et al. (1991) with the use of sodium sulfite and with the inclusion of heat-stable α -amylase. The ADF content was determined according to AOAC International (2005; method 973.18). The

NDF and ADF procedures were adapted for use in an Ankom200 Fiber Analyzer (Ankom Technology Corp., Fairport, NY). The concentration of starch was determined colorimetrically according to the procedure of AOAC International (2005; method 996.11). Gross energy was determined using an oxygen bomb calorimeter (model 6200, Parr Instrument Company, Moline, IL). The concentration of N in acidified urine samples was determined by micro-Kjeldahl analysis (AOAC International, 2005). Analysis of VFA was performed using a gas chromatograph equipped with a flame ionization detector and autoinjector (6850 network GC system, Agilent Technologies, Mississauga, ON, Canada) fitted with DB-FFAP column (30 m $\times$ 0.250 mm $\times$ 0.25 μ m; Agilent Technologies) as in Benchaa et al. (2015). Ammonia concentration was determined as in Weatherburn (1967). Protein, fat, lactose, urea N, and SCC in milk samples were analyzed by infrared spectroscopy (MilkoScan FT 6000; Foss Electric, Hillerød, Denmark). Milk composition was corrected for differences in milk yield between a.m. and p.m. milkings.

Statistical Analyses

All data were analyzed using the MIXED procedure of SAS. For the feeding trial data, the statistical model included frequency of diet delivery and period as fixed effects, and cow was considered as random effect. Ruminal fermentation characteristics (pH, VFA, NH_3) data were analyzed as repeated measures using the same model, with the addition of the fixed effects of day, sampling time (hour), and all interactions. The CH_4 data were also analyzed as repeated measures with the inclusion of the fixed effects of day and day $\times$ treatment interaction. The appropriate covariance structure used for repeated-measures analyses was chosen according to the type of repeated measurement (single or double) and sampling distance (equal or unequal) and to achieve lowest Akaike, corrected Akaike, and Bayesian information criteria values. The covariance structure used cow within treatment $\times$ period as subject of the repeated measures. For the manure trial, the statistical model included frequencies of diet delivery as fixed effects, and cow was the experimental unit. Significant effects of treatment were declared at $P \leq 0.05$, with tendencies at $0.05 < P \leq 0.10$, using the Tukey test for multiple comparisons.

RESULTS AND DISCUSSION

Intake and Apparent Total-Tract Digestibility

Frequency of diet delivery had no effect on intake of DM (26.3 kg/d), CP (4.6 kg/d), NDF (8.1 kg/d), or starch (6.7 kg/d), as presented in Table 2. Literature

Table 2. Intake and apparent total-tract digestibility of nutrients in lactating cows receiving the diet once, twice, or 4 times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
Intake (kg/d, unless otherwise noted)				
DM	26.6	26.1	26.2	0.47
DM (% of BW)	3.81	3.74	3.75	0.118
OM	24.7	24.0	24.0	0.48
CP	4.65	4.52	4.50	0.095
NDF	8.23	8.02	8.08	0.172
ADF	5.29	5.15	5.24	0.107
Starch	6.79	6.60	6.60	0.150
Gross energy (Mcal/d)	118	115	115	2.26
Digestibility (%)				
DM	70.6	70.5	70.5	0.32
OM	72.1	72.0	72.0	0.31
CP	68.1	68.3	67.9	0.62
NDF	52.3	51.7	52.1	0.83
ADF	50.3	49.5	50.1	0.94
Starch	96.9	97.1	96.9	0.20
Gross energy	69.0	69.0	69.0	0.33

data on the effect of feeding frequency on DMI have been variable among studies. In agreement with our findings, studies using cows in tiestalls (Robinson and Sniffen, 1985; Klusmeyer et al., 1990; Judy et al., 2018) reported no change in DMI among cows receiving diets once, twice, or 4 times daily. However, our results disagree with those of Shabi et al. (1999) and Crompton et al. (2011), who reported an increase in DMI when cows housed in tiestalls were fed 4 times versus once daily. On the other hand, when using cows in freestalls, some studies reported no effect of frequency of diet delivery on DMI (DeVries et al., 2005; Crossley et al., 2018), but others (Phillips and Rind, 2001; Mäntysaari et al., 2006) observed a decline in DMI when cows received diets 4 and 5 times daily versus once daily. Compared with a freestall system, social interaction between cows in tiestalls is minimal, which affects feeding activities and consequently DMI, explaining the lack of effect of feeding frequency on DMI observed in our study.

