Animal Microbiome

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Network analyses unraveled the complex interactions in the rumen microbiota associated with methane emission in dairy cattle

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Abstract

Background Methane emissions from livestock, particularly from dairy cattle, represent a significant source of greenhouse gas, contributing to the global climate crisis. Understanding the complex interactions within the rumen microbiota that influence methane emissions is crucial for developing effective mitigation strategies.

Results This study employed Weighted Gene Co-expression Network Analysis to investigate the complex interactions within the rumen microbiota that influence methane emissions. By integrating extensive rumen microbiota sequencing data with precise methane emission measurements in 750 Holstein dairy cattle, our research identified distinct microbial communities and their associations with methane production. Key findings revealed that the blue module from network analysis was significantly correlated (0.45) with methane emissions. In this module, taxa included the genera *Prevotella* and *Methanobrevibactor*, along with species such as *Prevotella brevis, Prevotella ruminicola, Prevotella baroniae, Prevotella bryantii, Lachnobacterium bovis,* and *Methanomassiliicoccus luminyensis* are the key components to drive the complex networks. However, the absence of metagenomics sequencing is difficult to reveal the deeper taxa level and functional profiles.

Conclusions The application of Weighted Gene Co-expression Network Analysis provided a comprehensive understanding of the microbiota-methane emission relationship, serving as an innovative approach for microbiota-phenotype association studies in cattle. Our findings underscore the importance of microbiota-trait and microbiota-microbiota associations related to methane emission in dairy cattle, contributing to a systematic understanding of methane production in cattle. This research offers key information on microbial management for mitigating environmental impact on the cattle population.

Keywords Rumen microbiota, Microbial networks, CH_4 emissions, WGCNA

Background

Methane (CH_4), a greenhouse gas (GHG), significantly contributes to global warming [1]. The livestock sector represents 14.5% of human-induced GHG emissions, where feed production/processing and enteric fermentation from ruminants are the two main sources representing 45% and 39% of emissions in the livestock sector, respectively [2]. Among all ruminant species, dairy cattle accounts for 30.1% of the sector's emission, and the emission in dairy cattle is mainly from ruminal CH_4 emission [3]. As one of the major contributors of GHG emissions,

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dairy sector plays a key role in realizing Agriculture Net Zero through mitigating enteric CH_4 emission [4]. Moreover, facing the high demand of milk and meat supply, CH_4 emissions from livestock is predicted to increase continuously by 2050 [5]. These highlight the pressing need for innovative strategies to mitigate CH_4 emissions to address environmental concerns while maintaining sustainable dairy production [6–9].

The rumen, largest digestive organ in ruminants, serves as an anaerobic ecosystem that fosters the growth of diverse microorganisms, including bacteria, archaea, fungi, and ciliate protozoa, which collectively influence CH_4 production [10, 11]. Major factors that affect CH_4 emissions from dairy cattle rumen include genetics [9, 12], management [13], feeding strategies [14], and lactation stages [15]. Ruminal methanogenesis, a key contributor of CH_4 emissions [16], contains complex biological processes with diverse metabolic pathways [17–19]. *Methanobrevibacter* and *Methanomassiliicoccus* are the dominant rumen archaeal genera responsible for a significant portion of CH_4 emissions [16]. Most rumen methanogens have hydrogenotrophic metabolisms, meaning they use electrons from H_2 to reduce CO_2 to CH_4, an efficient way to reduce H_2 concentrations in the rumen [20]. Meanwhile, ciliate protozoa from a vital component of the rumen microbial ecosystem, engaging in diverse metabolic pathways. Many of these functions arise from their close interactions with associated prokaryotic communities. For example, CH_4 production can be intensified through interspecies hydrogen transfer between protozoa and archaea [21]. Together with a diverse bacterial community that ferments and degrades complex carbohydrates and proteins, they form a complex network that ultimately determines CH_4 emissions. The effort to understand the complex microbial network is the key to developing mitigation strategies for ruminant CH_4 production [22–24].

Despite previous research on complex metabolic networks in rumen [25], a gap remains in the understanding of the interactions within rumen microbiota on CH_4 emissions. Previous studies have often focused on either the taxonomic composition of the microbiota or the host's genetic variations [12, 26–28]. The application of advanced system biological tools, such as Weighted Gene Co-expression Network Analysis (WGCNA) [29], to investigate the comprehensive relationship between the microbiota and CH_4 emissions remains unexplored. This study utilizes WGCNA to study the interactions within rumen microbiota and its link to CH_4 emissions in dairy cattle [30, 31]. WGCNA enables the identification of the microbial interactions associated with traits by generating modules [29]. In this study, we hypothesized that specific rumen microbial community as one module could be used to explain the CH_4 emissions in dairy cattle. To address this hypothesis, we integrate CH_4 emissions data and rumen 16S rRNA gene amplicon sequencing data from 750 Holstein cows to identify the microbial community structures and interactions that are associated with CH_4 emission in this study.

Results

Rumen microbiota compositions and structures

We obtained 191,035,652 and 115,632,617 total reads for bacteria (mean ± SD: 226,077.695 ± 209,835.229) and archaea (mean ± SD: 106,843.116 ± 90,783.265), respectively (Supplementary Fig. 1). Additionally, a total of 59,445 and 1805 observations (ASVs) for bacteria and archaea were retained as the raw data for downstream analysis (750 cows). The alpha diversity analysis showed richness (Chao1, richness) and evenness (Shannon 2, Simpson) metrics for archaeal and bacterial ASVs (Supplementary Fig. 1). Compared to archaeal communities, bacterial communities performed a better richness and evenness ($P < 0.001$) in Supplementary Fig. 1. A total of 35 and 1393 ASVs remained for archaea and bacteria in the final datasets after quality control (relative abundance > 0.01% among half samples) [12]. The diversity and distribution of these microbial communities was shown in Principal Component Analysis (PCA) plots confirmed with PERMANOVA, which shown herds influenced sample clustering, while lactations appear to distribute randomly across both archaeal and bacterial samples (Supplementary Fig. 2).

In terms of taxonomic classification, two predominant archaeal classes within the Euryarchaeota phylum — Methanobacteria and Thermoplasmata, identified (Fig. 1A). The Methanobacteriaceae family, which comprise the genera *Methanosphaera*, and *Methanobrevibacter*, was distinctly represented in the phylogenetic branches (Fig. 1A). In the bacterial domain, the Bacteroidetes phylum, enriched by genus *Prevotella*, and their species *Prevotella brevis,* and *Prevotella ruminicola* (Fig. 1C).

