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Article

Variability in Enteric Methane Emissions among Dairy Cows during Lactation

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Simple Summary: The objective of this study was to investigate variability in enteric methane (CH₄) emission rate and emissions per unit of milk among dairy cows on commercial farms in the UK. A large dataset of enteric CH₄ measurements from individual cows was obtained from 18 farms across the UK. We conclude that changes in CH₄ emissions appear to occur across and within lactations, but ranking of a herd remains consistent, which is useful for obtaining CH₄ spot measurements.

Abstract: The aim of this study was to investigate variability in enteric CH₄ emission rate and emissions per unit of milk across lactations among dairy cows on commercial farms in the UK. A total of 105,701 CH₄ spot measurements were obtained from 2206 mostly Holstein-Friesian cows on 18 dairy farms using robotic milking stations. Eleven farms fed a partial mixed ration (PMR) and 7 farms fed a PMR with grazing. Methane concentrations (ppm) were measured using an infrared CH₄ analyser at 1s intervals in breath samples taken during milking. Signal processing was used to detect CH₄ eructation peaks, with maximum peak amplitude being used to derive CH₄ emission rate (g/min) during each milking. A multiple-experiment meta-analysis model was used to assess effects of farm, week of lactation, parity, diet, and dry matter intake (DMI) on average CH₄ emissions (expressed in g/min and g/kg milk) per individual cow. Estimated mean enteric CH₄ emissions across the 18 farms was 0.38 (s.e. 0.01) g/min, ranging from 0.2 to 0.6 g/min, and 25.6 (s.e. 0.5) g/kg milk, ranging from 15 to 42 g/kg milk. Estimated dry matter intake was positively correlated with emission rate, which was higher in grazing cows, and negatively correlated with emissions per kg milk and was most significant in PMR-fed cows. Mean CH₄ emission rate increased over the first 9 weeks of lactation and then was steady until week 70. Older cows were associated with lower emissions per minute and per kg milk. Rank correlation for CH₄ emissions among weeks of lactation was generally high. We conclude that CH₄ emissions appear to change across and within lactations, but ranking of a herd remains consistent, which is useful for obtaining CH₄ spot measurements.



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Keywords: cattle; methane; measurements; farm

1. Introduction

In terms of sustainability management in ruminant production, low-productivity systems lose more energy per unit of animal product than high-productivity systems [1]. Global milk production, and the number of milking cows, has increased in recent decades to meet increasing demand for dairy products, and with this, monitoring and mitigation of greenhouse gas emissions associated with milk production has gained importance [2]. Herd fertility, disease incidence and replacement rate are major influencers of CH₄ emissions per kg of product [3,4]. Greenhouse gas emissions per unit product from dairy cows has been reducing by about 1% per annum over the last few decades with improved efficiencies of production [5]. However, due to increasing production per animal over this period, emissions per cow are estimated to have increased by 1.0% per annum [5].

Cattle are a notable source of CH₄ emissions from fermentation of food consumed, with enteric emissions accounting for approximately 50% of total greenhouse gas emissions from milk production [2]. Genetic selection on CH₄ could potentially help to mitigate emissions per cow and per unit product. A breath sampling or sniffer [4,6,7] approach to measure enteric CH₄ emissions from individual cows on commercial farms now provides the opportunity to explore differences among farms and populations of animals in their normal environment. Respiration chambers are the gold standard method to obtain precise and accurate measurements of enteric CH₄ emissions from individual animals. However, use of a mobile gas analyser approach to measure CH₄ emissions has been found to be correlated with respiration chamber measurements, and provides a cheaper and wider scope of application to measure large numbers of animals in their normal environment [8].

Frequent 'spot' measurements of CH₄ over several days from the same animal whilst being milked, along with application of signal processing to detect maximum amplitude of eructation peaks, has been found to provide a reliable and repeatable measure from individual animals using a mobile gas analyser [9,10]. Peaks in signal of CH₄ concentration are caused by eructations when sampling emissions in breath of cows. Few studies have investigated phenotypic variation in CH₄ emissions measured across commercial farms [6,7,11]. Such information is invaluable for quantifying normal variation and differences among systems with the potential for mitigating CH₄ emissions.