Apparent total-tract digestibility of OM, CP, NDF, and starch was not affected by frequency of diet delivery, averaging 72%, 68%, 52%, and 97%, respectively (Table 2). Previous studies (Robinson and Sniffen, 1985; Le Liboux and Peyraud, 1999; Judy et al., 2018) reported no effect of frequency of diet delivery on DM and nutrient digestibility in cows fed a high dietary forage proportion ($\geq 54\%$). The diet used in the current study contained 56% forage. Other studies using high concentrate proportion diets ($\geq 51\%$) observed an increase in DM, NDF, and CP digestibility when the diet was delivered 4 times versus twice daily (Shabi et al., 1999) or versus once daily (Dhiman et al., 2002).

Ruminal Fermentation Characteristics

Because interactions between treatment and measurement day were not significant for any of the ruminal fermentation characteristics, only averages of the 2-d measurements were reported. Diurnal evolution of ruminal pH (Figure 1) was altered by changing frequency of diet delivery (treatment $\times$ sampling time interaction, $P < 0.01$). Cows receiving the diet 4 times daily had higher ruminal pH at 3 and 4 h after the a.m. feeding compared with cows receiving the diet once or twice daily, but no difference was observed between cows receiving the diet once versus twice daily. Cows receiving the diet twice daily had higher ruminal pH at 9, 10, 11, and 12 h after the a.m. meal compared with cows receiving the diet once or 4 times daily, whereas no difference was observed between cows receiving the diet once versus 4 times daily. However, changing frequency of diet delivery had no effect on average (6.03), minimum (5.49), or maximum (6.49) daily ruminal pH (Table 3). Frequency of diet delivery had no effect on daily duration of ruminal pH < 6.0 (average 10.3 h/d) or duration of ruminal pH < 5.6 (average 2.1 h/d).

Except for molar proportion of propionate, significant treatment $\times$ sampling time interactions ($P \leq 0.02$) were observed for total VFA concentration (Figure 2) and molar proportions of individual VFA. At 3 h after the a.m. meal, ruminal fluid of cows receiving the diet 4 times daily contained lower ($P = 0.02$) total VFA concentration compared with cows receiving the diet once or twice daily. Total VFA concentration was also lower ($P < 0.01$) at 8 h after the a.m. meal in cows receiving

Figure 1. Ruminal pH ($\pm$ SEM) in lactating cows receiving the diet once (1X; ■), twice (2X; ●), or 4 times (4X; ▲) daily. ^{a,b} $P \leq 0.05$.

Figure 2. Ruminal total VFA concentration ($\pm$ SEM) in lactating cows receiving the diet once (1X; ■), twice (2X; ●), or 4 times (4X; ▲) daily. ^{a,b} $P \leq 0.05$.

the diet twice daily compared with cows receiving the diet once or 4 times daily.

Molar proportion of acetate was higher ($P \leq 0.02$) at 2, 3, and 4 h after the a.m. meal in cows receiving the diet 4 times a day compared with cows receiving the diet once or twice daily (Figure 3). These diurnal differences in ruminal pH, total VFA concentration, and acetate molar proportion coincided with the amount of fermentable OM ingested as affected by frequency of diet delivery at the specified time of the day. Indeed, at 2 and 3 h after the a.m. meal, cows receiving the diet 4 times daily had access only to 25% of their diet, which resulted in higher ruminal pH and lower total VFA concentration in the rumen, compared with cows receiving the diet once or twice daily who had access to 100% and 50% of their diet, respectively. At 8 h after the a.m. meal, cows receiving the diet twice daily had access to only 50% of their diet, which resulted in higher ruminal pH and lower total VFA concentration in the rumen, compared with cows receiving the diet

once or 4 times daily (access to 100% and 75% of the diet, respectively). Similar effects of frequency of diet delivery on diurnal variation of ruminal pH and total VFA concentration have previously been reported for steers fed twice versus 8 times daily (Bragg et al., 1986) and for cows fed twice versus 12 times daily (French and Kennelly, 1990).

