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Research article

Implications of dietary interventions (forage to concentrate ratio and forage type) in high or low methane-emitting dairy cows: Nutrient digestibility, rumen fermentation, and energy and nitrogen utilization

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ABSTRACT

Following the measurement of methane (CH_4) emissions from 100 Nordic Red lactating dairy cows, a subset of 10 cows was selected based on CH_4 yield (g CH_4/kg DM intake) being low (Low emitter = 22.9 ± 1.1; n = 5) or high (High emitter = 24.0 ± 0.9; n = 5) emitters to evaluate the relationship between CH_4 yield phenotypes and experimental diets [forage to concentrate (FC) ratio and forage type] on enteric CH_4 production, feed intake, lactation performance, ruminal digestion and passage kinetics, digestibility, and energy and nitrogen utilization. Ten lactating dairy cows fitted with rumen cannula were randomly allocated to experimental diets in a 3 times replicated 3 × 3 Latin square with an extra replicate that was randomly allocated to one of the diets in each 35-d experimental period, resulting in n = 10 for each diet. The experiment was split into 2 blocks (blocks A and B) of 4 or 6 cows over time (7-wk interval) to have all cows in the same lactation stage. Dietary treatments comprised grass silage-based diets containing high (70:30, HG) or low (30:70, LG) FC ratio or a red clover silage-based diet with FC ratio of 50:50 (RC) on DM basis. Intakes of DM and gross energy (GE) for cows fed LG were greater than HG and RC in both cow groups whereas NDF intake was lower for LG and RC than HG and no interaction was detected. Milk yield was 12% greater for LG and RC than HG, but ECM yield was the greatest for LG. The diets clearly affected all the rumen fermentation parameters while only propionate and butyrate proportions, and NH_3-N concentration were affected by the interaction; Low-emitting cows had greater ruminal propionate and lower butyrate proportions than High-emitting cows on LG and High-emitters had the highest NH_3-N concentration with RC. In addition, CH_4 intensity (g CH_4/kg milk) was greater for LG in Low- than High-emitting cows. Total-

Abbreviations: BW, Body weight; CH_4, Methane; CP, Crude protein; DE, Digestible energy; DM, Dry matter; DIM, Days in milk; DMI, DM intake; ECM, Energy-corrected milk; EE, Ether extract; FC ratio, Forage-to-concentrate ratio; GE, Gross energy; HG, High-grass silage diet; k_i, Rate of intake; k_d, Rate of digestion; k_p, Rate of passage; LG, Low-grass silage diet; INDF, Indigestible neutral detergent fiber; PdNDF, Potentially digestible NDF; OM, Organic matter; RC, Red clover silage diet; ME, Metabolizable energy; MRT, Mean retention time; MUN, Milk urea nitrogen; NDF, Neutral detergent fiber expressed exclusive of residual ash; NFC, Non-fibre carbohydrates; NRC, National Research Council; SD, Standard deviation; TMR, Total mixed ration; VFA, Volatile fatty acid.

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tract digestibility of DM, OM, and GE was greater for LG and HG relative to RC without any interaction effect and High-emitters had greater nutrient digestibility compared with Low-emitters. Diets but not cow group affected rumen contents and ruminal digestion and passage kinetics without any interaction whereas cows fed LG had shorter DM mean retention time than HG and RC. Feeding LG improved energy balance and affected other parameters of energy utilization whereas cow group and interaction effects were not significant. Overall, there were clear differences with respect to CH_4 yield phenotype on nutrients digestion and energy or N utilization in Low- vs. High-emitters whereas dietary treatments affected lactation performance, digestion, rumen fermentation and kinetics, and energy utilization of dairy cows. These findings indicate that dairy cows with low CH_4 yield may experience negative consequences on nutrient digestibility.

1. Introduction

Dairy cow methane (**CH_4**) emissions account for 46% of the total greenhouse gas emissions in the world dairy supply chain when expressed as carbon dioxide (**CO_2**) equivalents (Gerber et al., 2013). Considerable resources are devoted to the abatement of enteric CH_4 production by livestock (Beauchemin et al., 2020). The productivity of dairy cattle has risen considerably due to advances in nutrition, genetics, and management. Recently, the interest in including low CH_4 emission as a goal in breeding programs has substantially increased. This issue was triggered by studies showing that CH_4 emission is a heritable trait in ruminants (Pinares-Patiño et al., 2013; Difford et al., 2018). It appears that selecting for greater feed efficiency and lower CH_4 emission traits improve profitability and reduce environmental impact of dairy farms (Løvendahl et al., 2018). It was indicated that more efficient dairy cows produce less CH_4 emissions (Guinguina et al., 2020a,b). Nonetheless, the ranking of animals for CH_4 emissions trait can differ depending on the type and duration of experimental settings, the trait definitions and calculations used, the period in lactation examined and the production system, as well as interactions among these factors (Løvendahl et al., 2018). Therefore, if the between-animal differences are consistent over time with different diets, animal breeding might be a successful mitigation strategy.

Lower CH_4 yield (g CH_4/kg DMI) may arise due to one or more of the following i.e., less fermentation of organic matter in the rumen; a shift in volatile fatty acids (**VFA**) production towards alternative hydrogen-utilizing pathways (e.g., propionate) and an increased yield of microbial cells produced by fermentation (Beauchemin et al., 2020). Kennedy and Charmley (2012) reported that over 70% of the variation in daily CH_4 production can be explained by feed intake. According to a meta-analysis based on an inter-continental database, CH_4 represents an estimated loss of $6.0 \pm 1.18\%$ of energy intake from dairy cows (Niu et al., 2018). The CH_4 yield is negatively associated with feed intake and positively associated with digestibility (Blaxter and Clapperton, 1965; Yan et al., 2000). Further, if rumen retention time is increased, this would likely increase CH_4 emissions at a given daily intake of dry matter (Huhtanen et al., 2016). In addition, Shi et al. (2014) have proposed that a longer retention time of digesta in the rumen changes the expression of methanogenesis pathway genes leading to increased CH_4 production. However, this is not well known to which extent, enteric CH_4 production is influenced by the host animal genetics, diet, rumen microbiome and their interactions.

Forage type, i.e., grass vs. red clover forage having different composition, (i.e., protein and NDF concentrations) and also, substrate supply (i.e., dietary starch vs. forage NDF) by changing forage-to-concentrate (**FC**) ratio can affect nutrient digestibility. In addition, diet composition which is closely associated with digestibility influences passage and digestion kinetics of feed particles in the gastrointestinal tract, and finally CH_4 emissions when fed to dairy cows with high and low CH_4-yield phenotypes (Dewhurst, 2013; Cabezas-Garcia et al., 2017). Forage to concentrate ratio and dietary forage NDF concentration are important variables influencing CH_4 emissions per unit intake. Of note, low CH_4 yields have been reported when high-concentrate diets are fed to dairy cows (Bayat et al., 2017; Razzaghi et al., 2022), reflecting increased propionate production from the greater supply of starch in these diets. Conversely, forages increase CH_4 yield in ruminants by greater rumen mean retention time due to slower NDF digestion as explained by Huhtanen et al. (2016).

The objective of this study was to examine the role of CH_4 yield phenotype in regulating ruminal digestion, enteric CH_4 emissions, and nutrient utilization when challenged with dietary modifications including FC ratio and forage type. Differences in mean ruminal retention time can markedly contribute to variation in CH_4 yield. As such, we hypothesized that mean retention time may contribute to between-animal differences in CH_4 yield among animals fed grass silage-based diets containing high (70:30) or low (30:70) FC ratio or a red clover silage-based diet with a FC ratio of 50:50 on a DM basis. Elucidating these mechanisms may improve the ability to select for reduced CH_4 emissions. Our hypothesis was that CH_4 yield under specific dietary conditions results from animal physiology (controlled by animal genetics) via rumen fill, digestion and passage kinetics which are inherited to some extent. To test the hypothesis, CH_4 production was measured by the most accurate technical solution available, i.e., respiration chambers, together with the measures of whole-tract digestibility, ruminal digestion and passage kinetics measured using rumen evacuation technique, as well as energy and N utilization in multiparous dairy cows differing in CH_4 yield. Therefore, considering both CH_4 yield phenotype and dietary interventions shed light on the main factors involved in mitigation of CH_4 emission especially when variations in parameters such as ruminal digestion and passage kinetics are studied.

