Reducing dairy cattle enteric methane emissions using rumen metagenome

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Abstract

Ruminant digestion emits methane, a potent greenhouse gas contributing to global warming and reducing feed efficiency. Reducing enteric methane emissions (EME) via farming and breeding decisions is crucial, yet measuring these emissions on commercial farms is currently challenging and costly. It is common for EME to be measured using distinct technologies. However, different EME traits sometimes show weak correlations between countries, feeding systems or technologies, complicating the combination of reference populations. Here we show a methodology to predict and reduce EME with the use of the rumen microbiome. We identified a common *core* of 1,032 KEGG ontology identifiers (KO) from the rumen metagenome of 410 dairy cows located in Australia and 434 in Spain. This *core* explained 83% and 57% of EME (measured using SF6 in Australia and sniffers in Spain) with an accuracy of 0.38 and 0.19 respectively. This result suggest that the ruminal metagenome can be used to predict EME and make farming decisions to reduce these meissions. We also estimated reductions in EME of up to ~16% of the population mean per generation by selection on this *core*, being superior to direct selection on EME (~9 to 14%). A combination of direct selection on EME and indirect selection on the *core* would produce larger reductions (up to 19%). These results suggest that rumen metagenome features could be candidate for improvement with genomic selection in combination with EME traits. Combining reference populations through the ruminal metagenome can be used to predict EME irrespective of each population's EME trait. We propose a global effort to validate a common *core* of ruminal features associated with EME. If validated, our results could impact global ruminant emission reduction efforts.

Keywords: rumen microorganisms, metagenomics, methane production, phenotypic variation, genomic selection.
Presented at the ICAR Anual Conference 2024 in Bled at the Session 11: Methane Emission-Free Communications: Genetics, Environmental, and Life Cycle Assessment Studies4

Introduction

Ruminants have evolved in symbiosis with their rumen microbiota for over 50 million years, and for this reason can transform plant materials that humans cannot digest into vital nutrients and energy. This capability depends on a diverse microbial community that, unfortunately, produces methane—a potent greenhouse gas (GHG) that contributes to approximately 40% of global methane emissions (Moss *et al*., 2000) and makes up 40% of total GHG emissions from livestock (FAO, 2023). In addition, enteric methane emissions (EME) represent 2 to 12% of the energy loss in the ruminants' diet (González-Recio *et al*., 2023; Lassen and Difford, 2020).

Reducing enteric methane emissions (EME) through farm-management and breeding decisions is ideal. However, EME need to be measured for this purpose and recording these emissions in commercial farms is currently logistically challenging and expensive. Additionally, different EME traits are sometimes weakly correlated, complicating the combination of reference populations (Hristov *et al*., 2016). However, all EME traits have the same underlying biology – methane is mainly produced by the rumen microbiota (González-Recio *et al*., 2023).

The role of the host genetics and the rumen microbiome on EME remains unclear. The host genetics partially determines both EME (López-Paredes *et al*., 2020; Richardson *et al*., 2021) and ruminal microbial features associated with EME (Martínez-Álvaro *et al*., 2022; Saborío-Montero *et al*., 2021). For this reason, and according to the definitions of Pérez-Enciso *et al*. (2021), there could be two potential biological scenarios. Firstly, there is an indirect relationship where the host genome affects EME, but this is mediated by the microbiota. In the second scenario, a recursive model, both the host genetics and the microbiota exert influence on EME, and the host genetics also indirectly affects EME by modulating the microbiota (Saborío‐Montero *et al*., 2020).

In the last decade one of the most widely employed approaches to study the effect of the rumen microbiome on EME is estimating the variance in EME explained by a microbial relationship matrix (MRM) (Ross and Hayes, 2022). Additionally, recent studies have estimated reductions in EME by implementing breeding programs selecting on ruminal microbial features, which are heritable and genetically correlated with EME (Martínez-Álvaro *et al*., 2022).