The objective of the current study was to investigate variability in enteric CH₄ emission rate and emissions per unit milk across lactations among dairy cows on commercial farms in the UK.

2. Materials and Methods

Approval for this study was obtained from the Animal Welfare and Ethical Review Board of the University of Nottingham before the commencement of the study (approval number 30/3210).

2.1. Data

A total of 105,701 CH₄ spot measurements were obtained from 2206 dairy cows on 18 commercial farms using robotic milking stations in the UK (Table 1). Measurements were obtained between December 2009 and December 2013. Most cows in this study were Holstein-Friesian breed.

Table 1. Mean (s.e.) parity, milk yield, live weight, robot concentrate, dry matter intake (DMI) and number of milkings per day at each farm (A to R) for cows fed on diets consisting of a partial mixed ration (PMR), or PMR with grazing, plus concentrates.

| Farm | Diet | No. of Cows | Parity | Milk Yield (kg/day) | Live Weight (kg) | Robot Concentrate DMI (kg/day) | DMI ¹ (kg/day) | Milkings per Day (no. per Cow) |
|------|---------------|-------------|------------|---------------------|------------------|--------------------------------|---------------------------|--------------------------------|
| A | PMR + grazing | 55 | 3.1 (0.2) | 27.7 (1.2) | - | 6.0 (0.4) | - | 1.9 (0.07) |
| B | PMR | 70 | 3.2 (0.3) | 21.1 (0.8) | 621 (7.8) | 6.2 (0.2) | 17.6 (0.2) | 1.7 (0.05) |
| B | PMR + grazing | 66 | 4.0 (0.3) | 21.8 (0.9) | 597 (8.5) | 4.6 (0.2) | 17.1 (0.3) | 1.9 (0.07) |
| C | PMR | 41 | 2.1 (0.3) | 30.2 (1.3) | 634 (10.8) | 9.2 (0.6) | 18.9 (0.3) | 1.9 (0.09) |
| C | PMR + grazing | 34 | 2.9 (0.3) | 24.7 (1.6) | 634 (10.5) | 7.3 (0.5) | 19.3 (0.3) | 2.0 (0.07) |
| D | PMR + grazing | 47 | 2.2 (0.2) | 26.9 (1.4) | 610 (8.9) | 7.0 (0.5) | 17.9 (0.3) | 2.0 (0.09) |
| E | PMR | 73 | 2.6 (0.2) | 26.4 (1.0) | 647 (8.1) | 7.3 (0.4) | 18.8 (0.3) | 2.1 (0.06) |
| E | PMR + grazing | 71 | 3.9 (0.4) | 28.2 (0.9) | 629 (6.8) | 7.3 (0.3) | 18.5 (0.2) | 2.4 (0.08) |
| F | PMR + grazing | 45 | 3.6 (0.3) | 26.3 (1.2) | 601 (11.5) | 5.0 (0.3) | 17.6 (0.3) | 2.4 (0.09) |
| G | PMR | 116 | 2.6 (0.1) | 25.5 (0.7) | 627 (7.1) | 5.9 (0.2) | 18.2 (0.2) | 2.3 (0.06) |
| H | PMR + grazing | 85 | 3.0 (0.2) | 26.2 (1.1) | - | 7.9 (0.2) | - | 3.4 (0.17) |
| I | PMR | 110 | 2.9 (0.2) | 25.8 (0.7) | 602 (7.3) | 5.1 (0.2) | 17.6 (0.2) | 2.1 (0.05) |
| J | PMR | 329 | 2.7 (0.1) | 31.0 (0.6) | 669 (3.7) | 6.6 (0.2) | 19.9 (0.1) | 2.3 (0.04) |
| K | PMR | 199 | 2.2 (0.1) | 28.8 (0.7) | - | 5.9 (0.2) | - | 2.4 (0.06) |
| L | PMR | 63 | 3.7 (0.2) | 27.0 (1.1) | 697 (8.1) | 5.3 (0.3) | 20.1 (0.2) | 2.9 (0.10) |
| M | PMR | 119 | 2.4 (0.1) | 34.3 (0.9) | 611 (6.8) | 6.4 (0.2) | 18.7 (0.2) | 2.3 (0.06) |
| N | PMR | 129 | 2.0 (0.1) | 22.4 (0.8) | 605 (6.9) | 6.6 (0.4) | 17.4 (0.2) | 2.6 (0.08) |
| O | PMR | 81 | 2.9 (0.2) | 18.7 (0.8) | 580 (7.9) | 5.7 (0.2) | 16.4 (0.2) | 2.5 (0.08) |
| P | PMR | 26 | 2.4 (0.4) | 29.7 (2.0) | - | 4.0 (0.4) | - | 2.2 (0.12) |
| Q | PMR | 224 | 2.6 (0.1) | 35.4 (0.9) | - | - | - | 2.1 (0.03) |
| R | PMR | 223 | 2.4 (0.1) | 32.6 (0.6) | 608 (4.8) | 5.2 (0.1) | 18.5 (0.1) | 2.5 (0.06) |
| All | | 2206 | 2.6 (0.04) | 30.4 (0.2) | 617 (1.6) | 5.7 (0.05) | 18.5 (0.01) | 2.4 (0.02) |