The relative abundance of archaea highlighted 5 annotated genera, which were dominated by *Methanobrevibacter* and *Methanomassiliicoccus* (Fig. 1B). At the species level, there were 3 annotated species, including *Methanobrevibacter millerae, Methanosphaera stadtmanae,* and *Methanomassiliicoccus luminyensis* (Supplementary Fig. 3A). Bacterial community showed that the genus *Prevotella* was predominant. Other notable genera, like *Bacteroides, Fibrobacter, Ruminococcus* and *Ruminobacter* were also identified among the top abundant ASVs respectively (Fig. 1D). At the species level, dominant bacterial species included *Prevotella brevis*, *Prevotella maculosa*, *Prevotella ruminicola*, and *Alistipes shahii*, along

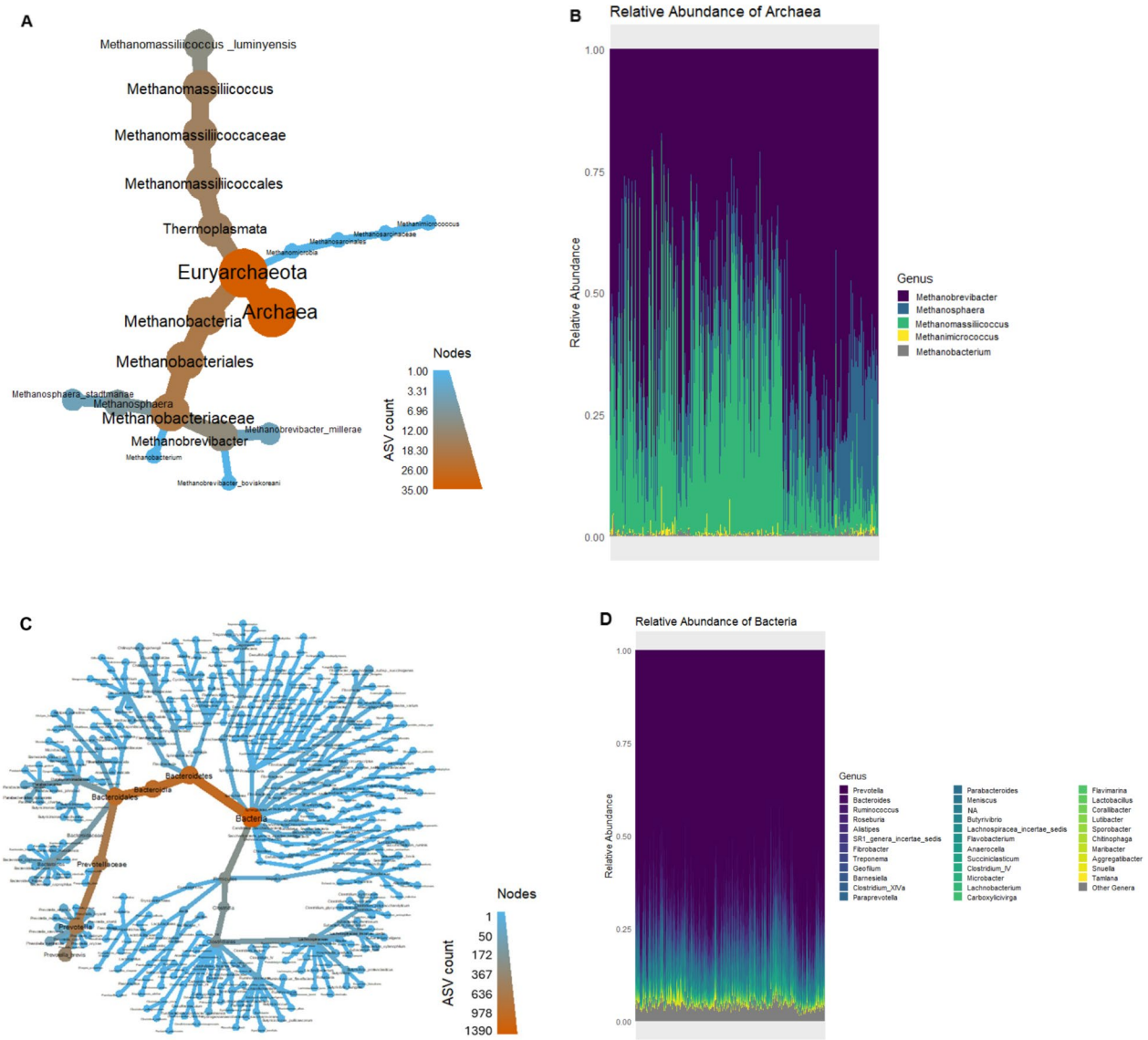

Fig. 1 Composition and abundance of rumen microbiota in cattle. Archaeal (**A**, **B**) and bacterial (**C**, **D**) community composition were annotated to species, genus, family, order, class, phylum, and domain levels. Node color represents the counts of ASV taxonomic annotation. Relative abundance of archaea (**C**) and bacteria (**D**) were summarized at genus level, respectively. NA means the non-annotations at genus level for bacterial communities in Fig. 1D

with *Bacteroides massiliensis, Bacteroides acidifaciens,* and *Bacteroides coprocola* (Supplementary Fig. 3B).

The WCNA clusters associated with CH_4 emission

The Module blue (MEblue) was notably correlated with CH_4 was notable, exceeding 0.45 with a significant P-value (7e-37) (Fig. 2), including genera *Methanobrevibacter, Prevotella*, and *Aminiphilus*; species *Prevotella brevis, Prevotella stercorea, Parabacteroides distasonis, Bacteroides massiliensis, SR1 genera incertae sedis, and Methanomassiliicoccus luminyensis* (Fig. 3). Additionally, Module brown (MEbrown) exhibited a relatively high correlation with the Herd (r = 0.43, p = 4e-34) (Fig. 2), with dominant taxa including *Prevotella*, *Rufibacter*, and species such as *Prevotella brevis*, *Prevotella albensis*, *Bacteroides coprocola*, *Parabacteroides distasonis*, and *Butyrivibrio proteoclasticus* (Supplementary Fig. 5).