2. Materials and methods

Animal care and all experimental procedures used in this study were approved by the National Ethics Committee (ESAVI/8858/

04.10.07/2015, Hämeenlinna, Finland) in accordance with the guidelines established by the European Community Council Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines (Kilkenny et al., 2010).

2.1. Cows, experimental design, and treatments

Feed intake and CH_4 emissions were measured on a group of 100 multiparous Finnish Nordic Red cows in respiration chambers during 5 d in established lactation (between 90 and 240 DIM) that had received a control diet based on grass silage with 55:45 FC ratio for at least 14 d. The cows were ranked according to their residual (observed – predicted) CH_4 yield (g/kg CH_4/DMI; Fig. 1 and Table 1), and 10 low and high CH_4 emitters (22.9 ± 1.1 vs. 24.0 ± 0.9 g/kg DM) were selected for further measurements (5 low-emitting and 5 high-emitting cows). The model used for identifying the residual was generated using Proc Reg of SAS using the same data collected from the 100 cows as CH_4 yield (g/kg DMI) = 29.41 – 0.34 × DMI (kg/d). The ranking of the cows was re-evaluated during the consecutive lactation period by measuring enteric CH_4 production and feed intake in the chambers for 5 d before the main experiment to ensure their ranking remains the same. The ranking remained the same even though CH_4 yield of the groups was slightly different (23.0 vs. 23.8 g/kg DM) from the original ranking. The selected cows (n = 10) were fitted with rumen cannula (i.d., 100 mm; Bar Diamond Inc., Parma, ID) and were randomly allocated to experimental diets in a 3 times replicated 3 × 3 Latin square with an extra replicate that was randomly allocated to one of the diets in each 35-d experimental period, resulting in n = 10 for each diet throughout the study. The study was conducted as two blocks of 4 or 6 cows with 7-wk intervals to have all the cows in the same lactation stage [block A consisting of 4 cows with (mean ± SD) 626 ± 34.4 kg BW, 4 ± 0.7 parity, 90.3 ± 17.9 DIM, and producing 39 ± 3.2 kg milk/d and block B consisting of 6 cows with 720 ± 64.1 kg BW, 4 ± 0.5 parity, 63.8 ± 15.6 DIM, and producing 43.2 ± 4.3 kg milk/d]. Details of diets and their chemical composition are presented in our previous study (Jaakamo et al., 2019). Briefly, treatments consisted of diets based on grass silage and cereal-based concentrate diets with either 70:30 (high-grass silage, **HG**), or 30:70 (low-grass silage, **LG**) FC ratio (on DM basis) and a red clover silage and cereal-based concentrate diet (**RC**) with 50:50 FC ratio (Table 2). Silages were prepared from mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) and from red clover (*Trifolium pratense*) swards treated with 5 L/t of formic acid-based additive (760 g of formic acid and 55 g of ammonium format, AIV 2 Plus, Kemira Ltd.). Diets were formulated based on Finnish Feed Tables (Luke., 2018) to meet metabolizable energy and protein requirements of the lactating cows producing 40 kg milk/d. The animals were housed in individual tie stalls in a dedicated metabolism unit with free access to water and salt blocks. The cows were milked 2 times daily at 0700 and 1645 h. The diets were fed as TMR at 0600, 0900, 1600, and 1900 for a targeted refusal rate of 10% to allow for *ad libitum* intake.

2.2. Measurements and chemical analysis

Feed intake and milk yield were recorded daily, but only measurements made between d 15 and 35 of each experimental period were used for statistical analysis. Representative samples of grass silage, red clover silage, and concentrates were collected during TMR preparation daily over 5 d on d 30–34 of each experimental period for chemical composition analysis as explained by Ahvenjärvi et al. (2018). Concentration of gross energy (**GE**) in samples of silage, concentrates, and excreta were determined by bomb calorimetry (1108 Oxygen bomb, Parr Instrument). Before GE analysis of urine sample, its pH was adjusted to 6.0 using 5 M NaOH solution and 70 g urine sample was freeze-dried over 4 d before being used in the calorimetric bomb. Nitrogen content of fresh urine sample was analyzed using Kjeldahl method. Urine sedimentations were collected by filtration using 4 layers of cheesecloth and analyzed for N by dumas procedure using Leco FP 428 nitrogen analyzer (Leco Corp., St Joseph, MI). Indigestible NDF (**iNDF**) concentration of silage, concentrates, and feces was determined by incubation of 1.0 g of sample DM in nylon bags (60 × 120 mm, pore size 0.017 mm) for 12

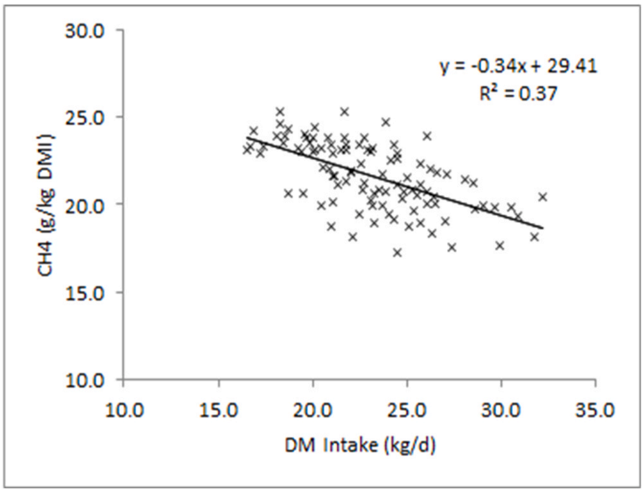

Fig. 1. Variation in CH_4 yield (g/kg DMI) as influenced by the level of DMI and used to select the low- and high-CH_4 emitting cows.

Table 1
Summary statistics of the dairy cows used in the experiment.

Item	High emitters	Low emitters	Difference (%)[1]	P value
Body weight, kg	729 ± 70	635 ± 47	14.7	0.038
Parity	4.6 ± 0.55	3.8 ± 0.45	17.4	0.55
Days in milk	81 ± 21	68 ± 24	13	0.68
DM intake, kg/d	24.2 ± 1.8	22.0 ± 1.4	10.0	0.34
DM intake, % BW	3.35 ± 0.42	3.49 ± 0.47	−6.8	0.74
DM intake, % $BW^{0.75}$	18.1 ± 1.7	18.7 ± 1.7	−3.2	0.75
Milk yield, kg/d	41.0 ± 6.1	42.0 ± 3.4	−2.54	0.26
Methane, g/d	581 ± 35	503 ± 41	15.5	0.69
Methane yield, g/kg DM intake	24.0 ± 0.9	22.9 ± 1.1	4.8	0.10
Methane intensity, g/kg milk	14.4 ± 1.6	12.0 ± 1.7	16.2	0.055

[1] Difference calculated as (high emitters – low emitters)/high emitters × 100.

Table 2
Formulation and chemical composition of experimental diets.