Overall, daily total VFA concentration, molar proportions of acetate and propionate, and the acetate:propionate ratio were not affected by frequency of diet delivery and averaged 125 mM, 62, 23 mol/100 mol, and 2.8:1, respectively (Table 4). Molar proportion of butyrate was lower ($P = 0.05$) in cows receiving the diet 4 times daily compared with cows receiving the diet twice daily, but not different from cows receiving the diet once daily. In agreement, several studies reported no effect of frequency of diet delivery on daily ruminal pH, total VFA concentration, or molar proportion of individual VFA (Le Liboux and Peyraud, 1999;

Table 3. Ruminal pH in lactating cows receiving the diet once, twice, or 4 times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
pH				
Mean	6.02	6.04	6.04	0.050
Minimum	5.52	5.49	5.48	0.053
Maximum	6.51	6.48	6.47	0.055
Time pH <6.0 (h/d)	10.94	10.50	9.48	1.718
Time pH <5.6 (h/d)	2.21	2.17	2.00	0.915
Time pH <5.2 (h/d)	0.07	0.01	0.05	0.039

Table 4. Total VFA concentration, VFA molar proportions, NH₃ concentration, and protozoa counts of ruminal fluid in lactating cows receiving the diet once, twice, or 4 times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
Total VFA (mM)	127	125	123	2.6
VFA (mol/100 mol)				
Acetate (A)	61.7	61.4	62.4	0.89
Propionate (P)	22.8	23.0	22.6	0.93
Butyrate	10.8 ^{ab}	11.0 ^a	10.4 ^b	0.23
Valerate	1.62 ^a	1.57 ^{ab}	1.54 ^b	0.033
Branched-chain VFA ¹	3.08	3.01	3.08	0.073
A:P ratio	2.77	2.75	2.85	0.143
NH ₃ (mM)	12.6 ^a	11.3 ^b	11.1 ^b	0.49
Protozoa (cells × 10 ³ /mL)				
Total	531 ^b	629 ^a	522 ^b	73.2
Entodiniomorphs	527 ^b	624 ^a	518 ^b	72.6
Holotrichs ²	4.65	4.79	3.68	1.160

^{a,b}Means within rows with different superscripts differ ($P < 0.05$).

¹Branched-chain VFA = isobutyrate + isovalerate.

²Holotrichs = isotrichs and dasytrichs.

Dhiman et al., 2002; Macmillan et al., 2017). Others reported a decline in ruminal pH associated with an increase in total VFA concentration and acetate and propionate molar proportions with increasing frequency of diet delivery (Froetschel et al., 1990; Klusmeyer et al., 1990).

Significant treatment × sampling time interactions ($P < 0.01$) were also observed for NH₃ concentration (Figure 4). At 1, 2, 3, and 4 h after the a.m. meal, NH₃ concentration was lower ($P \leq 0.02$) in cows receiving

the diet 4 times daily compared with cows receiving the diet once or twice daily, but not different between cows receiving the diet once or twice daily. Delivering the diet twice daily reduced ($P = 0.02$) ruminal NH₃ concentration at 6 h after the a.m. meal compared with delivering the diet once or 4 times daily, but no difference was observed between delivering the diet once or 4 times daily. At 8 h after the a.m. meal, NH₃ concentration was higher ($P < 0.01$) in cows receiving the diet 4 times daily compared with cows receiving the diet once or twice daily, and higher in cows receiving the diet once daily versus twice daily. Similar diurnal variation in molar proportion of branched-chain VFA were also observed (data not shown). These diurnal differences in ruminal NH₃ concentration and molar proportion of branched-chain VFA coincided with the amount of CP ingested as affected by frequency of diet delivery and at the specified time of day. Similar diurnal variation in NH₃ concentration have previously been reported in steers fed twice versus 8 times daily (Bragg et al., 1986) or fed twice versus 12 times daily (Cecava et al., 1990), and among dairy cows fed twice versus 6 times daily (Le Liboux and Peyraud, 1999) or fed concentrates once, twice, or 4 times daily (Yang and Varga, 1989).