The merged ASV dataset (archaea and bacteria) remained with 722 samples and 1428 ASVs. The lactation numbers of each cow (Lact), days in milk (DIM), and farms (Herd) were correlated with ASVs to dissect the effect of the cow's physiological status or the

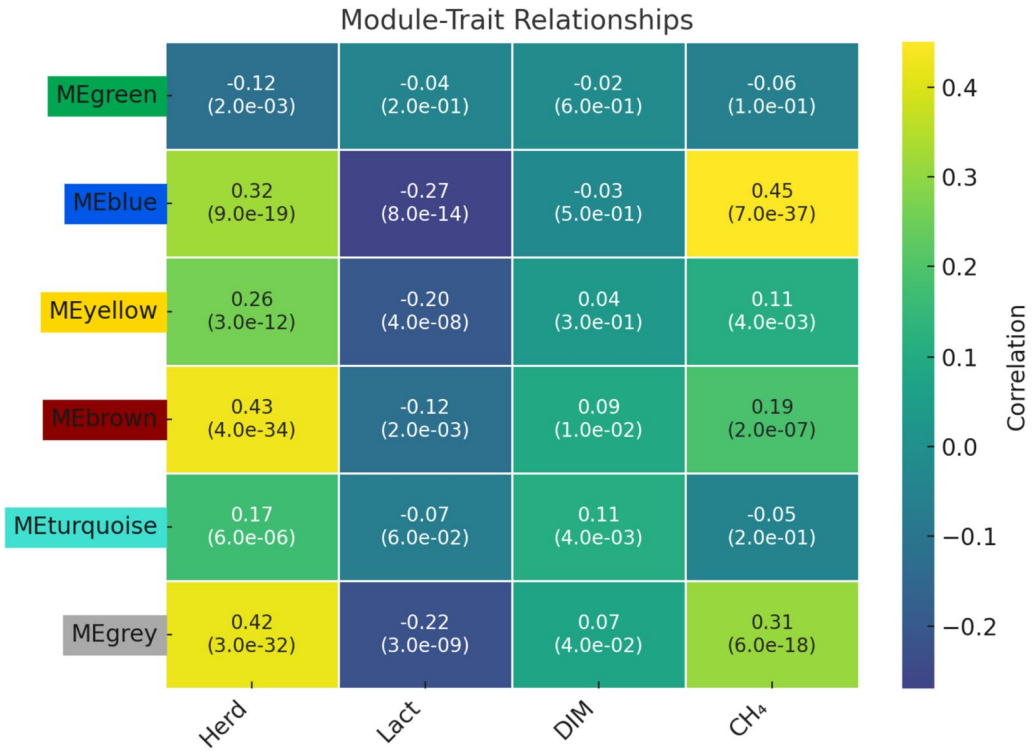

Fig. 2 Modules-traits relationships of merged rumen microbiota community. Herd: The distinct groups of animals sampled from different farms. Lact (Lactation): The lactation number for each cow. DIM (Days in Milk): The number of days a cow has been in milk production during the sampling period. CH_4 (Methane emissions): The corrected methane emissions for each cow per day. The *P*_values of correlation estimates are shown in brackets. The color of the rocks indicates the value of the correlation estimates

farm management. CH_4 emissions were corrected with ASVs to reveal the effect of rumen microbiota on the CH_4 emission. The resulting module dendrograms displayed the clustering of ASVs, identifying 6 modules for ASVs (Supplementary Fig. 4). Each module represents a group of highly interconnected microbial taxa, revealing co-abundance relationships and their links to CH_4 emissions. The grey module was excluded due to unclassified co-expression patterns. The heatmap (Fig. 2) displays the correlations between each module and trait combination, emphasizing the strong links between MEblue-CH_4 emissions and MEbrown-Herd. In contrast, Lact and DIM showed no significant module correlations ($r < 0.3$), suggesting that CH_4-associated microbial dynamics are more influenced by host-independent factors like diet and farm conditions rather than individual physiological stages.

The Hub ASVs and their interactions

To gain deeper insight into the key species in the identified modules (Fig. 2), we identified hub ASVs in significant modules. In MEblue, 56 hub ASVs were identified as hub ASVs (Fig. 3), including two archaeal ASVs (arc asv8-arc asv15) annotated to *Methanobrevibacter* and *Methanomassiliicoccus luminyensis*. Most hub ASVs were annotated to species within *Prevotella* (Fig. 3). Notably, 17 microbial interactions were identified in MEblue, with the most complex network involving 18 ASVs (asv539-asv334-asv930-asv1004-asv133-asv118-asv16-asv787-asv500-asv231-asv644-asv517-asv577-asv1139-asv156-asv307-asv1675-asv1184), which belonged to the species *Prevotella brevis, Prevotella ruminicol, Prevotella ruminicola,* and *Prevotella baroniae* (Fig. 3). There were 12 two microbiota interactions, 3 three microbiota interactions and 1 five microbiota interactions. These simple interactions included *Prevotella brevis-Prevotella ruminicola-Prevotella baroniae, Prevotella brevis-Lachnobacterium bovis, Prevotella ruminicola-Prevotella bryantii,* and *Prevotella ruminicola-Prevotella brevis-Barnesiella viscericola*, as well as *Paraprevotella xylaniphila* within MEblue (Fig. 3).

Similarly, MEbrown interactions, driven by the high correlation between Herd ($r = 0.43$, $p = 4e\text{-}34$) (Fig. 2), including 33 significant ASVs clustered into seven interactions. These interactions prominently involved genera *Prevotella, Rufibacter* and species such as