¹ Estimated by the equation: DMI (kg/day) = 0.025 × live weight (kg) + 0.1 × milk yield (kg/day) [12].

Cows were fed ad libitum and diets fed (Table 2) were classified as either a partial mixed ration (PMR; i.e., conserved forage and concentrate feed) or a PMR with grazed pasture. Pasture was predominantly perennial ryegrass swards. All cows received concentrate feed during milking allocated according to each cow's daily milk yield. Milk yield, live weight and intake of robot concentrate were recorded automatically at every milking. Dry matter intake (DMI) of cows was estimated from individual milk yield and live weight using the equation DMI (kg/day) = 0.025 × live weight (kg) + 0.1 × milk yield (kg/day) [12]. Eleven farms fed PMR and 7 farms fed a PMR with grazing (farms A to R). Cows at farms B, C and E were sampled during two separate periods when the cows were fed either a PMR or PMR with grazing. Percentage of forage and concentrate in each diet were obtained, and all nutrient analysis was conducted by a commercial analytical laboratory (Scianteq Analytical Services, Cawood, UK). (Table 2). Farms all fed a commercial concentrate blend.

2.2. Gas Sampling

Methane concentration (parts per million, *v/v*) in breath samples collected during milking was measured using an infrared gas analyser (Guardian SP; Edinburgh Instruments Ltd., Livingston, UK) and recorded at 1 s intervals using a data logger (Simex SRD-99; Simex Sp. z o.o., Gdańsk, Poland). Breath samples were collected via a tube positioned at the rear of the feed bin in each robotic milking station, and sampling was carried out for at least 7 days at each farm. Raw logger data for CH₄ concentration were analysed using MatLab Signal Processing Toolbox (version R2018a, The MathWorks, Inc., Natick, MA, USA). Peak analysis tools were used to identify eructation peaks and extract maximum amplitude within each milking. See Garnsworthy et al. [4] and Hardan et al. [10] for a full description of the sampling approach used.

Table 2. Forage and concentrate percentage and nutrient content in the dry matter (DM) of the diet for each farm (A to R).

| Farm | Forage (% DM) | Concentrate (% DM) | DM (g/kg) | Starch (g/kg DM) | Neutral Detergent Fibre (g/kg DM) | Crude Protein (g/kg DM) | Oil (g/kg DM) | Metabolisable Energy (MJ/kg DM) |
|------|---------------|--------------------|-----------|------------------|-----------------------------------|-------------------------|---------------|---------------------------------|
| A | 68.4 | 31.6 | 231 | 11 | 356 | 231 | 33 | 11.1 |
| B | 68.7 | 31.3 | 395 | 141 | 399 | 141 | 42 | 10.6 |
| C | 45.5 | 54.5 | 356 | 37 | 357 | 184 | 38 | 11.9 |
| D | 57.9 | 42.1 | 461 | 165 | 403 | 131 | 31 | 11.4 |
| E | 37.5 | 62.5 | 590 | 90 | 445 | 153 | 50 | 10.9 |
| F | 75.6 | 24.4 | 362 | 32 | 401 | 156 | 29 | 11.3 |
| G | 60.2 | 39.8 | 365 | 162 | 299 | 107 | 23 | 10.6 |
| H | 59.8 | 40.2 | 404 | 62 | 434 | 137 | 41 | 10.3 |
| I | 65.5 | 34.5 | 303 | 16 | 442 | 207 | 42 | 11.0 |
| J | 42.3 | 57.7 | 519 | 47 | 383 | 138 | 39 | 11.4 |
| K | 39.5 | 60.5 | 325 | 23 | 469 | 169 | 57 | 11.8 |
| L | 47.9 | 52.1 | 489 | 87 | 481 | 116 | 45 | 10.1 |
| M | 49.4 | 50.6 | 668 | 96 | 448 | 129 | 26 | 10.8 |
| N | 58.3 | 41.7 | 380 | 20 | 301 | 162 | 19 | 11.5 |
| O | 68.0 | 32.0 | 466 | 129 | 373 | 131 | 31 | 11.6 |
| P | 45.1 | 54.9 | 510 | 89 | 410 | 133 | 33 | 11.6 |
| Q | 42.9 | 57.1 | 360 | 19 | 470 | 155 | 50 | 11.2 |
| R | 56.0 | 44.0 | 361 | 68 | 411 | 131 | 49 | 10.5 |