	Treatments[1]		
Item	HG	LG	RC
Ingredient, % of diet DM			
Grass silage[2]	70.0	30.0	-
Red clover silage[3]	-	-	50.0
Barley grain	13.5	31.4	22.4
Molassed sugar beet pulp	6.8	15.8	11.3
Rapeseed meal[4]	8.6	20.2	14.4
Vitamin and mineral premix[5]	1.1	2.6	1.9
Chemical composition, % DM, unless stated			
DM, % as fed	45.0	70.2	56.0
OM	92.5	92.3	90.9
CP	14.4	16.5	18.0
Ether extract	3.64	3.38	3.85
NDF	37.1	29.9	32.5
Potential degradable NDF	31.8	24.1	22.8
ADF	21.7	17.1	20.8
Starch	8.58	19.2	14.1
Water soluble carbohydrate	4.57	5.69	3.84
Gross energy, Mcal/kg DM	4.23	4.28	4.25

[1] Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[2] Mean fermentation characteristics of experimental grass silage: pH, 3.91; in DM (g/kg) lactic acid, 67.4; acetic acid, 32.0; propionic acid, 0.57; butyric acid, 0.77; soluble N (g/kg total N), 557; ammonium N (g/kg total N), 40.2; gross energy (Mcal/kg DM), 4.21.
[3] Mean fermentation characteristics of experimental red clover silage: pH, 4.12; in DM (g/kg) lactic acid, 68.2; acetic acid, 25.8; propionic acid, 1.00; butyric acid, 2.91; soluble N (g/kg total N), 335; ammonium N (g/kg total N), 58.5; gross energy (Mcal/kg DM), 4.18.
[4] Solvent extracted of low glucosinolate content
[5] Vitamin and mineral premix (Mahti-Mira, Vilomix Finland Oy, Paimio, Finland) declared as containing: (g/kg) calcium, 220; magnesium, 64; sodium, 105; (mg/kg) zinc, 2.127; manganese, 1.025; copper, 1.18; iodine, 66; cobalt, 520; mineral selenium, 33; organic selenium, 37; (IU/g) vitamin A, 156; vitamin D_3, 52; and dl-α tocopherol acetate, 1.44.

d as previously described by Huhtanen et al. (1994). Potentially digestible NDF (**pdNDF**) concentration was calculated as NDF – iNDF. Milk samples were collected at each milking, from d 28–32, preserved with bronopol (Valio Ltd.) and analyzed for milk fat, crude protein, lactose and milk urea nitrogen (**MUN**) by infrared analysis (MilkoScan FT6000, Foss Electric).

2.3. Enteric CH_4 production

Four open-circuit respiratory chambers (W × L × H 288 × 396 × 220 cm) were used to measure enteric CH_4 emissions of dairy cows over 4 days in block A and 2–3 d in block B after at least 14 d of adaptation to the diet in each period and 1 d acclimatization to the respiratory chambers. More details on the respiration chambers and gas measurements are published by Bayat et al. (2022). The transparent chambers were in the main barn where the experimental cows could see each other and the other cows in the barn to reduce the stress level and potential negative effects on intake and production. Before running the main experiment, gas recovery tests were performed. Nominal amounts of CH_4 [0.2, 0.4, and 0.6 L/m equal to 288, 576 and 884 L/d set by flow meters (AGA, Espoo, Finland)] to represent the wide range of CH_4 production by the cows based on the previous experiments were released into every individual respiratory chamber for at least 2 h. The data recorded after achieving plateau in outlet air's CH_4 concentration (after about 1 h from initiation of gas release) were used to calculate the gas recovery ratio. For more accurate measurement of released gas, the gas cylinder was weighed by scale (20 kg ± 1 g) before and after releasing each gas, and the actual gas release rate was calculated. The measured versus released amount of gas was used to calculate whole system gas recovery (measured gas/released gas × 100). The recovery rates for CH_4 were 102 ± 2.73, 99.3 ± 1.08, 99.8 ± 0.34, and 101.3 ± 1.65% for chambers 1–4, respectively.

2.4. Rumen evacuation, digestion, and rumen kinetics

On d 35 of each experimental period, samples of rumen liquid (500 mL) were collected from each cow at 1.5-h intervals from 0600 to 1630 h. Immediately after collection, pH was measured using a portable pH meter. Samples were filtered through 2 layers of cheesecloth and stored at −20°C until analyzed for VFA and NH_3-N by procedures described in Huhtanen et al. (1998) and McCullough (1967), respectively. To evaluate the ruminal digestion and passage kinetics, the manual evacuation of whole rumen contents was conducted on d 24 at 0900 and on d 27 at 1500 of each experimental period. All ruminal contents were emptied, weighed and mixed well in a big container kept in 37 ℃ before taking a representative sample (500 g). Ruminal content was returned to the rumen immediately after sampling. The subsamples were used for DM determination (60 ℃, 48 h) and chemical composition. Concentrations of NDF and ADF were measured as described by Ahvenjärvi et al. (2018). The ruminal pool sizes of ADF, NDF and iNDF were determined by the multiplying DM pool size by the respective ADF, NDF and iNDF concentrations. Total-tract apparent digestibility coefficients were determined by total fecal collection over a 96-h interval for block A and 48- and 72-h intervals for block B starting at 1800 h on d 31 of each experimental period when the cows were in respiration chambers. Excreted feces were weighed, thoroughly mixed, subsampled (5%, wt/wt), and stored at −20°C until chemical analysis. Urine was separated from feces by means of a light-weight harness and flexible tubing attached to the vulva and collected in plastic canisters containing 500 mL of 5*M* sulfuric acid. Collection vessels were changed at 12-h intervals and daily samples (5%, wt/wt) were taken and stored at −20°C. The chemical composition of feces was determined using the same methods applied to feeds.

2.5. Calculations and statistical analysis

Total-tract digestibility coefficients of nutrients were calculated based on the difference of intake and fecal excretion. Digestion and passage kinetics of DM, NDF, and pdNDF and passage rate of iNDF were calculated as follows:

Rate of intake (k_i) = 1/24 × (intake, kg/d)/(rumen pool size, kg)

Rate of passage (k_p) = 1/24 × (omasal canal flow, kg/d)/(rumen pool size, kg)

Rate of digestion (k_d) = $k_i - k_p$

Methane production was calculated by multiplying the measured air flow (STP condition) and the difference in the concentration of CH_4 in the outlet and inlet air. To estimate the inflow air from measured outflow air, N in the air was used as a marker based on the Haldane principle. Energy intake and excretion in feces and urine were calculated by multiplying DMI, feces DM or urine DM by their respective GE contents. Daily ME intake was calculated as the difference between GE intake and energy excretions as feces, urine, and CH_4. Yield of ECM was calculated based on the yields of fat, crude protein, and lactose (ECM = milk (kg/d) × [38.3 × fat (g/kg) + 24.2 × crude protein (g/kg) + 16.54 × lactose (g/kg) + 20.7] / 3140; Sjaunja et al., 1990). Energy secretion in milk was calculated by multiplying ECM by 3.14. Heat production of the cows was calculated based on the exchanged oxygen, carbon dioxide, and CH_4 gases and urinary N excretion measured in the respiratory chambers (Brouwer, 1965). The energy of CH_4 was calculated using the conversion factor 55.24 kJ/g (Kriss, 1930). Energy balance was calculated as the difference between energy intake and energy excretion as feces, urine, CH_4, milk, and heat. Further, N balance was calculated as the difference between N intake and excretion in feces, urine, and milk.

All statistical analyses of data were performed by ANOVA, using SAS (version 9.4; SAS Institute Inc., Cary, NC). Energy and N utilization, digestion kinetics, milk production, and feed intake data were analyzed with PROC GLIMMIX model. The normality of analyzed variables was checked using box plot and scatter plot of residuals and fitted values generated using the Mixed procedure of SAS. The data was normally distributed, and no outliers were detected to be removed from the analysis. The experiment was split into 2 blocks of 4 or 6 cows over time to have all cows at the same lactation stage. The following model was used to analyze the data:

$$Y_{ijkl} = \mu + G_i + P_j + D_k + B_l + (G_i \times D_k) + (P_j \times B_l) + \varepsilon_{ijkl},$$

where Y_{ijkl} represents the dependent variable; μ, the overall mean; G_i, the fixed effect of animal group (i = low- or high-emitting cows); P_j, the fixed effect of period (j = 1–3); D_k, the fixed effect of diet (k = 1–3); B_l, the random effect of block (l = 1 and 2); $G_j \times D_k$, the interaction between group and diet; $P_j \times B_l$, the random effect of period and block interaction; and ε_{ijkl} the residual error. Least squares mean ± standard errors of the means are reported. *P*-values < 0.05 are considered significant, whereas *P*-values between 0.05 and 0.10 indicate a trend toward significance. A Tukey-Kramer multiple comparison test was used to evaluate significant differences.