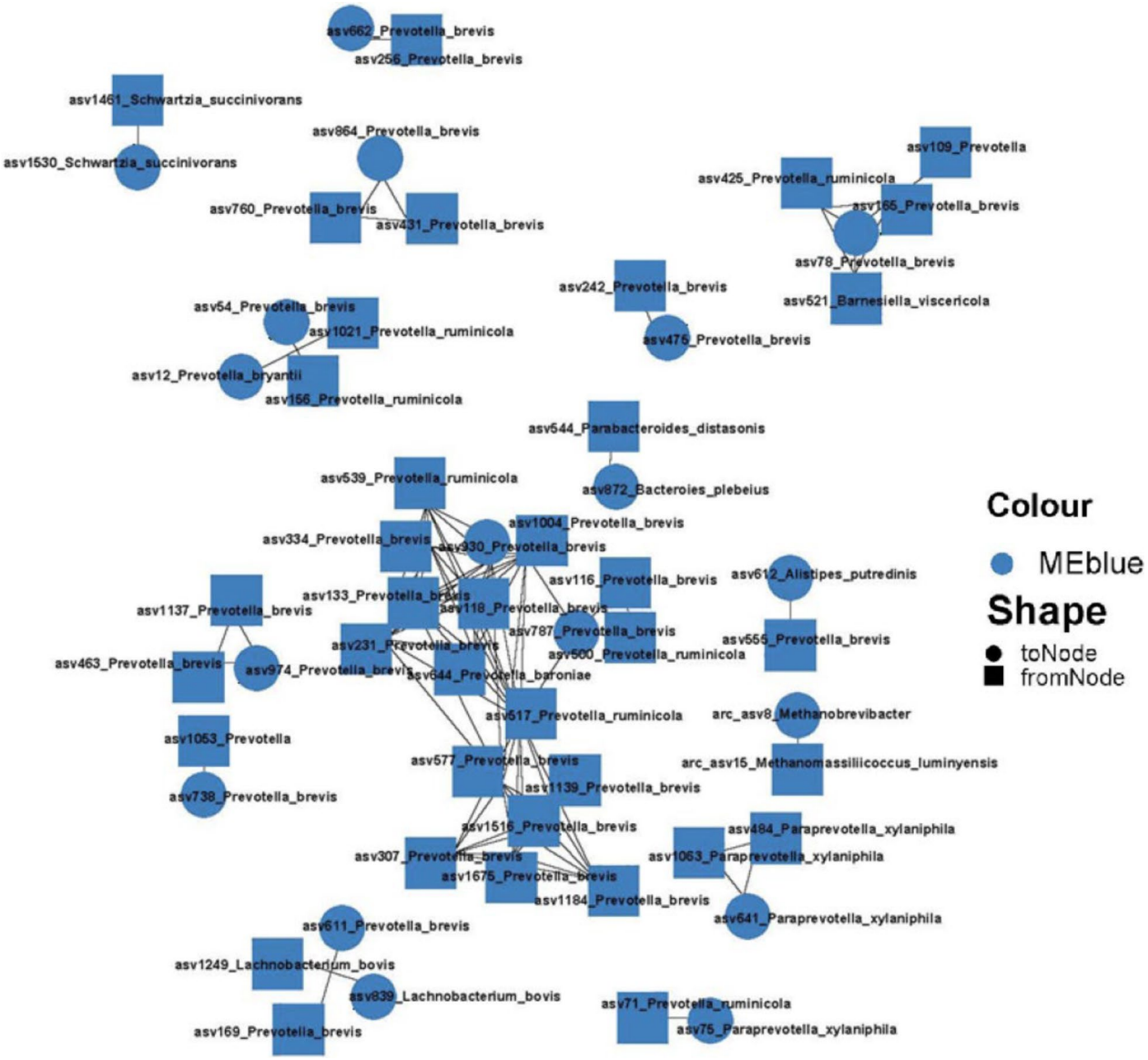

Fig. 3 WGCNA modules containing microbiota related to methane emissions with taxonomic annotations at highest level. These taxa were visualized alongside archaeal and bacterial species and genera nodes that significantly correlated to methane emissions. Nodes' color represents their modules generated by WGCNA. Node's shape represents the toNodes and fromNodes

Prevotella brevis, Prevotella ruminicola Parabacteroides distasonis, Parabacteroides merdae, Bacteroides coprocola, and *Flavonlfractor plautii* (Supplementary Fig. 5).

Pathways annotation and differential functions of archaea and bacteria

There were 363 archaeal and 312 bacterial level 3 pathways (Supplementary Table 2), respectively. The top 30 abundances of level 3 pathways were further annotated to "Methane metabolism", "Transporters", "Ribosome", and "Transfer RNA biogenesis" being the most notable in archaea as depicted in Supplementary Fig. 6A. Similarly, top abundant bacterial pathways were dominated by "Transporters", "DNA repair and recombination proteins", "Transfer RNA biogenesis", and "Ribosome" (Supplementary Fig. 6B).

For the MEblue and MEbrown modules, there were 314 and 302 level 3 KEGG pathways (Supplementary Table 3). The top abundant pathways of MEblue and MEbrown were similar, which was dominated by "Transporters", "Ribosome", "DNA repair and recombination proteins",