Average daily ruminal NH₃ concentration was higher ($P = 0.01$) in cows receiving the diet once daily compared with cows receiving the diet twice or 4 times daily, but no difference was observed when the diet was delivered twice or 4 times daily (Table 4). In accordance, Ulyatt et al. (1984) reported that increasing frequency of diet delivery to sheep reduced ruminal NH₃ concentration. Other studies reported no effect of frequency of diet delivery on ruminal NH₃ concentra-

Figure 3. Ruminal acetate molar proportion ($\pm$ SEM) in lactating cows receiving the diet once (1X; ■), twice (2X; ●), or 4 times (4X; ▲) daily. ^{a,b} $P \leq 0.05$.

tion (Froetschel and Amos, 1991; Dhiman et al., 2002; Macmillan et al., 2017). It is worth mentioning that differences in NH_3 concentration between cows receiving the diet once daily versus 4 times daily can also be related to the quantity of diet delivered to cows relative to the duration of ruminal fluid sampling in this study. Cows receiving the diet once daily had access to 100% of the diet during rumen fluid sampling (before to 8 h after the a.m. meal), resulting in greater consumption of dietary CP compared with cows receiving the diet twice or 4 times daily (i.e., access to 50% and 75% of the diet, respectively, for the same duration). Branched-chain VFA molar proportion averaged 3.1 mol/100 mol and was not affected by frequency of diet delivery (Table 4).

Populations of total protozoa and endodiniomorphs were higher ($P = 0.03$ and $P = 0.03$, respectively) in cows receiving the diet twice daily versus once or 4 times daily, but not different when cows received the diet once or 4 times daily (Table 4). Holotrichs populations (isotrichs and dasytrichs) were not affected by frequency of diet delivery (average of 4.37×10^3 cell/mL). Conversely, Bragg et al. (1986) reported that increasing frequency of delivery of a TMR to steers from twice to 8 times daily had no effect on the rumen protozoa population. Likewise, Yang and Varga (1989) also reported that increasing frequency of concentrates delivery to cows from once to 4 times per day had no effect on the rumen protozoa population.

Figure 4. Ruminal NH_3 concentration ($\pm$ SEM) in lactating cows receiving the diet once (1X; ■), twice (2X; ●), or 4 times (4X; ▲) daily. ^{a-c} $P \leq 0.05$.

Table 5. In sacco DM degradation kinetic parameters of TMR in lactating cows receiving the diet once, twice, or 4 times daily

Item ¹	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
a (%)	45.1	45.3	45.5	0.25
b (%)	44.2	43.7	43.5	0.49
c (per h)	0.042	0.038	0.041	0.0012
ERD (%)	62.8	62.0	62.8	0.15

¹a = soluble and rapidly degradable fraction; b = slowly degradable fraction; c = fractional rate of disappearance of the fraction b; ERD = effective ruminal degradability.

In Sacco Ruminal Degradability

Parameters of in sacco ruminal DM degradability of the diet are presented in Table 5. Soluble and rapidly degradable fraction, slowly degradable fraction, and fractional degradation rate averaged 45.3%, 43.8%, and 0.040/h, respectively, and were not affected by frequency of diet delivery. Consequently, frequency of diet delivery has no effect on effective ruminal DM degradability of the diet (averaged 62.5%). These results are consistent with the absence of an effect of frequency of diet delivery on ruminal total VFA concentration. Dehority and Tirabasso (2001) reported that increasing feeding frequency of sheep from 1 to 6 times had no effect on total and cellulolytic rumen bacteria population, which supports the lack of effect of diet delivery frequency on in sacco ruminal DM degradability parameters.

Milk Production and Milk Composition

Data of milk production and milk composition are presented in Table 6. Frequency of diet delivery had no effect on yield of milk, milk fat, or milk protein, which averaged 44.5, 1.65, and 1.44 kg/d, respectively. Consequently, production of FCM and ECM averaged 42.5 and 46.3 kg/d, respectively, and neither was affected by the experimental treatment. Concentrations of fat, protein, and lactose in milk were not affected by frequency of diet delivery. In this study, the lack of effect of frequency of diet delivery was consistent with no change in DMI. In agreement, other studies reported no effect of increasing frequency of diet delivery to dairy cows on yield of milk, milk fat, or milk protein (Robinson and Sniffen, 1985; Dhiman et al., 2002; Hart et al., 2014). However, an increase in milk fat or milk protein yield, or both, with increasing frequency of diet delivery has been reported when the concentrate percentage in the TMR exceeded 50% or the diet was