This study aimed to generate a methodology to: First, quantify the effects of an MRM constructed using a novel methodology on EME in two distinct dairy cattle populations of more than 400 animal each, one in Australia and one in Spain. Second, to estimate the response to selection on EME by indirectly selecting on ruminal metagenomic features. Third, to investigate whether these two dairy cattle populations with distinct EME traits could be connected through the rumen metagenome.

Material and methods

Ethical statement

The Australian experiments in this study were approved and undertaken in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). Approval to proceed was granted by the Agricultural Research and Extension Animal Ethics Committee of the Department of Energy, Environment and Climate Action (application number 2013-14 was approved on August 22nd, 2013, and application number 2016-12 was approved on August 22nd, 2016). The Spanish experiments in this study were conducted in accordance with Spanish Royal Decree 53/2013 for the protection of animals used for experimental and other scientific purposes and were approved by the Basque Institute for Agricultural Research and Development Ethics Committee (Neiker-OEBA-2017-004) on March 28, 2017.

Data

The Australian population included 410 Holstein lactating cows located at the Agriculture Victoria's Ellinbank SmartFarm (Ellinbank, Victoria, Australia). These cows were measured for dry matter intake (DMI) and grams per day methane production (MeP) in 11 cohorts between 2013 and 2017. MeP was considered the EME trait in Australia. At the beginning of the study, cows averaged 110 ± 19.4 (mean ± standard deviation) days in milk, 2.5 ± 1.25 lactations, and weighed 539 ± 69.8 kg. Over a 32-day period in an experimental facility, they had continuous access to feed, water, and a bare paddock (loafing area) for rest. The cows were outside except for twice-daily milking. Cows were fed with the diet described by Moate *et al*. (2021) and DMI was measured using feed bins equipped with load cells and electronic monitoring linked to individual cow identification (Gallagher Animal Management Systems, Hamilton, New Zealand Daily DMI was recorded over the 32 days. Daily MeP was obtained with the sulphur hexafluoride (SF_6) tracer method described by Deighton *et al*. (2014). Further details of the environment of the Australian dairy cattle population are provided by Moate *et al*. (2021).

The Spanish population included 432 Holstein cows, either in their first or second lactation, from 14 commercial farms across four Northern Spanish regions (Cantabria, País Vasco, Navarra, and Gerona). Following the methodology of Rey *et al*. (2019), EME were measured using a non-dispersive infrared methane detector (The Guardian® NG) from Edinburgh Sensors (Livingston, Scotland, UK), also termed "sniffer", installed in the feed bin of an automatic milking system. Individual methane concentration (MeC) in Spain as parts per million (ppm) was recorded for each cow during milking over a period of two to three weeks. The recorded eructation peaks were averaged to obtain a single record per cow. The Spanish population was under commercial milk recording schemes consistent with ICAR accredited recording protocols.

Cows located in Australia were genotyped with SNP arrays including custom genotyping-by-sequencing (GBS) and selected SNP (XT) panels (approximately 8,800 SNP of which at least 6,900 overlapped with the BovineSNP50 BeadChip, Illumina, San Diego, California, USA) and imputed to the Bovine 50K SNP chip panel using FImpute (Sargolzaei *et al*., 2014) as described by Haile-Mariam *et al*. (2020). Cows located in Spain were genotyped with the EURO12K SNP chip (Illumina, San Diego, California, USA) and imputed to 54,609 SNPs using BEAGLE (Browning *et al*., 2018) as described in Jiménez-Montero *et al*. (2013) and the Spanish reference population provided by the Spanish Holstein Association (CONAFE, Madrid, Spain) containing more than 200,000 genotypes. A panel with 39,058 (40K) SNP shared by both populations and with a minor allele frequency greater than 0.05 was selected for analyses.