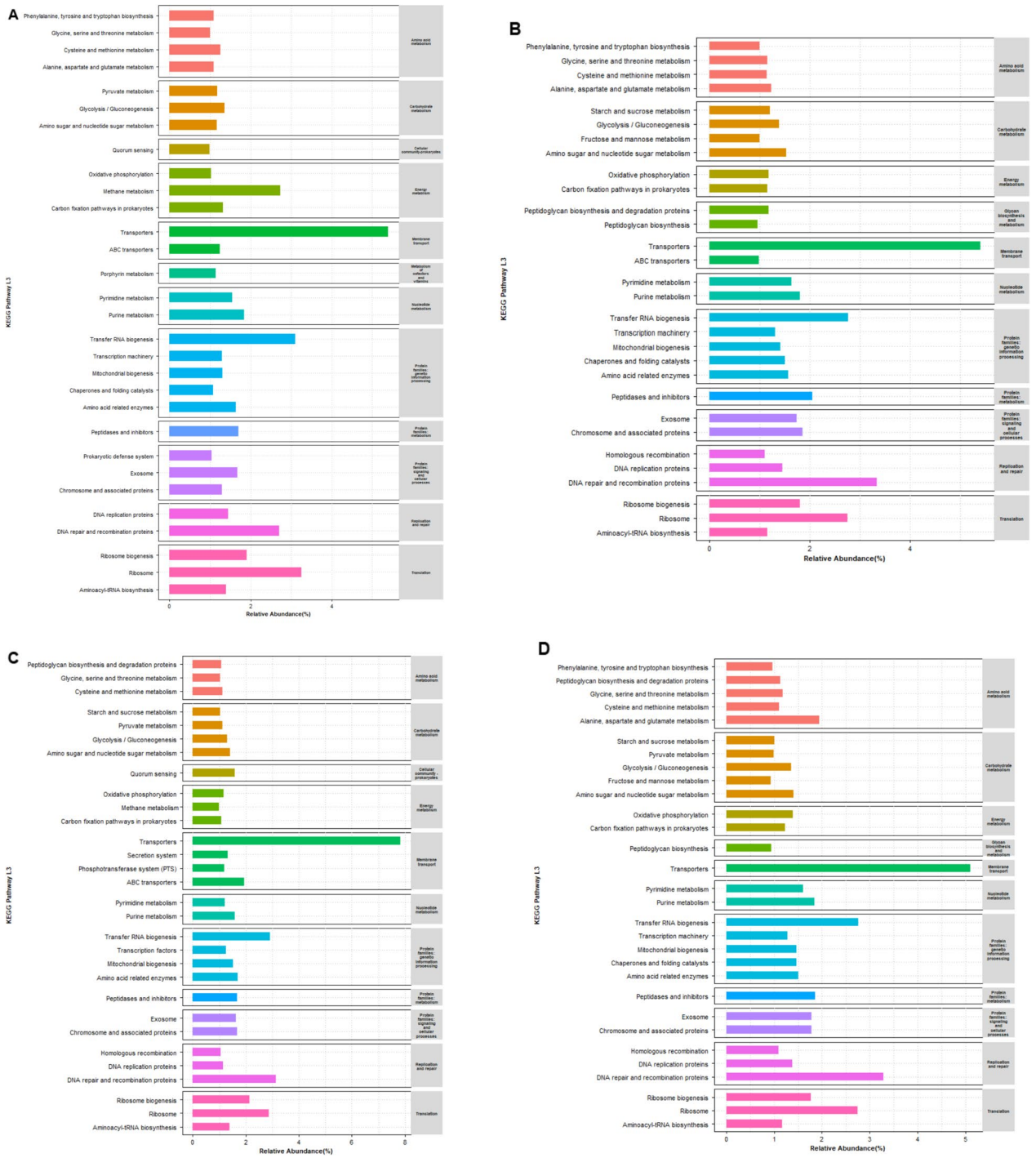

Fig. 4 Level 3 KEGG functional pathways of WGCNA high correlated Modules (Blue [**A**]; Brown [**B**]) and hub ASVs in module blue (MEblue) (**C**) and module brown (MEbrown) (**D**) characterized by Level 2 pathways. This figure displays the top 30 abundant KEGG pathways. To enhance clarity, the bar length means the relative abundance of KEGG pathways at level 3. The different colors of bar represent the different level 2 pathways, which are described in the grey box on the right side of the figure

and "Transfer RNA biogenesis" (Fig. 4A, B). Notably, in MEblue, "Methane metabolism" was observed in the top 30 abundant pathways (Fig. 4A). These two modules were enriched in the level 2 pathways "Protein families: genetics information processing", "Amino acid metabolism" and "Carbohydrate metabolism".

There were 264 KEGG level 3 pathways for hub ASVs in MEblue (Supplementary Table 4). The top abundant pathways were "Transporters", "DNA repair and recombination proteins", "Transfer RNA biogenesis", and "Ribosome", which was similar to bacterial community (Fig. 4C). Interestingly, "Methane Metabolism" was identified in the top 30 abundant pathways, belonging to "Energy metabolism" (Fig. 4C), which was observed in MEblue as well (Fig. 4A). Meanwhile, 265 KEGG level 3 pathways were annotated for hub ASVs in MEbrown (Supplementary Table 4). The top abundant pathways were similar to MEblue and MEblue KEGG pathways, including "Transporters", "DNA repair and recombination proteins", "Transfer RNA biogenesis", and "Ribosome" (Fig. 4D). The top abundant level 3 KEGG pathways of MEblue and MEbrown were all enriched in level 2 pathways "Protein families: genetics information processing", "Amino acid metabolism", and "Carbohydrate metabolism" (Fig. 4C, D).

Discussion

Based on the WGCNA results, MEblue was the most significant module, correlating moderately with CH_4 emissions (r = 0.45, p = 7e-37) (Fig. 2). This module includes both methanogenic archaea (*Methanobrevibacter* and *Methanomassiliicoccus luminyensis*) and carbohydrate-fermenting bacteria (*Prevotella brevis, Prevotella ruminicol, Prevotella ruminicola,* and *Prevotella baroniae)* (Fig. 3), forming a metabolic network that contribute to CH_4 emissions. *Methanobrevibacter* and *Methanomassiliicoccus luminyensis* are well-known for their roles in methanogenesis [32]. Both *Methanobrevibacter* and *Methanomassiliicoccus luminyensis* are methanogenic archaea that contribute to CH_4 production in rumen, utilizing hydrogen and CO_2 (hydrogenotrophy) or methylated compounds (methylotrophy) to produce CH_4 [33, 34]. Notably, *Methanomassiliicoccus luminyensis* specially requires hydrogen as an electron donor, reducing methanol, methylamines into CH_4 [35]. The co-occurrence of *Methanobrevibacter* and *Methanomassiliicoccus luminyensis* within MEblue suggests functional interactions, where *Methanobrevibacter* helps maintain low hydrogen partial pressure in the rumen, indirectly supporting *Methanomassiliicoccus* by creating favorable conditions for its methylotrophic methanogenesis [36].

In addition to archaea, MEblue also includes bacteria from genus *Prevotella*, particularly networks among *Prevotella brevis-Prevotella ruminicola-Prevotella baroniae*, *Prevotella* brevis-Lachnobacterium *bovis, Prevotella ruminicola-Prevotella bryantii,* and *Prevotella ruminicola-Prevotella* brevis-*Barnesiella viscericola*, as well as *Paraprevotella-xylaniphila* within MEblue (Fig. 3). The interactions between *Prevotella brevis*, *Prevotella ruminicola*, and *Prevotella baroniae* suggest synergistic relationships that enhance polysaccharide breakdown and hydrogen production, both of which are crucial for CH_4 production [37]. These bacteria was highly abundant and co-clustered, emphasizing their role in carbohydrate fermentation, converting into short-chain fatty acids (SCFAs) [38, 39]. Among SCFAs, acetate indirectly contributes to CH_4 production by methanogenic archaea. While acetate can serve as a substrate for methanogenesis in specific contexts, though its contribution to ruminal methane production is minimal due to the dominance of hydrogenotrophic and methylotrophic pathways and the rapid rumen passage rate, which limits acetogenic methanogens [39]. Furthermore, the cross-phylum microbial interaction (*Prevotella brevis-Lachnobacterium bovis*) is a suggests a synergistic metabolic network, where fermentative bacteria generate hydrogen as a byproduct, which is then utilized by methanogenic archaea for CH_4 production. Additionally, *Lachnobacterium bovis*, which was found to interact with *Prevotella brevis*, produces intermediates like lactate and acetate, further enhancing hydrogenotrophic pathways that contribute to CH_4 emissions [19]. Our results align with previous studies [17, 38] but provide more detailed insights into microbial interactions at the species level. Furthermore, these findings reinforce that MEblue represents a functionally cohesive module linked to CH_4 emissions through hydrogen-mediated microbial interactions.