The analyser sampled air at a flow rate of 1 L/min and measured CH₄ concentration in ppm every second. Measurements were converted to emission rate in grams per minute by multiplying by 60 and density of CH₄, assumed to be 0.706×10^{-6} g/L. Emission rates (grams per minute) were scaled to estimate emissions based on exponential rise time of eructation peaks and response time of the analyser (60 s) using Equation (1):

$$\text{CH}_4 \text{ emission rate (g/min)} = \text{maximum peak amplitude (ppm)} / [1 - \text{EXP}(-(\text{peak rise for amplitude in seconds}/60))] \times 60 \times 0.706 \times 10^{-6} \quad (1)$$

Emissions per kg milk were calculated by Equation (2).

$$\text{CH}_4 \text{ emission rate (g/kg milk)} = (\text{CH}_4 \text{ emission rate (g/min)} \times 1440) / \text{milk yield (kg/day)} \quad (2)$$

2.3. Statistical Analysis

Average values for week of lactation were used for analysis. A multiple experiment meta-analysis model in Genstat Version 21.1 (VSN International, Hemel Hempstead, UK) was used to assess effects of farm, week of lactation, parity, diet and dry matter intake on average CH₄ emissions per individual cow using Equation (3):

$$y_{ijklm} = \mu + F_i + W_j + P_k + D_l \times \beta \text{DMI} + C_m + E_{ijklm} \quad (3)$$

where y_{ijklm} is the dependent variable; μ is the overall mean; F_i is the fixed effect of farm (A to R); W_j = fixed effect of week of lactation (1 to 70); P_k = fixed effect of parity (1, 2, 3, 4 and 5 or more); D_l = fixed effect of diet (PMR or PMR with grazing); βDMI is the linear regression of Y on dry matter intake; C_m is random effect of individual cow; E_{ijklm} = residual error term.

Repeatability of gas concentration measures was assessed by σ^2 animal / (σ^2 animal + σ^2 residual), where σ^2 is the variance. Residual coefficients of variation (CV) were calculated from variance components as square root of residual σ^2 divided by estimated mean. Spearman's rank correlation was used to assess ranking of CH₄ emissions from cows across farms and weeks of lactation. Significance was declared at $p < 0.05$.

3. Results

Across the 18 farms studied, cows averaged 2.6 ± 1.9 lactations, were milked 2.4 ± 0.8 times per day at robotic milking stations with CH_4 measurements, and produced 30.4 ± 9.7 kg/day of milk (mean \pm s.d.; Table 1). Estimated mean enteric CH_4 emissions across the 18 farms were 0.38 (s.e. 0.01) g/min and 25.6 (s.e. 0.5) g/kg milk.

Factors with significant effects on CH_4 emission rate were: farm, week of lactation, parity, dry matter intake and the interaction between dry matter intake and diet (all $p < 0.001$) (Table 3). Significant effects on CH_4 emissions per kg milk were found for farm, week of lactation, parity, dry matter intake (all $p < 0.001$) and the interaction between dry matter intake and diet ($p < 0.05$).