Ruminal metagenome processing

Ruminal fluid samples from all animals were collected via an oesophageal probe placed into the rumen via the mouth. In Australia a probe similar to the one described by Geishauser (2019), and a vacuum pump were used to collect samples (Moate *et al*., 2014). In Spain, samples were obtained as described by Saborío-Montero *et al*. (2021). Following collection, ruminal fluid samples were frozen using liquid nitrogen vapours. Microbial genomic DNA was extracted from the ruminal fluid using a ZymoBIOMICS DNA miniprep kit (Zymo Research, Irvine, California, USA) in Australia, and with DNeasy Power Soil Kit (QIAGEN, Valencia, California, USA) in Spain. After DNA concentration and purity assurance, long-read sequencing with Oxford Nanopore Technologies (ONT) and R9.4.1 flow cells was used for metagenome sequencing (Oxford Nanopore Technologies, Oxford, United Kingdom).

Microbiome sequence data analysis

Basecalling was conducted using Guppy (Oxford Nanopore Technologies, Oxford, United Kingdom) with high accuracy mode (HAC) with the versions 5.0.16 and 4.2.2 in Australia and Spain, respectively. Reads with a quality score greater than 7 and sequence length greater than 150 base pairs were retained for analysis. The long reads were aligned to the KEGG database (Kanehisa and Goto, 2000) for KEGG ontology identifiers (KO) identification using the script SQM_longreads.pl of SqueezeMeta pipeline (version 1.4) (Tamames and Puente-Sánchez, 2019).

Relative abundance matrix of KOs

KOs not present in all animals or that included genes of *Bos taurus* (cow) were removed, retaining 1,032 KOs for downstream analysis. These KOs were used to construct two absolute abundance matrices, one per population, with dimensions animals ×KOs and populated with the number of reads assigned to each KO in each animal. Subsequently, a relative abundance (RA) matrix was created from each population as the proportion of each variable's absolute abundance compared to the total abundances in the same animal. These RA matrices were CLR-transformed to account for their compositional nature (Gloor *et al*., 2017) using the unweighted option of the *CLR* function of the easyCODA R package (Greenacre, 2018).

Relationship matrices

A genomic relationship matrix (GRM) was created with genotypes of the SNP markers shared by both populations, utilising the *Gmatrix* function from the R package *AGHmatrix* (Amadeu *et al*., 2016) following the approach of Yang *et al*. (2010). Additionally, a MRM was constructed as:

$$\mathbf{M} = (1/p)\mathbf{X}\mathbf{X}^{T} \quad (1)$$

Where M is the MRM, p is the number of KOs and X is the CLR-transformed matrix. A small constant value (1×10^{-8}) was added to the elements on the main diagonal of the MRM matrices to prevent singularity issues. Finally, the GRM and MRM were inverted with the function solve of R (R Core Team, 2022). We avoided the step of scaling and centring the KOs across animals as is widely used (Hess *et al*., 2023; Ross and Hayes, 2022; Ross *et al*., 2012) as this step decreases large effects of KOs on EME (López-García *et al*., 2022; Martínez-Álvaro *et al*., 2022; Roehe *et al*., 2016).

Variance in enteric methane emissions explained by the rumen microbiome

The variance in EME explained by the rumen microbiome was estimated with a microbiome BLUP (MBLUP) (Saborío-Montero *et al*., 2021) as:

$$y = 1'\mu + X\beta + Uh + Wm + e \quad (2)$$

Where m is the EME population mean; 1 is a vector of ones with the same length of γ ; β is a vector of fixed effects; u is a vector of random additive genetic effects; and m is a vector of random microbiota effects. X and W are incidence matrices. The

distribution of m is assumed N(0, MRMσm2); and e is a vector of random residuals distributed N(0,se2). EME of each country was included as the dependent variable (y) in this model. The fixed effects in Australia were cohort (11 levels), DMI, days in milk, energy corrected milk obtained with the methodology of Visscher *et al*. (1994), and daily body weight change during the experiment. In Spain, the fixed effects were lactation number (2 levels) and stage of lactation (3 levels). In Spain, the robots used to measure emissions nested within farms (24 levels) was used as a random effect and is represented by the effect h and the incidence matrix U. The models were conducted with the function asreml of the R package ASReml-R (version 3) (Butler *et al*., 2009). The proportion of EME variance explained by the rumen microbiome, microbiability (m2), was estimated as follow, where sp2 is the phenotypic variance on EME:

$$m^2=(\sigma_m^2)/(\sigma_p^2) \quad (3)$$

The accuracy of prediction was estimated with a 10 repetition, 5-fold cross-validation, where the phenotypes of the validation group were removed and the prediction was developed with the phenotypes from the remaining four groups and the rumen microbiome KOs of all animals. The prediction accuracy was calculated as the correlation between the random coefficient regressors from the MRM of the validation group and their EME. Then, the mean and standard deviation of the accuracies across the groups were calculated. This process was repeated 10 times, and the mean and standard deviations were averaged between repetitions to obtain the final accuracy mean and standard deviation.

Phenotypic and genetic parameters

Univariate genomic BLUP (GBLUP) were conducted to EME and each KO as the response variable in:

$$y = 1'\mu + X\beta + Uh + Zg + e \quad (4)$$

Where, g is a vector of random additive genetic effects with an assumed distribution N(0, GRM_g^2) and Z is an incidence matrix. The rest of Equation 4 are the same previously described for Equation 2.

The heritability (h^2) of the KOs was estimated as:

$$h^2=(\sigma_g^2)/(\sigma_p^2) \quad (5)$$

The phenotypic correlation between KOs and EME was calculated as the Pearson correlation between them and the genetic correlations as the correlations between the genomic estimated breeding values obtained with Equation (4).

Selection response of enteric methane emissions

We calculated three different scenarios to select against EME: (1) by directly selecting against EME only, (2) by indirectly selecting on the ruminal KOs only, and (3) by using a combination of scenarios (1) and (2), selecting on both EME and the KOs. The KOs used as indicative traits of MeP in Australia were 87 KOs in the *core* that had a heritability ≥ 0.20 in Australia and a genetic correlation with EME in Australia ≥ 0.20 (*core breeding Australia*). Similarly, the KOs used as indicative traits of MeC in Spain were 159 KOs in the *core* that had a heritability ≥ 0.20 in Spain and a genetic correlation with EME in Spain ≥ 0.20 (*core breeding Spain*). Additionally, we also used 15 KOs reported by Martínez-Álvaro *et al*. (2022) as associated with EME in beef cattle that were present in our *core*. We calculated the response to selection in all scenarios with a selection index approach (Cameron, 1997) incorporating the estimated heritability, and genetic and phenotypic correlations previously described using an in-house R script (R Core Team, 2022). Further, we calculated the response to selection when 30% to 1% of the population with lowest methane emissions were selected.

Results

The MRM explained 83 ± 7% of the variance in EME in Australia and 57 ± 20 % in Spain, with prediction accuracies of 0.37 ± 0.08 and 0.19 ± 0.11, respectively. The heritability of EME was 0.28 ± 0.12 and 0.11 ± 0.10 in Australia and Spain, respectively. The maximum KOs' heritability was 0.56 in Australia and 0.47 in Spain, the genetic correlations between EME and KOs were up to |0.54| in Australia and |0.43| in Spain (Figure 1), and phenotypic correlations up to |0.49| and |0.22| in Australia and Spain. These results agree with that reported by (Martínez-Álvaro *et al*., 2022). The *core breeding Australia* had a heritability of 0.27 ± 0.06 and a genetic correlation of 0.30 ± 0.07. The *core breeding Spain* had a heritability of 0.28 ± 0.07 and a genetic correlation of 0.24 ± 0.03.