Table 6. Milk production and milk composition in lactating cows receiving the diet once, twice, or 4 times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
Production (kg/d)				
Milk	44.6	44.7	44.2	1.48
4% FCM ¹	42.4	42.7	42.4	1.48
ECM ²	46.2	46.4	46.2	1.56
Composition (%)				
Fat	3.66	3.71	3.75	0.089
Protein	3.24	3.23	3.26	0.049
Lactose	4.64	4.63	4.64	0.038
MUN (mg/dL)	14.1 ^b	14.2 ^{ab}	14.7 ^a	0.41
SCC (10 ³ /mL)	70.6	63.3	56.7	18.63
Yield (kg/d)				
Fat	1.64	1.66	1.65	0.065
Protein	1.44	1.44	1.44	0.048
Lactose	2.07	2.07	2.05	0.075
Feed efficiency				
Milk/DMI	1.69	1.72	1.68	0.059
FCM/DMI	1.60	1.64	1.61	0.054
ECM/DMI	1.74	1.78	1.76	0.056
Milk N/N intake	0.304	0.312	0.312	0.0093

^{a,b}Means within rows with different superscripts differ ($P < 0.05$).

¹4% FCM (Tyrrell and Reid, 1965) = $0.4 \times \text{milk yield (kg/d)} + 15 \times \text{fat yield (kg/d)}$.

²ECM (Tyrrell and Reid, 1965) = $0.327 \times \text{milk yield (kg/d)} + 12.95 \times \text{fat yield (kg/d)} + 7.2 \times \text{protein yield (kg/d)}$.

based mainly on corn silage (Yang and Varga, 1989; Shabi et al., 1999). In this study, the dietary concentrates proportion was 44%, and corn silage was 45% of the dietary forage component. Thus it is possible that the effect of frequency of diet delivery on milk protein and fat contents is more pronounced if dairy cows are fed high-concentrate than low-concentrate diets.

Compared with once daily, delivering the diet 4 times daily increased ($P = 0.03$) MUN concentration, but no differences were observed when compared with deliver-

ing the diet twice daily. Previous studies reported no effect of frequency of diet delivery on MUN concentration (Dhiman et al., 2002; Mäntysaari et al., 2006; Macmillan et al., 2017). Milk production efficiency (kg of ECM/kg of DMI) and milk N efficiency (g of milk N/g of N intake) averaged 1.76 and 0.31, respectively, and were not affected by frequency of diet delivery. Other studies reported no effect of frequency of diet delivery on milk production efficiency (Dhiman et al., 2002) or milk N efficiency (Judy et al., 2018).

Table 7. Enteric methane production in lactating cows receiving the diet once, twice, or four times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
DMI ¹ (kg/d)	27.3	26.5	26.5	0.58
CH ₄				
g/d	526	536	540	17.2
g/kg of DMI	19.3 ^b	20.3 ^a	20.4 ^a	0.71
g/kg of OM digested ²	8.34 ^b	8.78 ^a	8.78 ^a	0.312
% of GE intake ³	5.75 ^b	6.06 ^a	6.06 ^a	0.206
g/kg of milk ⁴	11.9	12.2	12.6	0.70
g/kg of ECM ⁴	11.5	11.7	11.9	0.55

^{a,b}Means within rows with different superscripts differ ($P < 0.05$).

¹Determined when cows were in the chambers.

²OM digested determined over the 7-d collection period.

³GE = gross energy.

⁴Yields of milk and ECM were measured over 7 d of performance measurements.

Methane Production

Studies on the effect of frequency of diet delivery on enteric CH₄ emissions of dairy cows are scarce. In this study, interactions between treatments and measurement days were not significant for CH₄ emission measurements. Therefore, only averages across measurement days are presented in Table 7. Dry matter intake determined when cows were housed in the chambers averaged 26.8 kg/d and was not affected ($P = 0.19$) by feeding frequency.

Daily enteric CH₄ emissions averaged 534 g/d and were not affected by frequency of diet delivery. This level of emission is close to those reported in our previous studies for cows with similar DMI (Benchaa et al., 2014; Cherif et al., 2018). Relative to DMI, GE intake, or OM digested, CH₄ emissions were similar between cows receiving the diet twice or 4 times daily, averaging 20.4 g/kg of DMI, 6.06% of GE intake, and 8.78 g/kg of OM digested, respectively. However, delivering the diet once daily reduced ($P \leq 0.04$) CH₄ emissions to 19.3 g/kg of DMI, 5.75% of GE intake, and 8.34 g/kg of OM digested, respectively, compared with delivering the diet twice or 4 times daily. This decline in CH₄ emissions was not, however, associated with changes in nutrient intake and digestibility.