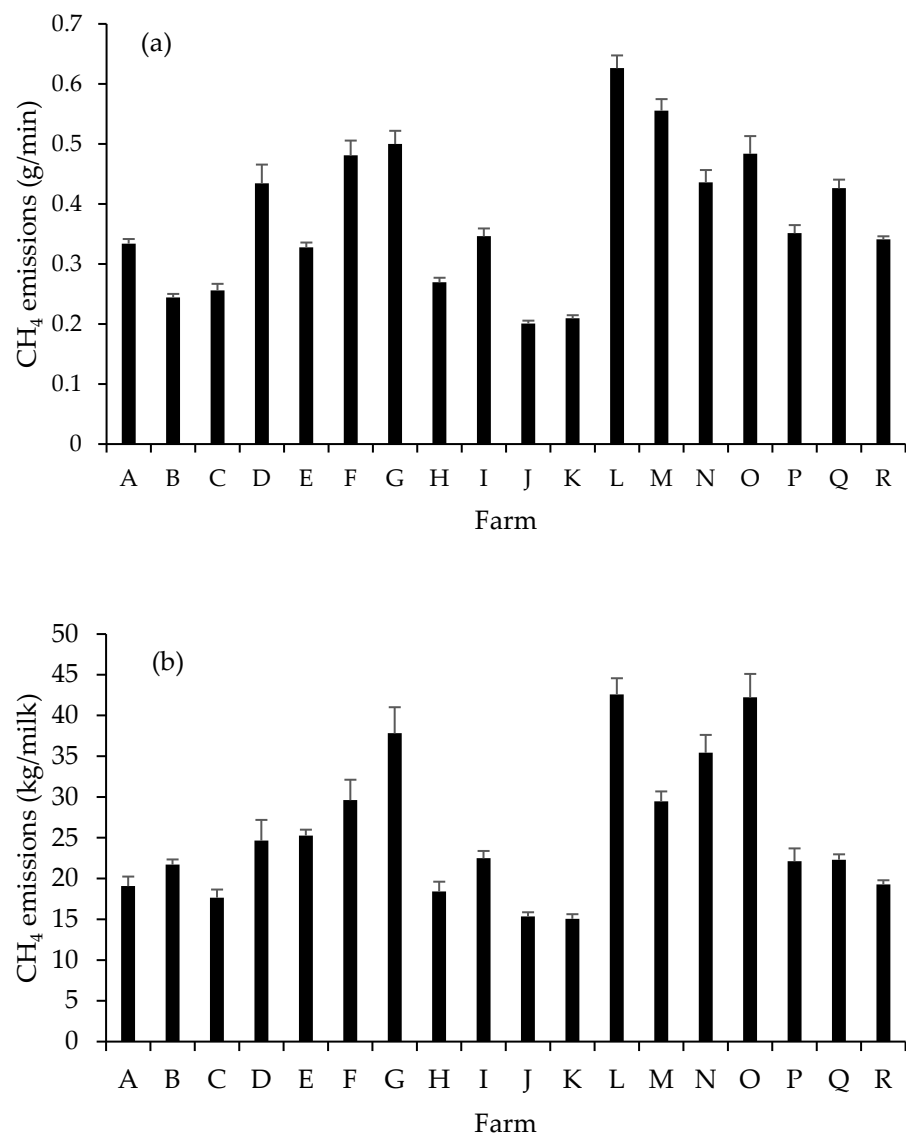


Figure 1. Estimated mean CH_4 emissions (with s.e. bars) in (a) grams per minute and (b) grams per kg milk for farms A to R.