Larger reductions were estimated with indirect selection on the KO cores compared with direct selection on EME, agreeing with a previous study (Martínez-Álvaro *et al*., 2022). The mean MeP in Australia was 462 g/d, and the mean MeC in Spain was 1,310 ppm. We estimated that, by selecting the top 1% of the population, a reduction in MeP of 13.6% of the population mean in Australia per generation with direct selection (Figure 2), 15.8% with indirect selection on the *core breeding Australia*, and 19.4% by combining direct and indirect selection on the *core breeding Australia*. Similarly, by selecting the top 1% of the population in Spain, we estimated a reduction in MeC of 8.9% of the population mean per generation with direct selection, 12.6% with indirect selection on *core breeding Spain*, and 14.4% by combining direct and indirect selection on the *core breeding Spain*. Fifteen KOs were shared between our core and the KOs reported in beef cattle by Martínez-Álvaro *et al*. (2022). Reductions of 7.0% and 4.8% of the EME population mean per generation were estimated in Australia and Spain, respectively (Figure 2). These 15 KOs were also estimated to increase the reduction on EME when combined with direct selection, compared to use only direct selection.

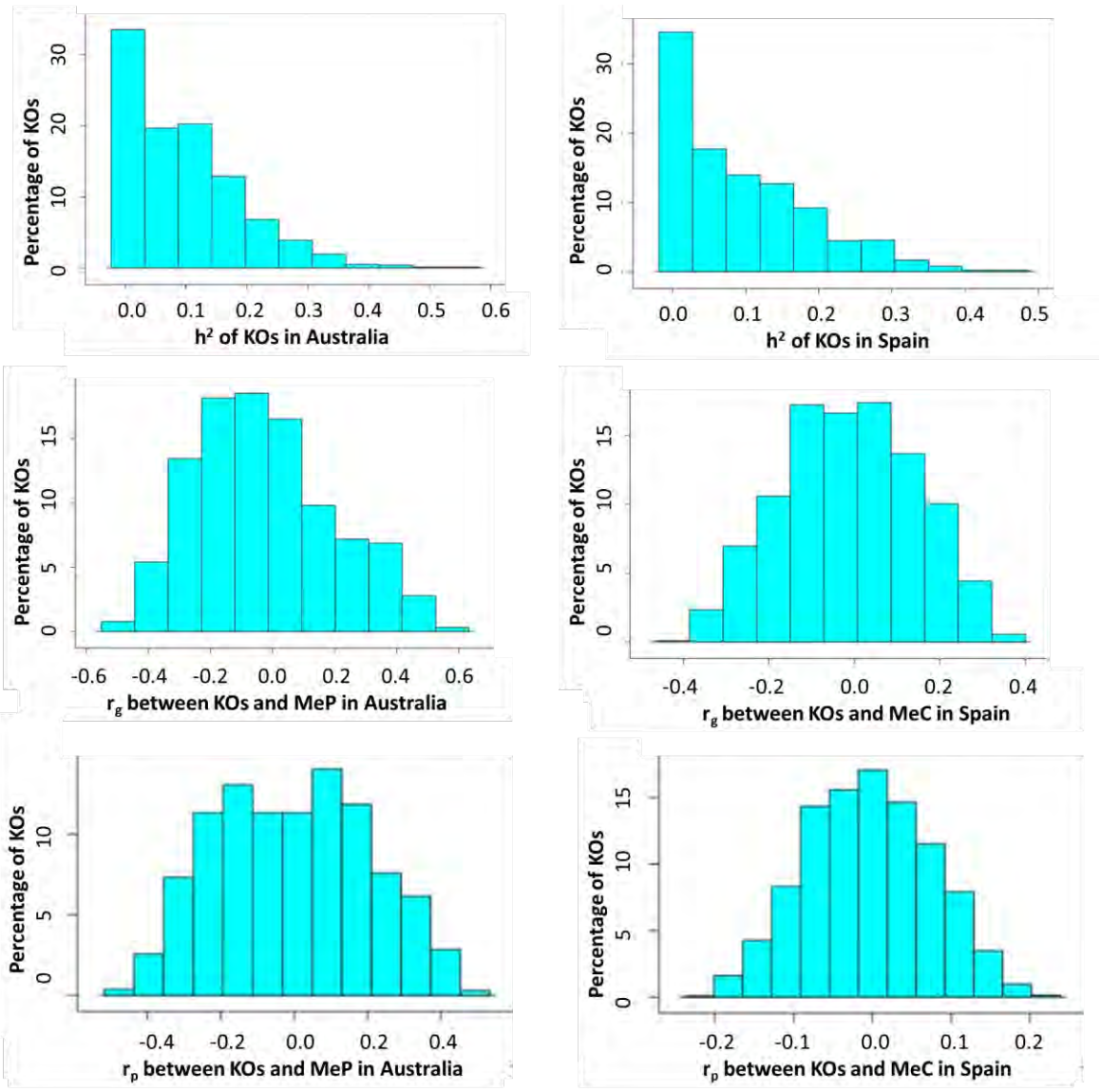

Figure 1. Heritability (h^2) of KEGG ontology identifiers (KOs) of a ruminal metagenome core shared between two dairy cattle populations located in Australia and Spain; and the genetic (r_g) and phenotypic correlation (r_p) between these KOs and methane production (MeP) in the Australian and methane concentration (MeC) in the Spanish populations.

