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ASSOCIATION BETWEEN RUMEN AND FAECAL MICROBIOME AND ENTERIC METHANE EMISSIONS IN DAIRY CATTLE

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SUMMARY

Ruminant livestock primarily contribute to global warming through enteric methane emissions, which is mainly produced by the rumen microbiota. The rumen microbiota could potentially be used as an indicator of enteric methane emissions. There is growing interest in evaluating the faecal microbiome as an indicator of enteric methane, as it is an easier-to-record alternative to the rumen microbiome. Here, we present the phenotypic correlations between methane production and the principal components from 60 rumen and 60 faecal microbiomes (120 samples in total) of 25 Holstein lactating dairy cows. Methane production exhibited phenotypic correlations of up to |0.37| ± 0.20 (mean 0.14) with the principal components of the rumen microbiome, and up to |0.35| ± 0.18 (mean 0.16) with the principal components of the faecal microbiome. The principal components of the faecal microbiome showed phenotypic correlations of up to |0.62| ± 0.12 (mean 0.22) with the principal components of the ruminal microbiome. These results require validation in larger populations, and genetic correlations need to be determined before implementation in commercial conditions.

INTRODUCTION

Enteric methane emissions (EME) from ruminants contribute to global warming. Methane emission reductions are expected to make a significant contribution to mitigating global warming, particularly in the short term (Forster *et al*. 2021). Enteric methane is the product of a complex interaction between ruminal microorganisms during the fermentation of feed (Gonzalez-Recio *et al*. 2023). Recent studies have identified features of the ruminal metagenome that could potentially be used as indicators of EME in breeding programs aimed at reducing these emissions (Gonzalez-Recio *et al*. 2023). However, recording EME and collecting ruminal samples on commercial farms is logistically challenging and cost-prohibitive on a large scale.

Faecal samples are easier and cheaper to collect than ruminal samples, making the faecal microbiome an attractive alternative to evaluate as an indicator of both EME and the ruminal microbiome. Earlier studies reported no association between ruminal and faecal microbiome features (Ross *et al*. 2012). However, technological advances in long-read sequencing over the past decade have enabled more detailed characterisation of microbiomes. This technology was recently used to calculate principal components (PCs) of ruminal microbiome feature matrices, which were associated with EME (Gonzalez-Recio *et al*. 2023).

The aim of this study was to investigate the phenotypic correlations between EME, and the PCs of matrices constructed with taxonomic and functional features from ruminal and faecal microbiomes obtained with long-read sequencing technology in dairy cattle.

MATERIALS AND METHODS

Twenty-five Holstein lactating cows located at the Ellinbank SmartFarm (Ellinbank, Victoria, Australia) were phenotyped for dry matter intake (DMI) and EME recorded as daily enteric methane production measured as grams per day (MeP; g/d). The animals were assessed in 2 experiments

between 2022 and 2023. Experiment 1 included 20 cows, experiment 2 included 10 cows, with 5 cows participating in both experiments. The cows in experiment 1 on average produced 32 ± 2.6 kg (mean ± standard deviation) energy-corrected milk (ECM), at 4.3 ± 1.97 parities, were 74 ± 21.1 days in milk (DIM), 584 ± 43.9 kg body weight and had a total dry matter intake of 18.5 ± 1.55 kg/d. The cows in experiment 2 had an average ECM yield of 23 ± 1.8 kg (mean ± standard deviation), 4.0 ± 1.56 parities, were 217 ± 15.7 DIM, 631 ± 40.5 kg body weight and had a total DMI of 22.4 ± 2.72 kg/d.

The cows had continuous access to feed, water, and a loafing area for rest. The cows were outside except for twice-daily milking at approximately 07:00 h and 15:30 h. All cows were offered 6.2 kg DM/d of grain and *ad libitum* vetch (*Vicia sativa* L.) hay. Individual cow DMI was measured using feed bins equipped with load cells. Methane production was obtained using the modified sulphur hexafluoride (SF_6) tracer method (Deighton *et al*. 2014). Methane production was measured over consecutive 5 days, with emissions transformed to MeP. The average ± standard deviation (SD) of MeP was 534 ± 66.5 g/d.