3. Results

Methane yield remained different between the Low- and High-emitting groups on re-testing (23.0 vs. 23.8 g/kg DM) after their initial selection (22.9 vs. 24.0 g/kg DM). There was no difference between cow groups for nutrient intakes (Table 3). However, the proportion of DM intake to $BW^{0.75}$ tended to be lower ($P = 0.07$) in High compared with Low emitting cows. Intakes of DM, OM, and GE were greater ($P < 0.01$) in LG than in HG and RC for both cow groups, whereas there was no difference between RC and HG diets for these variables. The CP intake was greater in LG and RC than HG ($P < 0.01$), and NDF intake was lower ($P < 0.01$) in LG and RC than HG diet. Also, starch intake was on average 53% greater in cows fed LG and RC than those fed HG diet ($P < 0.01$). Further, the EE intake was greater in RC and LG ($P = 0.039$) compared with HG, with no difference between cow groups. Furthermore, in the current study, interaction effects were not observed for the nutrient intakes.

Milk yield was on average 12% greater ($P < 0.01$) in LG and RC than in HG, but ECM yield was greatest ($P = 0.044$) for LG diet, and HG and RC diets were not different (Table 4). The treatments did not affect milk fat yield while milk fat concentration was lower for RC compared with HG diet. Also low-emitting cows when fed LG diet had lower milk fat concentration compared with high-emitting cows ($P = 0.013$ for Diet × Group interaction). Furthermore, milk protein concentration and yield were greater ($P < 0.01$) in LG compared with HG and RC in both cow groups. Feeding RC and LG increased ($P < 0.01$) the milk lactose yield compared with HG. Also, milk urea nitrogen increased ($P < 0.01$) by feeding RC compared with grass silage-based diets in both cow groups.

Ruminal pH was lower ($P < 0.01$) for LG compared with HG and RC diets in both Low- and High-emitting cows (Table 5). The ruminal concentration of total VFA was lower ($P < 0.01$) in cows fed HG relative to those fed LG and RC. The ruminal proportion of acetate decreased ($P < 0.01$) in LG compared with HG and RC. Interaction effects were observed for the ruminal molar proportions of propionate and butyrate, and ruminal NH_3-N concentration. Low emitting cows had the greatest ruminal proportion of propionate when received LG diet (231 vs. 190 mmol/mol; $P = 0.044$ for Diet × Group interaction). In High-emitting cows, LG diet had the greatest ruminal butyrate proportion ($P = 0.050$ for Diet × Group interaction). Feeding RC increased the NH_3-N concentration in the rumen by 3.7-fold compared with grass silage-based diets, especially High-emitting cows had greater ruminal NH_3-N concentration than Low-emitting cows when fed RC diet ($P = 0.021$ for Diet × Group interaction). Acetate to propionate ratio was lower ($P < 0.01$) for LG than other diets and Low emitters tended (P = 0.09) to have lower acetate to propionate ratio. Molar proportion of valerate decreased ($P < 0.01$) by 24% in RC compared with grass silage-based diets and molar proportion of isovalerate decreased ($P = 0.051$) by feeding LG compared with HG and RC diets. Moreover, molar proportion of caproate was lower ($P = 0.016$) in RC compared with LG and HG diets.

We detected a tendency ($P = 0.06$) for interaction between cow group and diet on daily CH_4 emissions, with a more pronounced decrease for Low- than High-emitting cows fed LG diet (Table 6). In addition, results showed a significant interaction between cow group and diet ($P = 0.013$) with greater CH_4 intensity as g/kg milk in Low than for High-emitting cows when fed LG but no other diets (15.6 vs. 11.4 g/kg milk). The LG reduced ($P < 0.01$) CH_4 emissions expressed per unit of OM intake, OM digested in the total-tract, ECM yield and percentage of GE intake relative to HG diet, while RC diet was not different from grass silage-based diets. CH_4 emissions expressed as g/kg OM intake or milk and percentage of GE intake tended to be greater in High- compared with Low-emitting cows ($P \leq 0.09$) while CH_4 production as g/d and per unit of ECM were lower ($P \leq 0.03$) for Low- compared with High-emitting cows.

Apparent total-tract digestibility of CP and EE remained unaffected by treatments but those of DM, OM, and GE were greater ($P \leq 0.02$) for grass silage-based diets relative to RC (Table 7). However, NDF and ADF digestibility was greater ($P \leq 0.02$) for HG compared with LG and RC, while digestibility of pdNDF was greatest ($P < 0.01$) for RC than other treatments. With respect to CH_4 emission phenotype, High-emitting cows had greater ($P \leq 0.02$) DM, OM, NDF, and GE digestibility compared with Low-emitting cows whereas starch digestion was not different between cow groups. Total-tract digestibility of CP, EE, and ADF tended ($P \leq 0.08$) to be higher in High- compared with Low-emitting cows. Moreover, the interaction response was not detected for apparent total-tract digestibility of nutrients in our study.

Rumen pool sizes of total digesta, DM, NDF, and ADF and the kinetic parameters estimated based on 2 rumen evacuations are shown in Table 8. Interaction response and group effects were not detected for rumen contents and kinetics. Both fresh and dried rumen contents were greater for rumen evacuation carried out at noon than in the morning (no statistical comparison was done). In cows fed HG, rumen contents of NDF and pdNDF were greater ($P < 0.01$) compared with LG and RC. Further, feeding RC increased ($P < 0.01$) ADF content in the rumen compared with the grass silage-based diets. The cows fed LG had greater ($P < 0.01$) rates of digestion and passage of DM compared with those fed HG and RC. The treatment effect on the passage rate of NDF was similar whereas

Table 3
Nutrient intake of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				*P*-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
DM	24.1	27.9	24.4	22.8	25.1	23.1	0.79	< 0.01	0.12	0.20
DM/BW$^{0.75}$	3.30	3.83	3.34	3.65	4.02	3.70	0.187	< 0.01	0.074	0.48
OM	22.3	25.8	22.2	21.1	23.1	21.0	0.72	< 0.01	0.12	0.19
CP	3.47	4.60	4.36	3.27	4.14	4.13	0.131	< 0.01	0.12	0.29
Ether extract, g/d	883	951	939	835	847	887	37.9	0.039	0.14	0.23
NDF	8.90	8.40	7.98	8.40	7.51	7.58	0.272	< 0.01	0.13	0.31
Potential degradable NDF	7.63	6.79	5.59	7.20	6.08	5.31	0.209	< 0.01	0.12	0.25
ADF	5.31	4.76	5.10	5.06	4.29	4.83	0.287	< 0.01	0.20	0.62
Starch	1.99	5.31	3.34	1.89	4.78	3.18	0.128	< 0.01	0.11	0.11
Water soluble carbohydrates, g/d	1107	1587	915	1069	1420	861	45.4	< 0.01	0.13	0.19
Total gross energy intake, Mcal/d	102	120	104	97	107	98	3.4	< 0.01	0.11	0.15

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane emitter cows.

Table 4
Milk yield and composition of high- and low-methane-emitter lactating fed cows grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				P-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
Yields, kg/d (unless otherwise noted)										
Milk yield	34.7	37.8	38.2	33.6	40.8	38.7	2.05	< 0.01	0.78	0.20
ECM yield[4]	35.9	38.3	36.3	35.3	39.0	36.4	1.7	0.044	0.98	0.81
Milk fat, g/d	1533	1546	1448	1516	1471	1405	80.1	0.14	0.65	0.80
Milk protein, g/d	1158	1360	1221	1113	1438	1255	58.5	< 0.01	0.75	0.25
Milk lactose, g/d	1531	1667	1714	1513	1848	1774	93.9	< 0.01	0.54	0.22
Milk composition, %										
Milk fat	4.44[a]	4.18[ab]	3.83[b]	4.54[a]	3.65[b]	3.68[b]	0.27	< 0.01	0.47	0.013
Milk protein	3.33	3.63	3.21	3.33	3.54	3.25	0.12	< 0.01	0.92	0.33
Milk lactose	4.40	4.39	4.48	4.51	4.53	4.57	0.06	0.32	0.11	0.74
Milk urea nitrogen, mg/100 mL	9.3	21.3	30.2	11.0	19.2	31.1	1.81	< 0.01	0.91	0.16
Feed efficiency (ECM/DMI)	1.49	1.38	1.49	1.53	1.57	1.58	0.05	0.56	0.11	0.27

[1]Values are least square means and pooled SEM for n = 5.
[2] Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.
[4]ECM, energy-corrected milk yield calculated as described by Sjaunja et al. (1990).
[a,b]Means with similar letters do not differ significantly.