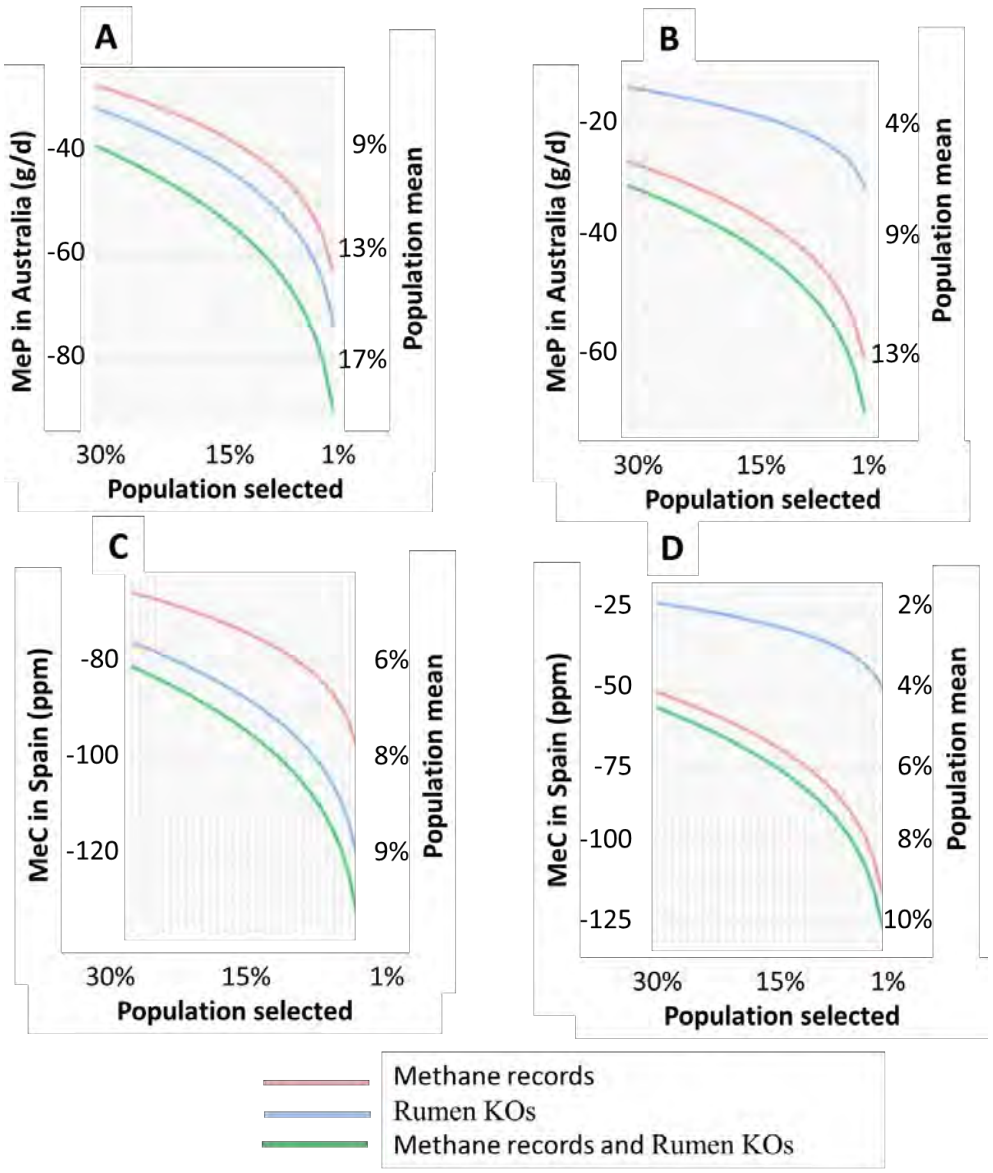

Figure 2. Estimated reduction by generation of enteric methane production (MeP) in Australian and enteric methane concentration (MeC) in Spanish dairy cattle populations. Red line: direct selection on enteric methane records. Blue: Indirect selection on ruminal microbial KEGG ontology identifiers (KOs). Green: Combination of direct selection on enteric methane records and indirect selection on KOs. In A and C, the KOs used are from a common core of 1,032 KOs shared between the populations located in Australia and Spain. A: Using 87 KOs with a heritability ≥ 0.20 in Australia and a genetic correlation ≥ with MeP. C: Using 42 KOs with a heritability ≥ 0.20 in Spain and a genetic correlation ≥ with MeC. B and D: Selection on 15 KOs shared between the Australian, Spanish dairy populations, and a beef cattle population (Martínez-Álvaro et al., 2022).

Discussion

The large variance in EME explained by our 1,032 KO *core* suggests that the ruminal metagenome could be used to reduce EME, for example, by identifying and removing high-emitter animals based on their ruminal microbiome profile or providing feed additives designed to reduce emissions exclusively to higher-emitting animals instead of the whole herd. Larger reductions on EME were estimated when using our *core* than when using direct EME and these reductions were even higher when combining the ruminal features and the EME records. These results are consistent with a previous study (Martínez-Álvaro *et al*., 2022). The large EME reductions by selecting on the KOs could be expected because EME is not an intrinsic animal trait, but a characteristic of the ruminal microbial community. This microbial community is heritable and genetically correlated