While MEblue is significantly associated with CH_4, other microbial modules also influence methane metabolism under different environmental conditions. For instance, MEbrown, another major module, contains similar fermentative bacteria (*Prevotella brevis* and *Prevotella ruminicola*), which are well-known contributors to carbohydrate fermentation and SCFAs production [39]. However, MEbrown networks exhibited greater diversity in bacterial taxa, including *Flavonifractor plautii*, *Parabacteroides merdae*, *Barnesiella viscericola*, and *Alistipes putredinis* (Supplementary Fig. 5). Unlike MEblue, which is moderately linked to CH_4 emissions, MEbrown is more influenced by herd effects (r = 0.43, p = 4e-34) (Fig. 3), suggesting that herd-specific factors such as feeding practices and diet composition [40]. Also, *Flavonifractor plautii* contributes to flavonoid degradation [41], influencing microbial dynamics and contributing to SCFAs production. PCA analysis (Supplementary Fig. 2) further revealed that the herd-specific factors significantly shaped microbial community structure, with lactation effects being less pronounced. This may be partly due to the fact that our study was conducted in commercial dairy cattle farms where cattle were reared with potentially different management conditions. Variations in feed composition, fiber content, and nutritional

balance across farms likely contributed to the microbial diversity within MEbrown. This underscores the importance of dietary and environmental factors in modulating microbial networks and their roles in CH_4 emissions [40]. Understanding these environmental influences can help develop targeted intervention strategies to manipulate microbial communities for CH_4 mitigation.

Since MEblue represents a microbial network closely associated with CH_4 production, dietary and management interventions targeting this module could be effective in reducing methane emissions. Recent studies reported that strategies such as dietary modifications, probiotics, and feeding additives can affect the CH_4 emissions by altering microbial community structure and metabolic pathways [42–44]. Probiotics can modulate gastrointestinal microbial. Their colonization in rumen improves feed efficiency, potentially reducing CH_4 emissions [23]. Similarly, dietary interventions, including high-lipid diets [45], nitrate supplementation [46], and plant secondary metabolites (e.g., tannins and saponins) [47, 48], have been explored as effective CH_4 mitigation strategies. Lipid supplementation can suppress methanogens by reducing hydrogen availability, while tannins can directly inhibit methanogens activity [45]. Furthermore, dietary nitrate supplementation provides an alternative hydrogen sink, outcompeting methanogenesis and reducing CH_4 emissions [46]. Beyond diet, farm management practices such as precision feeding, controlled grazing, and strategic supplementation can also influence microbial communities. Precision feeding strategies that optimize fiber and protein balance can reduce hydrogen accumulation and CH_4 formation [49]. Future studies should investigate the long-term effects of such interventions on microbial networks and rumen functionality.

Functional annotation of archaeal microbiota observed pathways associated with CH_4 production, which was enriched in “Methane metabolism”. This result provides an insight into the direct role of archaea in the rumen ecosystem. In contrast, bacterial ASVs exhibited different functional structures, including "Transporters", "Ribosome", and "DNA repair and recombination proteins". These differences reflect the complementary roles of bacteria and archaea in the rumen, where bacteria contribute to the breakdown and fermentation of complex carbohydrates, providing precursors like hydrogen and SCFAs for archaeal methanogens [17, 34, 39]. Interestingly, despite the variability in taxonomic composition, the rumen microbiota’s functional pathways appear conserved. Both the general bacterial ASVs, the WGCNA MEblue or MEbrown ASVs, and their hub ASVs were enriched in KEGG level 3 pathways "Transporters", "Ribosome", and "DNA repair and recombination proteins", which belong to level 2 pathways "Translators", "Membrane transport", and “Replication and repair” (Fig. 4, Supplementary Fig. 6). Our functional prediction results based on bacterial ASVs, WGCNA MEblue and MEbrown ASVs were similar. This similarity suggests functional redundancy among different bacterial taxa in the rumen, ensuring the stability and efficiency of microbial processes essential for host energy metabolism [16, 27, 50, 51]. Functional redundancy is a well-documented phenomenon in microbial communities and is thought to arise from environmental selection for critical biochemical processes, as observed in a recent study on cross-biome microbial networks [52]. To elucidate the specific roles of these interactions in CH_4 production, future studies could employ metagenomics or metatranscriptomics to identify active metabolic pathways and their gene-level regulation within CH_4 emissions.

Unlike SparCC [53], which focuses on pairwise correlations, WGCNA enables the identification of modules of highly correlated taxa or genes. This network-based approach provides deeper insights into the structure and potential roles of microbial or genes communities [17, 54]. Through WGCNA, hub ASVs—such as those annotated to *Prevotella brevis*, *Prevotella ruminicola*, *Prevotella bryantii*, *Methanobrevibacter* and *Methanomassiliicoccus luminyensi* were identified as key taxa driving module dynamics and contributing to CH_4-related metabolic pathways. Additionally, WGCNA excels in its ability to integrate multi-effects data, enabling the associations of microbial communities with external factors such as diet, management practices, or CH_4 emissions [29]. This comprehensive framework makes WGCNA a powerful tool for investigating complex relationships within microbial ecosystems and linking them to functional and environmental factors.

Additionally, our study employed both MiSeq and HiSeq sequencing platforms, two of the leading choices for short-read sequencing. These platforms are considered leading choices for various genomic and microbiome studies due to their robust data output and high-quality sequencing capabilities [55–57]. However, despite their widespread use, the potential influence of platform-specific differences on data analysis remains unclear in the context of our study. A study reported that HiSeq performed longer read sequences and better assigned taxa compared to MiSeq in the context of oral microbiome samples [58]. Moreover, long-read platforms such as Oxford Nanopore [59] and PacBio [60], may provide complementary strengths to short-read technologies by generating longer read lengths that can span complex genomic regions, improve genome assemblies, and resolve ambiguities in repetitive sequences [61].

While PICRUSt2 enabled functional predictions, its reliance on human-derived databases poses limitations

in accurately capturing the functional potential of rumen microbes [62]. To address this, COwPi [63]—a rumen microbiome-focused adaptation of PICRUSt [64]—offers a tailored database specific to the unique microbial communities and metabolic pathways of the rumen. This targeted approach reduces mismatches and improves the accuracy of functional predictions, particularly for KEGG pathway analysis [63]. However, it is important to note that the original PICRUSt has no longer developed, limiting its applicability to current datasets and novel discoveries in rumen microbiology and impeding the usage of COwPi. Despite these challenges, PICRUSt2 remains a practical and robust tool for predicting microbial functional genes, owing to its enhanced algorithm and extended database support. Incorporating rumen-specific features from CowPi into PICRUSt2 could further refine predictions, offering a comprehensive solution for rumen microbiome research.