Table 5
Rumen fermentation characteristics of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				P-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
pH	6.55	6.08	6.49	6.51	6.11	6.37	0.078	< 0.01	0.58	0.34
NH_3-N, mM	1.32[c]	0.95[c]	4.55[a]	1.42[c]	0.71[c]	3.46[b]	0.250	< 0.01	0.058	0.021
Total VFA, mM	118	131	135	122	131	136	4.6	< 0.01	0.67	0.79
Molar proportion of VFA, mmol/mol										
Acetate	637	577	665	626	563	659	10.1	< 0.01	0.42	0.79
Propionate	177[b]	190[b]	190[b]	188[b]	231[a]	193[b]	7.5	< 0.01	0.044	0.044
Butyrate	145[b]	191[a]	105[c]	143[b]	163[ab]	108[c]	8.9	< 0.01	0.24	0.050
Valerate	17.7	19.4	14.9	18.6	22.4	14.9	0.90	< 0.01	0.099	0.28
Isovlerate	7.5	6.8	10.3	8.9	6.4	8.1	1.10	0.051	0.65	0.22
Isobutyrate	7.60	6.04	7.80	7.62	5.63	6.73	0.343	< 0.01	0.18	0.26
Caproate	11.0	11.4	7.6	10.8	9.3	8.9	0.87	0.016	0.62	0.19
Acetate:propionate ratio	3.62	3.04	3.50	3.36	2.48	3.41	0.151	< 0.01	0.094	0.15

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.
[a,b,c]Means with similar letters do not differ significantly.

the mean retention time of DM was shorter with LG ($P < 0.01$) relative to HG and RC. Cows consuming diets containing RC showed higher mean retention time of iNDF than those consuming grass silage-based diets ($P < 0.01$). We did not detect interaction response on rumen kinetics and contents in the current study.

The effects of diets on variables related to energy and N utilization and excretion are reported in Table 9. Dietary treatments had no effect ($P \geq 0.12$) on milk energy secretion as a proportion of either GE intake, feces energy excretion as a proportion of N intake, and N balance. However, feeding LG diet increased GE intake, energy metabolizable (ME to GE intake), and energy balance ($P < 0.01$) while it decreased feces, urine, and methane energy excretion as a proportion of GE intake compared with HG and RC ($P \leq 0.02$). Heat production was greater ($P < 0.01$) for HG compared with LG and RC. Energy balance was positive and greater ($P = 0.01$) in LG cows compared with others. Further, using RC in the diets caused an increase in urine N excretion as a proportion of N intake compared with grass silage-based diets ($P < 0.01$) while cows received a greater ($P < 0.01$) dietary N with LG and RC than HG. In addition, interaction effect was observed ($P = 0.035$) between Diet and Group only for the milk N secretion as proportion of N intake where it was lower in High-emitting cows than its counterpart for cows fed with LG and RC.

4. Discussion

Heritability of CH_4 production on an absolute emissions basis (g CH_4/d) in cattle is estimated to be 0.40 versus a lower value of 0.19 when estimated based on CH_4 yield (g CH_4/kg DMI; Pickering et al., 2015). Enteric CH_4 emissions from ruminants are typically expressed in terms of absolute production (as g/d) and yield (as g/kg DMI; Beauchemin et al., 2020). But still, CH_4 intensity is

Table 6
Methane production of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				*P*-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
Ruminal CH_4 emissions										
g/d	581[a]	580[a]	547[ab]	514[ab]	466[b]	499[ab]	19.0	0.19	0.016	0.06
g/kg DMI	24.2	22.6	20.8	18.6	22.5	21.5	0.65	< 0.01	0.08	0.35
g/kg OMI[4]	26.2	22.6	24.7	24.4	20.2	23.7	0.71	< 0.01	0.08	0.37
g/kg DOM[5]	35.7	30.5	34.6	33.7	28.1	34.0	1.01	< 0.01	0.14	0.50
g/kg Milk	16.8[a]	15.6[ab]	14.4[bc]	15.5[ab]	11.4[c]	13.0[c]	0.85	< 0.01	0.08	0.013
g/kg ECM	16.3	15.2	15.1	14.7	11.9	13.8	0.69	< 0.01	0.034	0.12
% of GEI[6]	7.53	6.39	6.97	7.04	5.74	6.70	0.197	< 0.01	0.09	0.34

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.
[4]Organic matter intake (kg/d).
[5]Digestible organic matter in total digestive tract (kg/d).
[6]Gross energy intake.
[a,b,c]Means with similar letters do not differ significantly.

Table 7
Apparent nutrient digestibility (%) of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				*P*-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
DM	71.4	71.8	68.6	70.4	69.8	66.8	0.60	< 0.01	0.023	0.65
OM	73.4	74.0	71.5	72.5	72.0	69.7	0.70	< 0.01	< 0.01	0.63
CP	64.1	65.9	66.4	63.5	65.0	63.7	1.20	0.31	0.08	0.49
Ether extract	61.2	63.7	63.1	61.7	59.9	61.6	1.40	0.73	0.08	0.14
NDF	61.1	58.9	57.6	58.7	53.1	54.9	1.40	0.023	0.021	0.43
Potential digestible NDF	68.7	70.4	77.7	65.4	63.8	76.2	1.97	< 0.01	0.053	0.37
ADF	66.7	60.2	57.0	64.1	54.1	55.0	2.10	< 0.01	0.062	0.30
Starch	98.3	98.9	98.8	98.3	98.7	98.8	0.10	< 0.01	0.64	0.71
Gross energy	69.5	71.2	68.7	68.8	68.9	66.7	0.70	0.021	< 0.01	0.49

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.

increasingly recognized as the most useful economically and environmentally important index, as it directly relates emissions to productivity (Beauchemin et al., 2020). However, incorporating CH_4 production in a genetic selection program represents a major challenge because of the difficulty of measuring CH_4 as a long-term phenotype of the animal (Løvendahl et al., 2018). Although breeding strategies need more time to have an impact compared with feeding strategies, they are cumulative and permanent. However, it is not clear whether genetic selection for CH_4 traits would provide any further advantage over selection for production traits, such as milk production (Capper et al., 2009; Legesse et al., 2016) or especially feed efficiency (Guinguina et al., 2020b). In the current study, we strive to provide more details on possible differences between rumen kinetics, fermentation pattern, and energy and N utilization in Low- or High-emitting cows using typical Nordic diets differing in FC ratio and forage type. It is clear that compared with forage-based diets, concentrate-based diets are associated with lower CH_4 yield (g/kg DM intake; Razzaghi et al., 2022) due to changing rumen fermentation pattern (resulting in more propionate competing with methanogenesis for H^+; Wang et al., 2014), inhibiting methanogens (decreasing rumen pH and fiber digestion; Beauchemin et al., 2020), reducing number and activity of rumen protozoa (Martin et al., 2010), and differences in rumen kinetics (i.e., the rate of digesta passage from the rumen; Pinares-Patiño et al., 2003; Cabezas-Garcia et al., 2017). It is important to note that our experimental diets were not formulated to be isonitrogenous. Our objective was to evaluate the effect of FC ratio and forage type on aforementioned parameters; however, the FC ratio of red clover silage-based diet was different and equal to the average of other two grass silage-based diets (50:50 vs 70:30 and 30:70) to avoid increasing the number of treatments while having forage type as a dietary factor in the study. In addition, this arrangement was helpful to avoid providing extra N from red clover silage, which has a greater CP content. Special attention should be paid to the modest difference in CH_4 yield (i.e., 4.8%) between CH_4 yield groups selected in this experiment from a small population of 100 cows which might have limited our ability to detect distinct biological differences and interaction between animal grouping and dietary interventions.