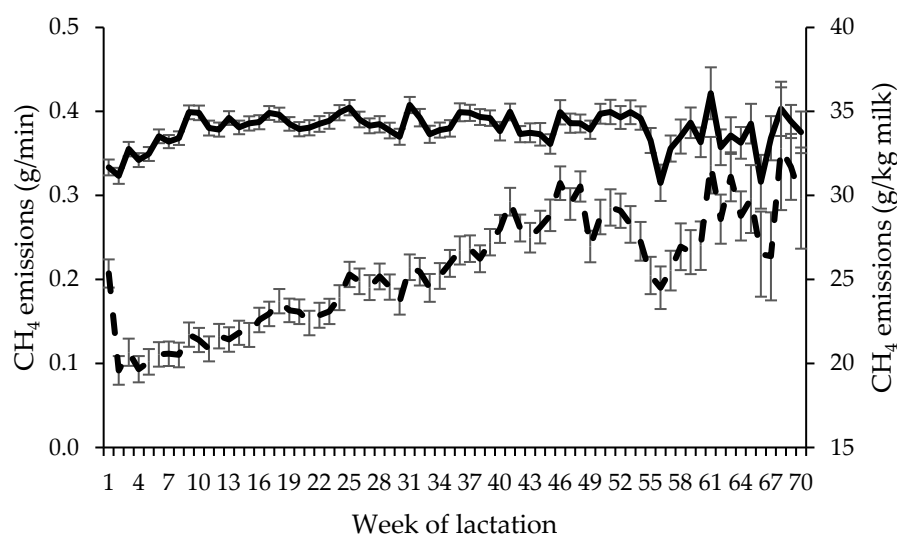


Figure 2. Estimated mean (with s.e. bars) CH₄ emission rate (g/min; solid line) and per kg milk (dashed line) from week 1 to 70 of lactation.

There was no effect of diet on CH₄ emission rate, but CH₄ emissions per kg milk were higher for cows fed PMR with grazing ($p < 0.001$). Estimated mean CH₄ emissions ranged from 0.2 g/min for cows at farms J and K to 0.6 g/min for cows at farms L and M (Figure 1a). Estimated mean CH₄ emissions ranged from 15 g/kg milk for cows at farms J and K to 42 g/kg milk for cows at farms L and O (Figure 1b).

Rate of CH₄ emissions increased to 0.4 g/min at week 9 of lactation, but were relatively constant from weeks 10 to 70 of lactation (Figure 2). Emissions of CH₄ per kg milk generally increased to a peak in week 46 of lactation (Figure 2). Variability in emissions was more notable in later lactation. Furthermore, older cows were associated with a lower emission rate and per kg milk (both $p < 0.001$; Table 3). Increasing dry matter intake increased emission rate, with the increase being higher in grazing cows (0.02 g/min per kg dry matter intake). Increasing dry matter intake reduced emissions per kg milk, with the reduction being higher for cows fed PMR (−0.82 g/kg milk per kg dry matter intake).

When CH₄ emissions had been adjusted for significant fixed effects, considerable residual variation in CH₄ emissions remained among cows within farms. Coefficient of variation in CH₄ emissions ranged from 5.7 to 75.1% for g/min and from 19.6 to 80.2% for g/kg milk for cows across farms.

Profile of CH₄ emission rate throughout lactation was consistent among herds. Rank correlation for CH₄ emission rate among weeks of lactation was generally high across lactations with 80% of rank correlations being greater than 0.5 (Figure 3).

Rank correlation for CH₄ emission per kg milk among weeks of lactation were generally lower than for emission rate with 71% of rank correlations being greater than 0.5 (Figure 4).

Table 3. Effect of farm, week of lactation, parity, diet and dry matter intake (DMI) on enteric methane emission rate (g/min) and per kg milk for dairy cows across commercial farms studied.

| Variable | Effect (s.e) | Mean | Emissions (g/min) | | | | p Value | Effect (s.e) | Mean | Emissions (g/kg Milk) | | | |
|--------------------------------|-----------------|-----------------|-------------------|------|-------|--------|-----------------|--------------|------|-----------------------|------|--------|---------|
| | | | F-Statistic | d.f. | SED | | | | | F-Statistic | d.f. | SED | p Value |
| Farm ¹ | | | 129 | 17 | 0.02 | <0.001 | | | 54 | 17 | 2.2 | <0.001 | |
| Week of lactation ² | | | 4.2 | 69 | 0.01 | <0.001 | | | 10 | 69 | 1.4 | <0.001 | |
| Parity | 1 | 0.39 | 9.7 | 4 | 0.004 | <0.001 | | 26 | 16 | 4 | 0.48 | <0.001 | |
| | 2 | 0.39 | | | | | 26 | | | | | | |
| | 3 | 0.38 | | | | | 25 | | | | | | |
| | 4 | 0.37 | | | | | 25 | | | | | | |
| | 5+ | 0.36 | | | | | 25 | | | | | | |
| Diet | PMR | 0.38 | 0.8 | 1 | 0.007 | 0.385 | | 24 | 22 | 1 | 0.78 | <0.001 | |
| | PMR + grazing | 0.38 | | | | | 28 | | | | | | |
| DMI | 0.01 (0.001) | | 105 | 1 | 0.001 | <0.001 | −0.82 (0.08) | | 101 | 1 | 0.08 | <0.001 | |
| Diet × DMI | PMR | 0.01 (0.001) | 17 | 1 | 0.002 | <0.001 | −0.82 (0.08) | | 5 | 1 | 0.32 | 0.024 | |
| | PMR + grazing | 0.02 (0.003) | | | | | −0.10 (0.40) | | | | | | |