Despite providing valuable insights into microbial interactions related to CH_4 emissions, our study has several limitations. One key limitation is our reliance on inferred functional data from 16S rRNA gene amplicon sequencing. While predictive tools such as PICRUSt2 offer valuable insights, they lack the resolution of direct metagenomic or metatranscriptomic approaches, which could provide more accurate functional annotations. Integrating multi-omics data, including metabolomics and metaproteomic, would enhance our understanding of the active metabolic pathways driving CH_4 emissions.

Another limitation is the absence of longitudinal measurements. Our study provides a snapshot of microbial interactions at a single time point, but microbiota composition and CH_4 emissions fluctuate over time due to factors such as diet changes, lactation stage, and seasonal variations. Longitudinal studies tracking microbial shifts across different feeding regimes and environmental conditions would be crucial to fully understanding the stability and adaptability of CH_4 -related microbial networks. Additionally, while herd-specific factors were considered, more detailed environmental metadata—such as individual feeding behaviors, rumination time, and precise nutrient intake—could further clarify the external influences on microbial community structures.

Conclusions

This study employed WGCNA to investigate the interactions within the rumen microbiota and their associations with CH_4 emissions using a population-level of 750 Holstein cows. The MEblue was significantly correlated with CH_4 emission, revealing the critical roles of taxa such as *Prevotella*, *Methanobrevibacter*, and *Methanomassiliicoccus luminyensis*. These taxa underscore the complex interactions in carbohydrate fermentation and methanogenesis, key processes contributing to CH_4 production. Additionally, MEbrown was strongly associated with herd factors, revealing microbial networks influenced by farm management practices, diet composition, and feeding strategies. Functional predictions emphasized the complementary roles of bacteria and archaea in the rumen ecosystem, where bacteria provide substrates such as short-chain fatty acids (SCFAs) and hydrogen for methanogenic archaea, which are enriched in pathways linked to CH_4 production. The present study highlights the microbiota-trait and microbiota-microbiota interactions related to CH_4 emission in dairy cattle, contributing to a systematic understanding of CH_4 production in cattle and offering key information on microbial management for mitigating environmental impact in cattle population.

Methods

Data preparation

All microbial data is same in this publication [12]. The detailed process is below. Rumen samples were drawn from individual cows using "Flora Rumen Scoop" [65], an oral insertion probe, to collect approximately 40 ml of liquid for 750 cows individually [12, 28]. To avoid cross-contamination, the "Flora Rumen Scoop" was carefully rinsed during each sampling to minimize cross-contamination. Then samples were labeled, promptly stored on ice, and transported with liquid nitrogen to the laboratory within two hours for the next steps. Each collected samples were vortexed, and a subsample of 1.2 ml liquid was contained in 1.5 ml tube and frozen in liquid nitrogen until transfer to the sequencing company (GATC, Biotech, Constance, Germany) [12]. The corresponding CH_4 emission records of these 750 lactating cows are described in detail earlier [12, 28]. Fourier Transform Infrared unit (FTIR; Gasmet DX-4000, Gasmet Technologies, Helsinki, Finland) [66] and non-dispersive infrared (NDIR; Guardian NG/Gascard Edinburgh Instruments Ltd., Livingston, UK) [67] which were fitted within automatic milking machines were applied to record the CH_4 emissions from the studied cows. All herds practiced indoor feeding strategies with ad libitum access to feed and water. A total mixed ration (TMR) was provided, consisting primarily of rolled barley, corn silage, grass clover silage, rapeseed meal, soybean meal, and up to 3 kg of concentrate supplement administered during milking. Although the TMR formula across all commercial herds were similar, variances in ingredients of farms were anticipated to impact the nutritional values and fiber content of the TMR across different herds.

16S rRNA gene sequencing and bioinformatics

A Qiagen QIAamp stool kit (Valencia, United States of America) was used to obtain total DNA from each rumen liquid sample [68]. Subsequently, sequencing library construction and sequencing were conducted by GATC Biotech (Constance, Germany). The variable regions V1-V3 of 16S rRNA gene for bacteria, while the variable regions V4-V6 of 16S rRNA gene for archaea were amplified using two primer sets to analyze rumen microbial profiles. Archaeal primers were S-D-Arch-0519-a-S-15: 5'-CAGCMGCCGCGGTAA-3' and S-D-Arch-1041-a-A-18 5'-GGCCATGCACCWCCTCTC-3' (expected amplicon size 542 bp), whereas bacterial primers were 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 534R: 5'-ATTACCGCGGCTGCTGG-3' (expected amplicon size 508 bp) [69, 70]. To analyze regional amplicons, GoTaq Green polymerase was used for PCR amplification, and Illumina Miseq and Hiseq platforms were used to generate paired-end sequencing (2 × 300 bp). Sequencing data were processed using Usearch11 [71], Vsearch [72] and QIIME2 [73]. Initially, paired-end reads were merged by their overlapping sequences using Usearch11 (using command -fastx merge). Subsequently, primers and homopolymer sequence runs from merged sequences were trimmed by Usearch11 -search oligodb algorithm. Quality control was executed by Vsearch –fastq eestats2 and Usearch11 -fastx truncate. Only sequences ≥ 300 bp and ≥ 450 bp in length for archaea and bacteria, respectively, were retained for subsequent analyses. The identification of amplicon sequence variants (ASVs) was conducted using DADA2 [74] in QIIME2. The ASVs table for archaea and bacteria were filtered by QIIME2 using feature-table command line with filter-samples to eliminate low counts (> 2,000 reads for bacteria and > 1,000 reads for archaea) and using filter-features to trim low relative abundance (> 0.0001 for each). Subsequently, QIIME diversity subsample-table was used to normalize archaea and bacteria ASV tables. Then the QIIME diversity alpha was conducted to estimate the α-diversity indices (Chao1, Shannon 2, Simpson, richness, and reads). β-diversity (Principal Component Analysis [PCA]) was calculated using Bray–Curtis dissimilarity matrices using phyloseq package in R [75], confirmed with PERMANOVA. Moreover, the representative sequences for archaea and bacteria ASVs were annotated at domain, phylum, class, order, family, genus, and species levels to the database Greengenes2 [76] using QIIME greengenes2 taxonomy-from-table. We then predicted functional characteristics of general archaea and bacteria communities, WGCNA modules, and their hub ASVs using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2 V2.2.0) [62] with Kyoto Encyclopedia of Genes and Genomes (KEGG) [77] database. It predicted the microbial function according to the proportion of marker gene sequences in samples [62]. Only taxa with a relative abundance > 0.01% among half samples were considered as detected taxa and included in the downstream analyses [12]. Subsequently, ASVs units were visualized using phylogenetic trees generated through the application of the Metacoder package in the R software [78]. The relative abundance of taxonomic annotation and KEGG pathways for archaea and bacteria were plotted by ggplot2 in R [79]. After quality trimming, the ASVs of archaea and bacteria were disseminated by principle component analysis (PCA) using Vegan [26] package in R, considering the influence of herds and lactations.