According to Nocek and Braund (1985), delivering the diet once daily to dairy cows did not affect DMI, but increased daytime (0600 to 1800 h) rate of intake (1.01 kg of DM/h) compared with delivering the diet 4 times daily (0.91 kg/h). Similarly, DeVries et al. (2005) reported that increasing frequency of diet delivery reduced duration of feed consumption after diet delivery and increased daily feeding time, without affecting DMI. Also, Mäntysaari et al. (2006) reported that delivering the diet once daily induced a large peak of feed intake after diet delivery, whereas delivering the diet 5 times daily induced smaller peaks of feed intake that coincided with diet delivery. Based on the findings of these studies, it can be speculated that delivering the diet once daily may have increased the first meal size, which would have increased passage rate and reduced extent of rumen fermentation of the diet compared with cows receiving the diet twice or 4 times daily. Consequently, this reduced CH₄ emissions expressed on DM, GE, or digested OM intake bases in cows receiving the diet once daily compared with cows receiving the diet twice or 4 times daily. Other studies have demonstrated an inverse relationship between enteric CH₄ production (g/kg of DMI or % of GE intake) and ruminal passage rates (Okine et al., 1989; Goopy et al., 2014; Hammond et al., 2014). In agreement, Crompton et al. (2011) reported numerically lower CH₄ emissions (g/kg of DMI)

in cows receiving a diet ad libitum once per day versus twice or 4 times daily. However, studies that restricted DMI in cows reported no effect of frequency of diet delivery on CH₄ production expressed in grams per day or grams per kilogram of DMI (Jonker et al., 2014; Judy et al., 2018).

In the current study, the lower enteric CH₄ (g/kg of DMI) in cows receiving the diet once daily compared with cows receiving the diet twice daily was associated with lower numbers of protozoa in cows receiving the diet once daily. Enteric CH₄ production has been positively associated with total protozoa and entodiniomorph populations in the rumen (Hegarty, 1999; Guyader et al., 2014). An increase in passage rate would reduce protozoa population in the rumen according to Franzolin and Dehority (1996) and Janssen (2010). In the present study, the decline in CH₄ yield (g/kg of DMI) in cows receiving the diet once daily versus 4 times daily was associated with a decreased acetate molar proportion at 2, 3, and 4 h after the a.m. meal. Delivering the diet 4 times daily compared with once daily may have increased rumen retention of OM and allowed more extensive fiber degradation in the rumen, resulting in higher acetate molar proportion at 2, 3, and 4 h after the a.m. meal. Increased acetate production in the rumen would liberate more hydrogen for methanogenesis (Boadi et al., 2004; Beauchemin et al., 2009).

When expressed on a milk production basis, CH₄ emissions were not affected by frequency of diet delivery (average of 11.7 g of CH₄/kg of ECM). Similarly, Judy et al. (2018) reported no effect of frequency (once or twice daily) of diet delivery to Jersey cows on CH₄ emissions expressed on a milk production basis. Crompton et al. (2011) reported a numerical increase in CH₄ emissions (g/kg of milk) when frequency of diet delivery to cows increased.

Effect of frequency of diet delivery on diurnal variation in rate of CH₄ production (g/h) is presented in Figure 5, and diurnal enteric CH₄ production (g/h) kinetic parameters are presented in Table 8. Initial rate of CH₄ production before the a.m. meal (0930 h) was similar between treatments and averaged 18.1 g/h. Regardless of frequency of diet delivery, rate of CH₄ production increased after each diet delivery. Similarly, Crompton et al. (2011), Jonker et al. (2014), and Judy et al. (2018) reported that peaks of CH₄ production rate coincide with frequency of diet delivery. In cows receiving the diet once per day, the rate of CH₄ emission peaked (25.8 g/h) 1.79 h after the a.m. meal (0930 h) and then declined at a rate of 0.017/h. In cows receiving the diet twice daily, CH₄ production rate peaked (24.5 g/h) 2.21 h after the a.m. meal and then

Figure 5. Daily enteric CH₄ emissions in lactating cows receiving the diet once (1X; ■), twice (2X; ●), or 4 times (4X; ▲) daily.