Two ruminal and faecal samples were collected from each cow during the EME recording periods. Ruminal fluid samples were collected via an oesophageal probe placed into the rumen via the mouth (Moate *et al*. 2014). Faecal samples were collected opportunistically from a fresh pat over days 1 to 2 of the methane measurement period, with any missing samples collected via rectal tickling on day 3 of the methane measurement period. Rumen samples were allowed to drain freely through a cheesecloth layer, separating rumen solids from the ruminal fluid, and then frozen at -80°C. Collected faeces were subsampled (~30 g) then frozen at -80°C.

The microbial genomic DNA from the ruminal and faecal samples was extracted using the ZymoBIOMICS DNA Microprep Kit (Zymo Research) with a reduced volume of binding buffer. Sequencing libraries were prepared using the Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies) and sequenced on the PromethION 24/2 (Oxford Nanopore Technologies) according to the manufacturer's instructions. Basecalling was conducted using Dorado software version 0.5.3 with the module FAST. Reads with quality score less than 7 or length less than 250 base pairs were removed. Taxonomic and functional annotation was conducted with the SqueezeMeta software (Tamames and Puente-Sánchez 2019) with version 1.5.2 to obtain the number of reads assigned to taxonomic genera, KEGG Orthology groups (KOs) and Clusters of Orthologous Genes (COGs). The number of reads assigned to the same feature in the two samples from the same animal-experiment combination was summed.

For the ruminal and faecal samples separately, the metagenome features (genera, KOs and COGs) that were not present in at least 90% of the animals were removed, and the remaining missing values were imputed to non-zero values with the geometric Bayesian-multiplicative method from the cmultRepl function of the zCompositions R package (Palarea-Albaladejo and Martín-Fernández 2015). Relative abundance matrices were constructed with the proportion of the reads assigned to each feature relative to the sum of reads assigned to all features of the same type (genera, KOs or COGs) within the same animal-experiment combination. These relative abundance matrices were then transformed with the centered log-ratio transformation with the unweighted option of the CLR function from the easyCODA R package (Greenacre 2018) to account for the compositional nature of the dataset. Then, the first five PCs were calculated with the function prcomp of the R package stats (R Core Team 2022) with the options centre and scale. The phenotypic correlations (r_p) between MeP and the PCs were estimated with bivariate linear models with the structure $\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{e}$ using ASReml-R version 3.0, where $\mathbf{y}$ was a vector of MeP and PCs or two PCs; $\boldsymbol{\beta}$ is a vector of fixed effects; $\mathbf{e}$ is a vector of random residuals with an assumed distribution $N(0, \mathbf{I}\sigma_e^2)$; and $\mathbf{X}$ is an incidence matrix. The effects of the experiment (2 levels), DMI, DIM, parity, ECM, and body weight were evaluated for each combination of two traits, and significant effects were included as fixed effects in the respective model for that combination.

RESULTS AND DISCUSSION

The first five principal components derived from the metagenomes explained between 54 and 69% of the matrices' variance. MeP exhibited phenotypic correlations between zero of and |0.37| ± 0.20 with the PCs of the rumen microbiome (Table 1). The phenotypic correlations between MeP and the PCs from the faecal metagenome ranged from zero to |0.35| ± 0.18. Twenty-one out of 75 (28%) of the phenotypic correlations calculated between the PCs from the rumen and faeces had a magnitude of at least 0.30. Using the KOs, the PCs from faeces showed phenotypic correlations ranging from |0.22| ± 0.18 to |0.62| ± 0.12 with the first PC from the rumen, which accounted for 46% of the variation in the rumen.