¹ Farms A to R with estimated means shown in Figure 1. ² Weeks 1 to 70 with estimated means shown in Figure 2.

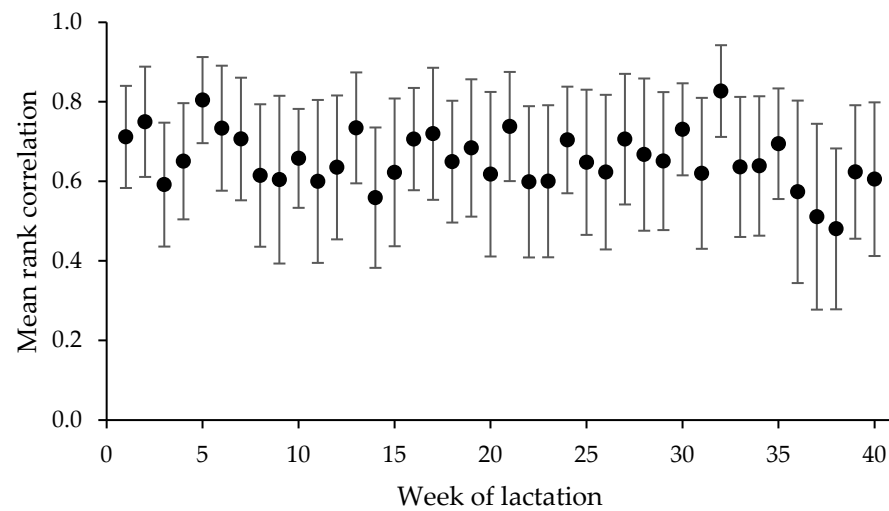


Figure 3. Rank correlation of CH₄ emissions (with s.e. bars) in grams per minute across lactations.

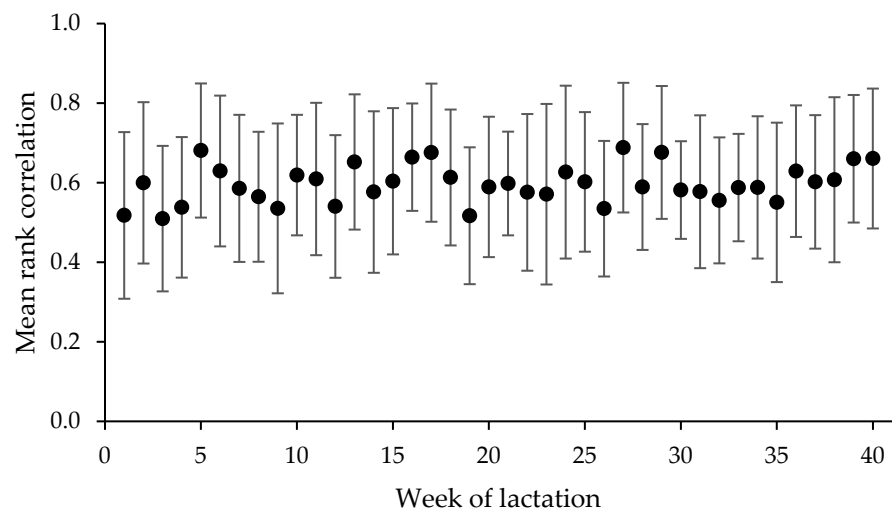


Figure 4. Rank correlation of CH₄ emissions (with s.e. bars) in grams per kg milk across lactations.