with EME (Figure 1). The *core breeding Australia* and *core breeding Spain* used to estimate the selection response was heritable (~0.27) genetically correlation with EME (rg = 0.30 in Australia; rg = 0.24 in Spain). Based on our results, the *core breeding Australia* and *core breeding Spain* could be considered as target traits for improvement in emissions reduction genomic selection programs, in combination with EME records.

Genomic selection on a common ruminal metagenome *core* shared between Australia and Spain would lead to reductions in EME in both populations. These results indicate the potential for combining geographically diverse? reference populations in breeding programs through their ruminal metagenome, irrespective of each population's EME trait (Figure 3). Additionally, 15 out the 30 KOs reported as associated with EME in beef (Martínez-Álvaro *et al*., 2022) were used to estimate reductions of up to 7% of EME's population mean our dairy cattle populations (Figure 2). Further research could evaluate whether a common *core* between dairy and beef cattle, and other ruminants such as sheep, would reduce EME in all ruminant populations. Generating a reference population with EME measurements, ruminal metagenome and host genomics is costly and time consuming. Based on the results of this study, fostering international collaboration among the dairy, beef and other ruminant industries to combine diverse populations, EME traits, and environments through the rumen metagenomecould be beneficial for reducing global methane emissions. A common methodology is recommended for this purpose and based on our results, we present a methodology that (1) predicts most of the variance in EME, (2) potentially leads to significant EME