CH4 records and correction

The mean of 750 Holstein CH_4 concentrations were corrected for environmental factors, including diurnal variation and day to day differences using a linear mixed model following a previous study [67]. Then the CH_4 emission records were corrected by a linear mixed model including fixed effects of herd (Herd), lactation (Lact), and days in milk (DIM) using R package lme4 [80]. The models are described below.

1) Fixed effect model for testing effects of herd (Herd), lactation (Lact), and days in milk (DIM) on CH_4 emission:

$$y_{ijk} = \mu + Herd_i + Lact_j + \beta.DIM_{ijk} + \varepsilon_{ijk}$$

y_{ijk}: Observed CH_4 emissions for the *k-th* individual in the *i-th* Herd and *j-th* Lactation.

μ: Overall mean.

$Herd_i$: Fixed effect of the *i-th* Herd (i = 1,2,...,6).

$Lact_j$: Fixed effect of the *j-th* Lactation (j = 1,2).

β: Regression coefficient for Days in Milk (DIM).

DIM_{ijk}: Days in Milk for the *k-th* individual in the i-th Herd and j-th Lactation.

ε_{ijk}: Residual error term, assumed to follow $N(0, \sigma_e^2)$, σ_e^2 is the random error variance.

2) Mixed model to get correct phenotype for CH_4

$$y_{ijk} = \mu + Herd_i + Lact_j + \beta.DIM_{ijk} + u_k + \varepsilon_{ijk}$$

y_{ijk}: Corrected CH_4 emissions for the *k-th* individual in the *i-th* Herd and *j-th* Lactation.

μ: Overall mean.

$Herd_i$: Fixed effect of the *i-th* Herd (i = 1,2,...,6).

$Lact_j$: Fixed effect of the *j-th* Lactation (j = 1,2).

β: Regression coefficient for Days in Milk (DIM).

DIM_{ijk}: Days in Milk for the *k-th* individual in the i-th Herd and j-th Lactation.

u_k: Random effect of the *k-th* individual, assumed to follow $N(0, \sigma_u^2)$, σ_u^2 is the random effect of the cows.

ε_{ijk}: Residual error term, assumed to follow $N(0, \sigma_e^2)$, σ_e^2 is the random error variance.

Co-expression network of rumen microbiota and traits association.

Co-expression network among detected bacterial and archaeal at genus-level taxa were inferred using the Weighted Gene Co-expression Network Analysis (WGCNA) implemented in R [29]. Before starting analyses, we merged archaea and bacteria ASVs. We then used WGCNA to identify the modules of ASVs with the highest relevance to lactations (Lact), Herd, DIM, and CH_4 emissions from cows. The normalized rumen microbial data were log 10 transferred and the combined dataset was quality-checked before analyzing. The low-quality samples and ASVs were removed from the combined dataset using function goodSamplesGenes(). A threshold power of 10 for microbial ASVs data were chosen in the quality control considering that they were the smallest threshold that results in a scale-free R^2 fit of 0.8. After quality control, WGCNA was applied to the combined dataset to generate the network, using blockwiseModule() function. The blockwiseModule() function was used to calculate topologic overlap matrix (TOM) with correlation function, followed by ASVs being hierarchically clustered using 1-TOM (dissTOM) as the distance measure. Original module assignments were determined by using dynamaic tree-cutting, using default parameters mergeCutHeight = 0.25, and minModulesize = 30. The clustered modules were plotted by function plotDendroAndColors() with clustering dendrogram and module colors. The Lact, Herd, DIM, and CH_4 data were used to select the highly correlated modules. The moduleEigengenes() function was used to calculate the module eigengenes (MEs) for each ASV. We then calculated the correlations of MEs with Lact, Herd, DIM, and CH_4 using corPvalueStudent() function. The hub ASVs were selected by function intramodularConnectivity() using dissTOM and moduleColors as input. All hub ASVs interaction were then displayed by networks using Cytoscape [81]. Subsequently, the significant modules ASV sequences and their hub ASV sequences combined with their feature tables were inputted to PICRUSt2 to predict the microbial functions based on KEGG database, as in the previous step. The relative abundance of KEGG level-3 pathways were plotted by ggplot2 package in R [79].

Abbreviations

ASV	Amplicon sequence variant
CH_4	Methane
DIM	Days in milk
GHG	Greenhouse gas
Lact	Lactation numbers of each cow
MEs	Module eigengenes
PCA	Principal component analysis
TOM	Topologic overlap matrix
WGCNA	Weighted gene co-expression network analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-025-00386-z.

Additional file 1.
Additional file 2.
Additional file 3.
Additional file 4.
Additional file 5.
Additional file 6.
Additional file 7.

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Author contributions
XY analyzed and interpreted data. XY wrote the manuscript. BL, ZC modified analyzing process and manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All microbiota sequence data is freely available at https://www.ebi.ac.uk/ena/data/view/PRJEB28065. The data underlying this article are available in G. F. Difford and Q. Zhang, at https://doi.org/https://doi.org/10.1371/journal.pgen.1007580 and https://doi.org/https://doi.org/10.1038/s41396-020-0663-x.

Declarations

Ethics approval and consent to participate
All animals handlings were conducted according to "Metagenomics in Dairy Cows" protocol. The protocol was approved by The Animal Experiments Inspectorate, Danish Veterinary and Food Administration, Ministry of Environment and Food of Denmark (Approval number 2016–15-0201–00959).

Consent for publication
The dataset used in this study was generated previously. No animal handling was involved in this study.

Competing interests
The authors declare no competing interests.

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