4. Discussion

Although CH₄ emissions increased overall with increasing dry matter intake, emission per kg milk decreased as dry matter intake increased. The increase in CH₄ emission rate during early lactation reflects increased feed intake and milk production of cows during early lactation. Change in CH₄ emission rate with week of lactation was similar to results reported in previous studies [4,6]. Diet composition and feed intake are important factors driving variation in enteric CH₄ emissions from ruminant animals [13]. In the current study, there was considerable variability in enteric CH₄ emissions among farms, however, ranking of cows for emission rate and emissions per kg milk were fairly consistent among herds. This supports implementation of farm-level spot measurements, which are repeatable at different stages of lactation. Farms with the lowest CH₄ emission rate were Farms J and K, where cows were fed a PMR only. However, some farms, such as Farms A and B, fed cows a PMR with grazing and CH₄ emissions were relatively low, which may be influenced by higher average age of cows in these herds. The farm with highest CH₄ emissions was farm L, which may be due to farm L having heavier cows with higher feed intakes and milk production. In the current study, first and second-parity cows had higher emission rates than later parity cows. This may be due to the physiology of younger cows as they develop towards maturity, and as passage rate, digestibility and retention time of substrate

in the rumen may be longer [14]. CH₄ emissions in earlier parities were higher due to the digestive system of younger cows still developing [14,15].

Across farms, lower emissions per kg milk with a PMR diet than with PMR plus grazing may be due to a combination of higher proportion of concentrate feed in the diet reducing CH₄ per unit of feed intake [16,17] and increased efficiency of energy utilisation from dilution of maintenance energy requirements. To meet their genetic potential for milk production, cows must maximise feed intake [18], which is more likely to be achieved with a highly digestible mixed ration than with pasture. In the current study, cows received a commercial concentrate feed during milking in addition to the concentrate component of PMR. Concentrate allowance fed during milking varied with individual milk yield. Because concentrate feed has a curvilinear effect on fibre digestion, a higher concentrate allowance would lower CH₄ emissions per unit intake [19]. The higher emission rate in grazing cows can be explained by higher proportions of forage in diets of grazing cows (0.59) compared to those fed solely PMR (0.51).

The remarkable decline in CH₄ intensity in dairy production during recent decades has been achieved through better nutrition and breeding [5,20]. Future technologies for further reducing CH₄ emissions include genetic selection, feed additives [21,22] and on-farm CH₄ monitoring. Frequent CH₄ spot measurements from individual dairy cows during a day and over at least seven days after week 10 of lactation can provide a suitable estimate of individual animal emissions. Spot sampling of dairy cows whilst milking or feeding has been found to relate to total daily CH₄ emissions [4,23–25] from the same cows when in a respiration chamber.

5. Conclusions

This study found significant effects of farm, week of lactation, parity, dry matter intake and the interaction between dry matter intake and diet, on CH₄ emission rate and emissions per kg milk. Increasing dry matter intake increased emissions per minute, with the increase being higher in grazing cows. In addition, increasing dry matter intake reduced emissions per kg milk, with the reduction being higher for cows fed PMR. In terms of rank correlation, the profiles for CH₄ emission rate and CH₄ emission per kg milk during a lactation appear consistent among herds. Rank correlations for CH₄ emissions among weeks of lactation was generally high across lactations.

Author Contributions: Conceptualization, A.H., M.J.B. and P.C.G.; methodology, A.H., M.J.B. and P.C.G.; software, A.H. and M.J.B.; formal analysis, A.H.; investigation, A.H.; resources, A.H., M.J.B. and P.C.G.; data curation, A.H.; writing—original draft preparation, A.H., M.J.B. and P.C.G.; writing—review and editing, A.H., M.J.B. and P.C.G.; supervision, M.J.B. and P.C.G.; project administration, M.J.B.; funding acquisition, A.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal work was carried out under the authority of the UK Animals Scientific Procedures Act (1986), within Project Licence number 30/3210. Approval for the work was obtained prior to commencement, from the University of Nottingham Animal Welfare and Ethical Review Body.

Informed Consent Statement: Although this study did not involve human subjects, informed consent was obtained from all farmers for use of their anonymised farm data in analyses.

Data Availability Statement: The datasets analysed are available from the corresponding author on request.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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