Table 8
Rumen contents and ruminal kinetics of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				*P*-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
Fresh rumen content, kg										
Morning evacuation	89.7	83.5	84.6	82.5	76.7	77.3	5.03	0.092	0.23	0.99
Noon evacuation	109	95.2	107	100	89.2	96.5	8.84	0.003	0.21	0.75
Average	99.3	89.3	95.7	91.3	82.9	86.8	6.72	< 0.01	0.21	0.87
Rumen content, kg										
Morning evacuation, DM	11.0	10.8	10.7	10.9	9.9	10.1	0.81	0.46	0.58	0.77
Noon evacuation, DM	14.2	12.6	14.7	13.5	12.4	13.5	1.50	0.20	0.48	0.63
Average, DM	12.6	11.7	12.7	12.2	11.2	11.8	1.10	0.48	0.53	0.77
NDF	7.35	6.39	7.04	7.35	6.55	6.50	0.656	< 0.01	0.82	0.35
Potential digestible NDF	11.4	10.4	8.84	11.1	9.97	8.40	0.872	< 0.01	0.62	0.99
ADF	3.80	3.33	4.63	3.83	3.47	4.27	0.394	< 0.01	0.86	0.13
Rumen kinetics										
DM, /h										
k_i	0.082	0.102	0.081	0.079	0.096	0.083	0.0079	< 0.01	0.63	0.48
k_p	0.024	0.028	0.025	0.023	0.029	0.027	0.0022	< 0.01	0.57	0.54
k_d	0.059	0.074	0.055	0.056	0.067	0.055	0.0059	< 0.01	0.44	0.46
MRT[4], h	12.4	10.0	12.5	12.8	10.8	12.2	1.00	< 0.01	0.66	0.48
NDF, /h										
k_i	0.053	0.056	0.048	0.049	0.050	0.049	0.0050	0.20	0.40	0.28
k_p	0.020	0.023	0.020	0.020	0.023	0.022	0.0022	0.26	0.63	0.68
k_d	0.032	0.033	0.027	0.029	0.026	0.027	0.0031	0.078	0.22	0.11
MRT[4], h	19.6	18.2	21.2	21.0	21.1	20.6	1.91	0.40	0.40	0.17
Potential digestible NDF, /h										
k_i	0.054	0.057	0.072	0.04	0.049	0.071	0.0046	< 0.01	0.28	0.49
k_p	0.017	0.016	0.016	0.017	0.018	0.017	0.0016	0.93	0.51	0.93
k_d	0.037	0.040	0.056	0.033	0.032	0.054	0.0036	< 0.01	0.16	0.36
MRT[4], h	19.2	18.0	13.8	20.7	21.4	14.1	1.62	< 0.01	0.25	0.32
Indigestible NDF, /h										
k_i	0.045	0.053	0.027	0.043	0.051	0.029	0.0050	< 0.01	0.86	0.74
k_p	0.039	0.047	0.024	0.037	0.047	0.027	0.0054	< 0.01	0.97	0.63
MRT, h	22.5	19.4	38.7	23.1	20.2	35.9	2.80	< 0.01	0.78	0.25

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.
[4]Mean retention time.

4.1. Intake, milk production and composition

The ranking of cows as Low and High emitter by respiration chambers showed no differences in DM and nutrient intakes except DMI/BW$^{0.75}$ where this variable tended to be greater in Low-emitting cows. Similarly, others did not observe differences in DMI between High- and Low-CH_4 emitting sheep (Robinson et al., 2015) or dairy cows (Stepanchenko et al., 2023) based on CH_4 yield. However, one study, conducted by Denninger et al. (2020), reported greater DM intake and ECM yield for Low-emitting animals with a greater number of Brown Swiss cows (n = 30) compared with the current experiment.

Greater DMI of cows fed LG diet is consistent with the previous studies that evaluated alfalfa silage-based diets with 35:65 and 60:40 FC ratios (Yang and Beauchemin, 2007) or grass silage-based diets with 30:70 and 70:30 FC ratios (Saliba et al., 2014). In the diet formulated with high grass-silage, lower DMI may be related to increased NDF concentration and the filling effects of forages in ration (Allen, 2000). Lack of interaction between Diet and Group with respect to nutrient intake indicates that Low- and High-emitting cows followed their intake potential regardless of the dietary interventions.

In this study, Low- and High-emitting cows had similar production performance and milk composition in line with results from Stepanchenko et al. (2023) who investigated the relation of high and low CH_4-yield phenotypes with DMI and lactation performance in 10 Holstein cows. It should be noticed that performance and milk composition are highly variable and require a large sample size to reach the required statistical power, meaning that observations related to milk yield and milk composition reported here must be interpreted with caution. However, cows fed LG diet had a greater milk yield compared with HG diet, while RC acted as an intermediate, as it was not different from grass silage-based diets. The observed increase in milk yield with LG compared with HG could be explained by a greater feed intake having greater nutrient density (19.2 vs. 8.6% starch, 16.5 vs. 14.4% CP, and 29.9 vs. 37.1% NDF on DM basis). Moreover, in the current study, milk lactose yield was 16.4% greater with feeding the LG than HG that can explain partially greater milk yield. High-emitting cows had numerically greater NDF intake and different rumen fermentation pattern (a tendency for greater acetate-to-propionate ratio), which provides more substrate to milk fat synthesis than their counterparts which can explain partially their higher milk fat concentration compared with Low-emitting cows (4.18 vs. 3.65%) especially those fed a LG diet (for interaction of Diet × Group). Therefore, it appears that increased NDF intake and digestion in high-emitting cows would be expected to

Table 9
Energy and N metabolism of high- and low-methane-emitter lactating cows fed grass silage or red clover silage-based diets[1].

	High emitter			Low emitter				*P*-value[3]		
Item	HG[2]	LG	RC	HG	LG	RC	SEM	Diet	Group	Diet × Group
Energy										
Gross energy intake, Mcal/d	102	120	104	97	107	98	3.4	< 0.01	0.11	0.15
Proportion of energy intake, Kcal/Mcal										
Feces	305	288	313	312	311	333	7.1	0.021	< 0.01	0.49
Urine	42.3	33.9	39.6	40.9	34.3	38.0	1.36	< 0.01	0.53	0.71
Methane	75.3	63.9	69.7	70.4	57.4	67.0	1.97	< 0.01	0.091	0.34
Milk	265	242	263	272	275	278	10.7	0.37	0.14	0.26
Heat	348	331	331	329	319	316	7.0	0.048	0.079	0.83
Metabolizable energy	577	615	577	576	597	562	7.3	< 0.01	0.065	0.48
Milk energy/metabolizable energy intake	460	391	457	473	458	495	19.2	0.027	0.071	0.26
Energy balance, Mcal	−3.08	4.88	−1.49	−2.21	0.26	−3.08	1.625	0.01	0.25	0.27
Nitrogen (N)										
N intake, g/d	556	736	697	523	663	662	21.0	< 0.01	0.12	0.29
Proportion of N intake, g/kg										
Feces	359	341	336	365	350	363	12.7	0.31	0.082	0.49
Urine	258	281	330	244	243	300	8.8	< 0.01	0.039	0.13
Milk	328[a]	291[b]	274[c]	330[a]	342[a]	297[b]	10.0	< 0.01	0.054	0.035
Nitrogen balance, g/d	30.6	61.8	39.7	31.3	42.9	26.8	10.85	0.12	0.26	0.67

[1]Values are least square means and pooled SEM for n = 5.
[2]Refers to diets containing 70 (HG) or 30% DM (LG) grass silage and 50% DM red clover silage (RC).
[3]Statistical comparisons: Diet = effect of experimental diets; Group = effect of high- and low-methane-emitter cows; and Diet × Group = interaction between experimental diets and high- and low-methane-emitter cows.
[a,b,c]Means with similar letters do not differ significantly.

result in greater milk fat content as explained by Daley et al. (2022) who evaluated the role of variation sources in cow's milk fat.