Table 1. Phenotypic correlations (r_p) ± standard error (SE), and corresponding *p*-value between the first five principal components (PC1 to PC5) calculated from the content of features from the faecal and ruminal metagenome and methane production (MeP). FE: Fixed effects with significant effect fitted in the prediction model. KO: KEGG Orthology groups. COG: Clusters of Orthologous Genes. Variance explained by each principal component is shown in parenthesis

Features	**Faeces**	**Rumen**	**FE**	**r_p ± SE**	***p*-value**
Genera	PC1 (16%)	PC3 (6%)	-	-0.52 ± 0.16	1.2×10^{-3}
Genera	PC2 (13%)	PC2 (15%)	parity,ECM	-0.48 ± 0.15	1.4×10^{-3}
Genera	PC4 (7%)	PC3 (6%)	experiment,DMI, parity, ECM	-0.43 ± 0.17	1.1×10^{-2}
Genera	PC5 (6%)	PC1 (29%)	-	-0.40 ± 0.18	2.6×10^{-2}
Genera	PC5 (6%)	PC2 (15%)	experiment,DMI, parity, ECM	-0.47 ± 0.16	3.3×10^{-3}
Genera	PC5 (6%)	PC5 (4%)	parity	-0.37 ± 0.17	3.0×10^{-2}
KO	PC2 (11%)	PC3 (6%)	experiment,ECM	-0.35 ± 0.17	4.0×10^{-2}
KO	PC3 (7%)	PC1 (46%)	experiment,DMI,ECM	-0.35 ± 0.17	4.0×10^{-2}
KO	PC3 (7%)	PC2 (9%)	DMI, parity	-0.46 ± 0.16	4.0×10^{-3}
KO	PC5 (4%)	PC1 (46%)	experiment,parity	-0.62 ± 0.12	2.4×10^{-7}
COG	PC2 (11%)	PC3 (7%)	DMI,parity,ECM	-0.37 ± 0.18	4.0×10^{-2}
COG	PC4 (5%)	PC3 (7%)	DMI,parity	-0.39 ± 0.17	2.2×10^{-2}
COG	PC5 (5%)	PC1 (29%)	experiment	-0.34 ± 0.17	4.6×10^{-2}
Features	**Faeces**	**Final trait**	**FE**	**r_p ± SE**	***p*-value**
Genera	PC5 (6%)	MeP	experiment	0.35 ± 0.18	5.2×10^{-2}
Features	**Rumen**	**Final trait**	**FE**	**r_p ± SE**	***p*-value**
Genera	PC4 (5%)	MeP	-	0.37 ± 0.20	6.4×10^{-2}

This study investigated the phenotypic correlations between EME and the PCs from taxonomic and functional features of the ruminal and faecal microbiomes in dairy cattle. Some PCs from the faeces exhibited phenotypic correlations with MeP up to |0.35| and other PCs up to |0.62| with the first principal component of the ruminal KOs content, which explained 46% of the variation in the ruminal microbiota. Using a short-read sequencing technology, a previous study found no stronger correlation between the ruminal and faecal microbiomes of the same cow compared to correlations between different cows (Ross *et al*. 2012), which could indicate no association between ruminal and faecal samples. A possible reason for this difference could be the long-read sequencing technology used here, which has shown unprecedented advances over the past decade in taxonomic and functional characterisation of metagenomes (Nature Methods 2023).

While the laboratory and computational costs of processing the faecal microbiota are approximately the same as those for processing the ruminal microbiota, faecal sampling is easier with less animal welfare implications and is more cost-effective than collecting ruminal samples.

This suggests that faecal microbiota could be a suitable proxy to estimate EME and infer the ruminal microbiota if the correlations can be improved. The correlations estimated in this study still require validation in larger populations in varying environments and production systems. Furthermore, a biological interpretation of the association between the features with larger weights in the PCs and EME requires further investigation. Additional data is also needed to estimate the genetic correlations between these traits. Determining the genetic correlations between EME and the ruminal and faecal microbiota in Australian dairy cattle is essential to assess the viability of using these microbiomes in breeding programs to reduce EME.

CONCLUSIONS

The faecal microbiome showed promise as a proxy for estimating both enteric methane emissions and the ruminal microbiome. However, the results are limited by the small number of animals analysed and therefore, it is essential to validate the results in larger and more diverse populations. Additionally, it is necessary to explore the biological basis of the statistical associations between the principal components of the microbiomes and enteric methane emissions. Furthermore, estimating the heritability of microbial features and their genetic correlations with enteric methane emissions is necessary to establish these features as reliable genetic indicators in breeding programs designed to reduce emissions.

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