declined at a rate of 0.023/h. Rate of CH₄ production then peaked again (28.1 g/h) 1.06 h after the p.m. diet delivery (2130 h) and declined at a rate of 0.064/h. In cows receiving the diet 4 times a day, CH₄ production rate peaked (24.6 g/h) 1.05 h after the first diet delivery (0930 h) and then declined at a rate of 0.099/h. Methane production rate peaked (25.2 g/h) 0.87 h after the second diet delivery (1300 h) and then declined at a rate of 0.030/h. After the third diet delivery (1630 h), CH₄ production rate peaked (27.1 g/h) 0.61 h after diet delivery and then declined at a rate of 0.029/h. Methane production rate peaked (26.2 g/h) 0.60 h after the fourth diet delivery (2130 h) and then declined at a rate of 0.098/h. In our study, time (h) from diet delivery to peak CH₄ production ranged from 0.60 to 2.21 h, which is similar to the range of 0.66 to 2.00 h reported by Crompton et al. (2011). In accordance with our observations, Crompton et al. (2011) and Jonker et al. (2014) reported a decrease in time (h) to reach peak CH₄ production after diet delivery, whereas CH₄ production rate (g/h) after the peak declines more rapidly as frequency of diet delivery increases. In summary, for

CH₄ production, time (h) to reach peak declined with increasing frequency of diet delivery and averaged 1.79, 1.64, and 0.78 h when diets were delivered once, twice, and 4 times daily, respectively. Peak CH₄ production rate (g/h) averaged 25.8, 26.3, and 25.7 g/h when diets were delivered once, twice, and 4 times daily, respectively. Rate of decline of CH₄ production after the peak (per h) increased with increasing frequency of diet delivery and averaged 0.017, 0.044, and 0.064/h when diets were delivered once, twice, and 4 times daily, respectively.

Nitrogen Excretion

Daily fecal and urinary outputs averaged 7.7 kg DM/d and 27.2 kg/d, respectively, and were not influenced by frequency of diet delivery. Fecal N excretion averaged 233 g/d and 32% of N intake and was not affected by frequency of diet delivery (Table 9). Nitrogen excretion in urine expressed in grams per day tended ($P = 0.08$) to be higher for cows receiving the diet 4 times daily compared with twice daily, but was not different from cows receiving the diet once daily. When expressed as a percentage of N intake, urinary N excretion was higher ($P = 0.05$) for cows receiving the diet 4 times daily versus once and twice daily, with no differences between delivering the diet once or twice daily. Total N excretion expressed as grams per day was not affected by frequency of diet delivery (averaged 483 g/d). When expressed as a percentage of N intake, total N excretion was higher ($P = 0.04$) when cows received the diet 4 times daily versus once or twice daily, with no differences between delivering the diet once or twice daily. In agreement, Judy et al. (2018) reported that diet delivery to cows once or twice per day had no effect on fecal or urinary N excretion.

The higher urinary and total N excretion (as a proportion of N intake) in cows receiving the diet 4 times daily compared with cows receiving the diet once or twice daily is in agreement with higher MUN in cows receiving the diet 4 times daily. Increasing frequency of diet delivery increases salivary secretions (Ruckebusch,

Table 8. Diurnal enteric CH₄ production (g/h) kinetic parameters in lactating cows receiving the diet once, twice, or 4 times daily

Daily frequency of diet delivery	Time of diet delivery (hhmm)	Initial CH ₄ production rate (g/h)	Time to peak (h)	Peak CH ₄ production rate (g/h)	Rate of decline (per h)
Once	0930	18.2	1.79	25.8	0.017
	2130	17.9	2.21	24.5	0.023
Twice	0930	20.3	1.06	28.1	0.064
	2130	18.1	1.05	24.4	0.099
4 Times	0930	21.7	0.87	25.2	0.030
	1300	24.0	0.61	27.1	0.029
	1630	24.1	0.60	26.2	0.098
	2130				

Table 9. Nitrogen excretion in feces and urine of lactating cows receiving the diet once, twice, or 4 times daily

Item	Frequency of diet delivery			SEM
	Once	Twice	4 Times	
Intake N (g/d)	744	723	720	15.2
Fecal N				
g/d	238	229	231	7.6
% of N intake	31.9	31.7	32.1	0.62
Urinary N				
g/d	251 ^{AB}	245 ^B	254 ^A	6.8
% of N intake	34.0 ^b	33.9 ^b	35.6 ^a	0.93
Total N excretion				
g/d	490	474	486	11.3
% of N intake	65.9 ^b	65.6 ^b	67.7 ^a	1.02
Retained N				
g/d	27.89	23.26	9.39	7.342
% of N intake	3.65	3.17	1.07	0.977

^{a,b}Means within rows with different lowercase superscripts differ ($P < 0.05$).