Regardless of CH_4 phenotype, the yield and concentration of milk protein increased in cows fed LG compared with HG and RC. This may be related to greater starch intake and the greater CP concentration or intake. However, considering similar CP intake in both LG and RC, cows fed RC had intermediate response to diet. Although the lower proportion of NH_3-N and soluble N in the red clover silage compared with grass silage (mediated by polyphenol oxidase (PPO) and isoflavones present in red clover tissues) has been found consistently (Vanhatalo et al., 2006; Broderick et al., 2007), in our case, it did not hold true. The concentration of NH_3-N in silage and rumen was greater for RC compared with grass silage-based diets. In addition, a greater MUN concentration in RC cows supports absorption of excess ammonia released in the rumen. Previous studies (Spek et al., 2013; Hynes et al., 2016) have reported a positive relationship between urinary N output and MUN in dairy cows, which is consistent with our findings when dairy cows were fed RC. Therefore, it appears that both lower energy intake (i.e., starch) and a different fermentation quality can justify an intermediate response for milk protein in RC cows. Moreover, there should be consideration of diet characteristics in our study (anisonitrogenic diets and also different FC ratio in comparison to grass silage-based diets) that affects the results.

Milk urea nitrogen concentrations varied from 93 mg/L for the HG to 311 mg/L for the RC in the present study. This demonstrates that the lowest value was close to 93 mg/L, which has been suggested to indicate an adequacy of RDP in the diet, and the highest value was < 450 mg/L, which seems to be approximately the upper limit for milk and milk protein yield responses (Nousiainen, 2004).

4.2. Rumen fermentation and gas emissions

In the current study, a more comprehensive sampling protocol covering multiple time points after feeding (7 time points) was used to detect daily rumen pH from ruminally cannulated cows. As expected, mean rumen pH was lower (6.09 vs. 6.48) in cows fed LG in both High- and Low-emitting groups. No effect of CH_4 yield phenotype on mean ruminal pH was found, which agrees with the results reported by Stepanchenko et al. (2023), where rumen fluid was collected using the ororuminal tubing technique at 8 different time points from high or low CH_4 emitters fed with a similar ration (FC ratio of 48:52 and diet NDF = 30% of DM).

Intake of DM, substrate type in rumen, and fractional outflow rate of feed particles from the rumen are well known to be among the main factors responsible for CH_4 production variations. The High-emitting cows in the present study had numerically greater DM intake than Low-emitting cows. In addition, the differences in rumen fermentation profile including a greater molar propionate concentration (204 vs.186 mmol/mol) and a tendency for lower acetate to propionate ratio in Low-emitting cows provides some evidence to explain the group differences in CH_4 emissions in line with Denninger et al. (2019). The extent of rumen fermentation, as reflected by total VFA concentration, being greater with RC and LG compared with HG can be due to more OM intake and digestion, specifically its starch fraction, as shown by apparent total-tract digestibility measurements. With cow group analysis, no difference was observed for the molar proportion of acetate, but the acetate-to-propionate ratio tended to differ between High- and Low-emitting cows, indicating that the dominant fermentation pathway may differ between microorganisms within these two groups.

Dietary strategies promoting propionate production in the rumen impede ruminal methanogenesis by reducing the availability of H^+ for CH_4 production by methanogens (Martin et al., 2010). Moreover, feeding LG resulted in greater increases in butyrate molar proportion in rumen fluid when fed to High-emitting cows (Diet × Group interaction). A greater dietary starch concentration favors the proportion of propionate, whereas dietary sugars increase the proportion of butyrate (Jentsch et al., 2007), resulting in H^+ surplus and increased CH_4 production (Ungerfeld and Kohn, 2006). In the current study, dietary sugar concentration and intake was the greatest in cows fed LG than other diets while sugar intake was similar for High- and Low-emitting cows. However, the effect of sugar on CH_4 production is pH dependent, as sugars increase CH_4 production only when rumen pH is maintained at a high level (Hindrichsen and Kreuzer, 2009) as this condition holds true for our study. Stepanchenko et al. (2023) explored the relationship between high and low CH_4-yield phenotypes and rumen fermentation parameters in lactating dairy cows. Their research revealed that the low-emitting phenotype results in rumen fermentation shifts like those observed when CH_4 production is chemically inhibited. Specifically, this shift includes a decreased relative proportion of acetate and an increased relative proportion of propionate. This study suggests that host genotype may influence selective bacterial-archaea networks, which govern energy-partitioning mechanisms, diverting hydrogen (H_2) away from methanogenesis toward alternative H_2-utilizing pathways such as succinate and propionate production in low CH_4-emitting cows (Stepanchenko et al., 2023).

It is interesting to note that the ruminal concentration of NH_3-N was greater for cows fed RC diet compared with cows fed grass silage-based diets, with more pronounced increase in High-emitting cows (Diet × Group interaction), which indicates an increase in ruminal protein degradation when RC diet was fed. This is contrary to Benchaar et al. (2015) with lactating cows, who reported the ruminal concentration of NH_3 was greater when included in diets based on corn silage (a warm-season grass) than red clover silage where polyphenols inhibit ruminal protein degradation, and consequently reduce ruminal NH_3 production. Increased ruminal concentration of NH_3-N in RC cows when fed to High-emitting group can be due to rumen fermentation pattern and silage quality of red clover silage compared with grass silage. Additionally, it should be noticed that CP intake from RC (from red clover silage) and LG (from concentrate) was greater compared with HG diet as reported in Table 3.

By screening enteric CH_4 emissions using the GreenFeed system, Stepanchenko et al. (2023) reported that high CH_4-yield phenotype differed by 21% in daily CH_4 emissions, 24% in CH_4 yield, and 22% in CH_4 intensity compared with low CH_4-yield phenotype. Daily CH_4 production and CH_4 intensity (g/kg ECM) in High-emitting cows were greater in the present experiment compared with a meta-analysis of previous experiments conducted in our institute (Adjassin et al., unpublished), where cows produced on average 506 g CH_4/d and 13.9 g CH_4/kg ECM. On the other hand, CH_4 production and intensity were lower for Low-emitting cows than those obtained by Adjassin et al. (Unpublished data). There is a positive relationship between CH_4 production and ruminal NDF digestion in cattle (Niu et al., 2018) which is also observed for High-emitting cows in our study. Less efficient fiber degradation or faster passage of digesta through the rumen, reduce H^+ accumulation and consequently CH_4 production. The lower daily CH_4 production for the LG diet in Low-emitting cows compared with other diets could partly be explained by the lower NDF intake, NDF total-tract digestibility, and greater starch intake. When CH_4 production was corrected for the amount of OM intake, Low-emitting cows tended to have lower CH_4 yield than the High-emitters (22.4 vs. 24.5 g/kg OM intake). One explanation for this difference is the lower total-tract apparent digestibility of OM in Low-emitting cows associated with alterations in rumen fermentation without any differences in passage kinetics in both groups.

4.3. Total-tract nutrient digestibility

The lack of interactions between dietary interventions and CH_4-yield phenotype (as selected based on CH_4 yield) on nutrients digestion measured herein showed that the effects of diets and cow group were independent. Low-emitting group had lower digestion values for DM, OM, and NDF. It is possible that between-cow differences in OM digestibility result mainly from the digestion of the slowly digestible NDF fraction, which can produce more acetate and CH_4. Positive relationships of OM and NDF digestibility to molar proportion of acetate, and negative relationships to propionate were suggested by Cabezas-Garcia et al. (2017). However, Ahvenjärvi et al. (2024) comparing 2 groups of dairy cows selected based on residual feed intake (RFI) reported that low-RFI group had higher CH_4 yield without any differences in ruminal molar proportion of acetate and propionate, and OM and NDF digestibility. Analysis of the different groups in our study showed a lower CH_4/kg ECM for Low-emitting cows compared with High-emitting cows (13.4 vs. 15.5). This result shows that low CH_4 emitters utilized the feed more efficiently for milk production.