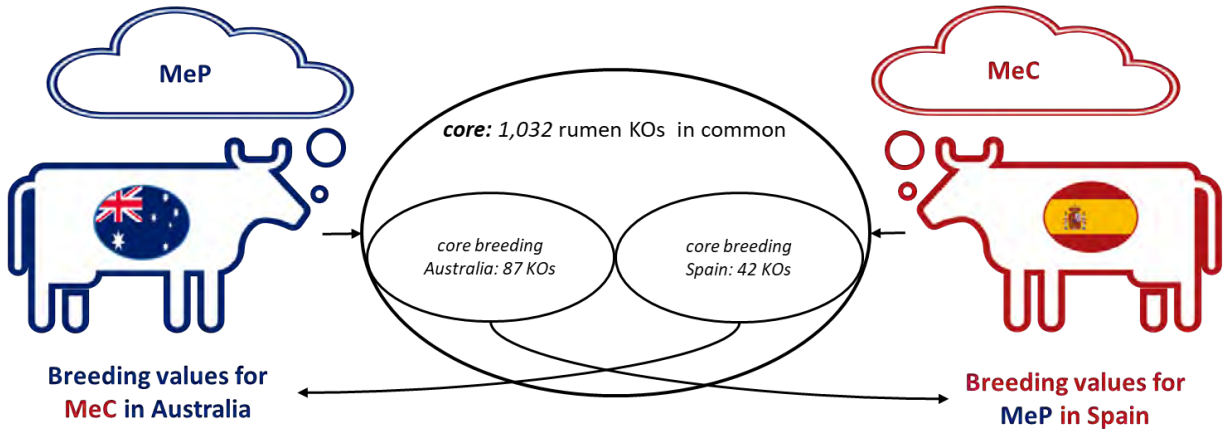

Figure 3. Sharing reference populations of the ruminal metagenome core facilitates prediction of enteric methane production (MeP) in Australia and enteric methane concentration (MeC) in Spain. Core breeding Australia: KOs with heritability ≥ 0.20 in Australia and genetic correlation with MeP ≥ 0.20. Core breeding Spain: KOs with heritability ≥ 0.20 in Spain and genetic correlation with MeC ≥ 0.20.

reductions through informed farming and breeding decisions, and (3) could potentially connect reference populations irrespective of their EME traits.

Conclusion

- We have developed a methodology to predict enteric methane emissions (EME) from ruminants. Using this methodology, we detected a common *core* of 1,032 KEGG ontology identifiers (KO) in the rumen metagenome of 834 dairy cows from Australia and Spain. This *core* explained up to 83% of the variation in EME with an accuracy of up to 0.38, which could potentially facilitate farming decisions aims to reduce methane emissions.
- Large reductions in EME, of up to ~16% of the population mean per generation, could be achieved by selection on this *core*, being superior to direct selection on EME. A combination of direct selection on EME and indirect selection on the *core* would produce larger reductions (up to 19% of the population mean). These results suggest that rumen metagenome features could be candidate traits to improved-genomic selection programs along with EME records.
- Sharing reference populations of the ruminal metagenome *core* facilitates prediction of EME irrespective of each population's EME trait. For this reason, we propose a global effort to validate a common *core* of ruminal features associated with EME.
- If validated, our results could impact global ruminant emission reduction efforts.

Acknowledgements

- The authors are grateful for the support by DairyBio (Melbourne, Australia), funded by Dairy Australia (Melbourne, Australia), the Gardiner Foundation (Melbourne, Australia), and Agriculture Victoria (Melbourne, Australia) for financial support. Likewise, the authors are grateful for the financial support of the METALGEN project (RTA2015-00022-C03) from the national plan for research, development, and innovation 2013–2020 and the Department of Economic Development and Competitiveness (Madrid, Spain).
- The authors also thank the staff at Ellinbank SmartFarm (Ellinbank, Australia), regional Holstein associations, and farmers of Spain for their technical expertise and assistance. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- The authors thank Sunduimijid Bolormaa, of Agriculture Victoria Research, for imputing the genotypes of the Australian population. The authors extend their gratitude to Josie B. Garner and Peter J. Moate from Agriculture Victoria Research (Ellinbank), as well as to William J. Wales, who is affiliated with both the Faculty of Veterinary and Agricultural Science of The University of Melbourne and Agriculture Victoria Research (Ellinbank), for their valuable contributions in developing the Australian datasets used in this study.

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