^{A,B}Means within rows with different uppercase superscripts tended to differ ($P = 0.08$).

1988; Beauchemin, 2018) and consequently increases urea recycling into the rumen. According to Lapierre and Lobley (2001), 67% of the ruminal NH_3 pool originates from BUN, of which 47% are provided through saliva secretions. If urea N is not used for microbial protein synthesis, it will be reabsorbed, transferred to the liver, and excreted in urine. Because the diet used in this study provides a sufficient concentration of RDP (Table 1; NRC, 2001), excess contribution of urea from saliva generated by treatment effect is likely to be excreted in urine. However, the differences between treatments in MUN and urinary N excretion ($\leq 4\%$) are too small to have any biological significance.

Cabrera et al. (2006) reported that increasing frequency of diet delivery would reduce N excretion in urine through reduced daily fluctuation of ruminal NH_3 concentration. The contrary was observed in this study when cows received the diet 4 times daily versus once or twice daily. It should be recognized that ruminal fluid sampling in this study encompassed the first 8 h after the a.m. meal and did not include the entire daily fluctuation of NH_3 concentration. This may explain different responses to treatments in ruminal NH_3

concentrations, MUN, and urine N excretion. Nitrogen retention was not affected by frequency of diet delivery (averaged 20 g/d and 2.6% N intake).

Chemical Composition of the Mixture of Feces and Urine and Manure Methane Emissions

Amounts excreted and chemical composition of feces and urine mixture are presented in Table 10. Quantity of VS averaged 6.9 kg/d and was not affected by frequency of diet delivery. This is consistent with the absence of treatment effect on OM intake and digestibility. Quantities of VS observed in this study are within the values predicted by equation 10.24 in Tier 2 of IPCC (2006), which averaged 7.3 kg/d.

Maximal CH_4 emission potential averaged 148 L/kg of VS and was not affected by frequency of diet delivery, which was consistent with the absence of treatment effect on VS and NDF excreted (Table 10). In this study, the observed values of maximal CH_4 production potential of manure are within the range of values (85 to 197 L/kg of VS) observed in our previous studies (Massé et al., 2016; Benchaa and Hassanat, 2019; Hassanat and

Table 10. Volatile solids of the mixture of urine and feces, maximal methane production potential of incubated manure of lactating cows receiving the diet once, twice, or 4 times daily

Item	Daily frequency of diet delivery			SEM
	Once	Twice	4 Times	
Volatile solids				
kg/d	6.85	7.16	6.64	0.354
% of DM	83.7	83.1	82.3	0.55
NDF (% of DM)	44.8	44.6	44.5	1.18
Maximal CH_4 production (L/kg of volatile solids)	155	164	125	14.7

Benchaar, 2019) under similar experimental conditions (17 wk of anaerobic incubation at 20°C). According to Environment Canada (2015), maximal CH₄ emission potential of manure from dairy cattle varies widely (100 to 240 L/kg of VS).

CONCLUSIONS

Under the experimental condition of the present study, diet delivery to dairy cows once, twice, or 4 times per day had no effect on DMI, nutrient digestion, ruminal pH, milk production, or milk composition. Methane production, expressed as a percentage of GE intake was lower for cows fed once daily versus cows fed twice or 4 times daily. Enteric CH₄ production peaked after each meal and then declined after, with a more rapid decline rate when frequency of diet delivery increased. Diet delivery 4 times daily resulted in higher urinary N excretion (% of N intake) compared with delivering the diet once or twice daily. Manure maximal CH₄ emission potential was not affected by frequency of diet delivery.

ACKNOWLEDGMENTS

The authors are grateful for the efforts of Lisa Croteau and Sylvie Provencher (technical assistance), Steve Methot (help with statistical analysis), and the barn crew (care for animal) from the Sherbrooke Research and Development Centre (Agriculture and Agri-Food Canada, Sherbrooke, Quebec). Funding from Agriculture and Agri-Food Canada (Ottawa, Ontario) made this work possible. The authors have not stated any conflicts of interest.

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ORCIDS

- C. Benchaa <https://orcid.org/0000-0002-8644-6892>
 F. Hassanat <https://orcid.org/0000-0002-2311-8234>