Cows consuming the RC had a slower passage rate of iNDF than those consuming the HG and LG (2.5 vs. 4.2%/h). This translated into a longer mean retention time of iNDF for cows consuming the RC diet relative to cows consuming the grass silage-based diets (37.3 vs. 21.3 h). In general, legumes contain less fiber and more protein than grasses, and we observed that the NDF intake was greater in the grass silage-based diets compared with the RC. Unlike the results of Vanhatalo et al. (2009) who showed a greater digestibility of OM and DM in the rumen and in the total tract of cows consuming red clover compared with grass silage diets, in our study there was a negative effect from feeding red clover silage on digestibility of DM and OM in both cow groups. Even though the digestibility of NDF was not different for RC and grass silage diets, digestibility of the pdNDF was greater for RC than for grass silage-based diets as also was reported by Vanhatalo et al. (2009). Further, we tried to have the forage ratio in RC diet (50:50) as the average of both grass silage-based diets (70:30 and 30:70) in order to be able to compare them together. However, the difference in FC ratio may have affected ruminal digestion and passage kinetics of NDF, we also highlight that this is inevitable when formulating diets.

4.4. Rumen digestion and passage kinetics

We hypothesized that increased ruminal nutrient digestibility in High-emitting cows may contribute to greater CH_4 production compared with their counterparts. The link between ruminal digestibility and enteric CH_4 emissions has been shown in our study where total-tract OM, DM, and NDF digestibility were greater in High- compared with Low-emitting cows. A study utilizing the sulfur hexafluoride tracer technique revealed that CH_4 yield was strongly correlated with OM intake, rumen fill, and cellulose digestibility, while being inversely related to the ruminal particulate outflow rate (Pinares-Patiño et al., 2003). These findings were later corroborated by experiments conducted in respiration chambers. Pinares-Patino et al. (2011) observed that animals with low CH_4-yield had a shorter ruminal retention time for the soluble fraction, though only a numerical decrease was noted for the particulate fraction. However, in the current study, we did not observe any variation in rumen mean retention time of DM and NDF in Low- and High-emitting cows. In the current study, despite similar DM intake and digesta passage in both cow groups, we believe that main reason for between-animal variance in CH_4 emissions is digestibility (greater DM, OM and NDF digestibility) and rumen fermentation pattern (greater acetate-to-propionate ratio) observed in High-emitting cows. These findings suggest that caution is warranted when selecting for low CH_4-emitting animals, as this could inadvertently favor smaller animals with lower OM and NDF digestibility. This concern aligns with early research in sheep showing that ruminal passage rate is a heritable (Ørskov et al., 1988) and repeatable trait (Smuts et al., 1995).

Red clover silage has high PPO activity, which reduces proteolysis and slows the breakdown of cell wall proteins. This indirectly affects the retention time of pdNDF as protein-bound fibers take longer to digest. In contrast to literature, we found a shorter mean retention time for pdNDF in cows fed RC compared with grass silage-based diets while a longer retention time for iNDF was detected due to greater iNDF concentration in red clover silage (up to 2.5-fold) compared with grass silage based-diets.

Ørskov et al. (1988) reported a strong negative correlation coefficient (−0.80) between passage rate of Cr-mordanted straw and digestibility for 22 cows. However, in our study, low and high CH_4-yileding groups had no difference in rumen contents and ruminal kinetics. Despite this, results from Huhtanen et al. (2016) and Cabezas-Garcia et al. (2017) demonstrated that variations in rumen digesta passage and mean retention time contribute to between-animal variation in CH_4 emissions more than rumen fermentation pattern.

4.5. Energy and N utilization

In our study, BW changes are not reported because changes in BW may not necessarily reflect changes in tissue energy and due to the short experimental periods, the measured BW changes are not reliable. Interaction effects were not observed between diet and cow group for the variables of energy utilization. Feeding LG increased GE intake, energy metabolizability (ME to GE intake), and energy balance while it decreased feces, urine, and CH_4 energy excretion as a proportion of GE intake compared with HG and RC. We observed a reduced CH_4 energy excretion as a proportion of energy intake when more concentrate was substituted for forage. In agreement with our results, Bayat et al. (2017) reported lower CH_4 energy excretion as a proportion of energy intake from cows fed high- compared with low-concentrate (304 vs. 126 g/kg starch on a DM basis, respectively). In the current study, the main changing factor in experimental diets is grass silage ratio whereas changes in starch content are marginal (8 vs. 14.5% diet DM). Therefore, changes in grass silage ratio and fermentation quality can affect NDF digestion, rumen fermentation, CH_4 production, and finally energy utilization (Huhtanen et al., 2009). The greater proportion of concentrate in the diets changed energy partitioning toward milk synthesis as evaluated by milk energy as a proportion of ME intake contributing to the greater milk yield by cows fed with LG.

Nitrogen balance was not different among the diets, whereas previously positive effects of greater proportion of concentrates or dietary starch on N balance have been observed (Aguerre et al., 2011; Morris et al., 2020). However, milk N secretion as proportion of N intake was lower in High-emitting cows than their counterparts fed LG and RC (Diet × Group interaction). It appears that CH_4 yield phenotype has an imperative effect on daily N excretion in dairy cows (Low-emitting cows had greater daily N excretion in feces and urine as a proportion of N intake compared with High-emitting cows despite their lower N intake). Because of the much higher N intake of RC, the total N excretions of RC cows were greater than those fed grass silage-based diets. Also, a greater proportion of N was excreted in urine rather than in feces on RC, whereas the opposite was true for HG and LG. Lavery and Ferris (2021) also reported a linear relationship between the intake of dietary N and urinary N excretion in dairy cows. This corresponds well with the calculation that excretion of N in the urine increases exponentially as soon as N intake exceeds 400 g/d or dietary CP content exceeds 15% in diet DM as explained by Castillo et al. (2000). The efficiency of dietary N conversion into milk N of 28.5% (on average for Low- and High-emitting cows) for RC cows agrees with other studies (Dewhurst et al., 2003; Broderick et al., 2007) and was relatively low compared with 32.3% for grass silage-based diets in the present study. Nonetheless, the feed N efficiency of the grass silages used in the various studies (Dewhurst et al., 2003) varied in the range of 22–26% with the CP content of the silages varying from 13.9% to 20.6% in DM. Overall, this demonstrates that the CP content of the silage is not the only determinant of feed N efficiency. It should be noted that an efficient nutritional strategy for carbohydrate and protein supplementation of red clover-based diets to improve dietary N conversion into milk N would be used.

5. Conclusions

There were clear differences between Low- and High-emitting cows (selected based on methane yield, g/kg DMI) in DM, OM and NDF digestion, CH_4 production, and energy or N utilization whereas dietary interventions (FC ratio and forage type) affected lactation performance, digestion, rumen fermentation and kinetics, and energy utilization of dairy cows. The lack of interactions between dietary interventions and CH_4 yield phenotype on measured nutrient intakes and digestion, kinetics, and energy utilization showed that the effects of diets and CH_4-yield phenotype were independent. In the case of rumen fermentation pattern, the interaction response was detected for greater ruminal molar proportion of propionate and butyrate in cows fed low-grass silage in Low- and High-emitting cows, respectively. Replacing grass silage with red clover silage and change in dietary concentrate proportion (70:30 and 30:70 vs. 50:50) resulted in increased N intakes and ammonia N concentrations in the rumen fluid but also increased N excretions. Despite the importance of phenotypically CH_4 emitters with respect to selection for lower CH_4 emissions, unfavorable efficiency responses, especially negative effects on OM and NDF digestion were observed in Low-emitting cows. This highlights the importance of paying attention to the association between selection for low CH_4 production and reduced OM and NDF digestibility in any attempt to reduce environmental impact of dairy cows. However, rumen pool sizes of DM and NDF and rumen kinetics were affected by dietary modifications while were not different between High- and Low-emitting cows in our study.

CRediT authorship contribution statement

Razzaghi, A: Formal analysis, Writing – original draft, Writing – review & editing. **Kairenius, P:** investigation. **Ahvenjärvi, S:** Methodology, investigation, Writing – review & editing. **Stefanski, T:** investigation. **Tapio, I:** investigation, formal analysis, investigation, methodology, Writing – original draft, Writing – review & editing. **Vilkki, J:** funding acquisition, Writing – review & editing. **Bayat, AR:** conceptualized and supervised the project, Writing – original draft, Writing – review & editing.

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Declaration of Competing Interest

The authors have not stated any conflicts of interest.

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