RESEARCH ARTICLE

Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows

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Abstract

Cattle and other ruminants produce large quantities of methane (~110 million metric tonnes per annum), which is a potent greenhouse gas affecting global climate change. Methane (CH_4) is a natural by-product of gastro-enteric microbial fermentation of feedstuffs in the rumen and contributes to 6% of total CH_4 emissions from anthropogenic-related sources. The extent to which the host genome and rumen microbiome influence CH_4 emission is not yet well known. This study confirms individual variation in CH_4 production was influenced by individual host (cow) genotype, as well as the host's rumen microbiome composition. Abundance of a small proportion of bacteria and archaea taxa were influenced to a limited extent by the host's genotype and certain taxa were associated with CH_4 emissions. However, the cumulative effect of all bacteria and archaea on CH_4 production was 13%, the host genetics (heritability) was 21% and the two are largely independent. This study demonstrates variation in CH_4 emission is likely not modulated through cow genetic effects on the rumen microbiome. Therefore, the rumen microbiome and cow genome could be targeted independently, by breeding low methane-emitting cows and in parallel, by investigating possible strategies that target changes in the rumen microbiome to reduce CH_4 emissions in the cattle industry.

Author summary

Methane is a potent greenhouse gas and ruminant livestock contribute a substantial amount of total methane from human activities. Variation between cows' methane production has been found partly due to their genetics (heritable), making genetic selection a promising strategy for breeding low methane emitting cows. We hypothesized that the

total methane production by a cow is affected by rumen microbes which are directly responsible for production of methane, as well as the cows' own genetics and their interaction. We sampled the rumen contents of 750 dairy cows and found the relative abundance of some bacteria and archaea to be heritable and associated with methane production, but the majority of variation in relative abundance of rumen bacteria and archaea is due to non-genetic factors. We compared the amount of variation in methane production associated with host genetics as well as rumen bacteria and archaea and found the host genetics to explain 21% and rumen microbes 13%. Importantly, the two were largely independent of each other, so breeding for low methane emitting cows is unlikely to result in unfavorable changes in the rumen microbiome. However, further functional annotation of rumen microbiota is needed to confirm this. Strategies that target each source of variation can be conducted in parallel to optimize reduction in methane production from dairy cows.

Introduction

Methane (CH_4) is a potent greenhouse gas (GHG) with a climate change potential ~32 times greater than carbon dioxide (CO_2)[1] and an atmospheric half-life of 12 years, which is substantially shorter than CO_2 (> 100 years)[2]. Therefore, reducing CH_4 emissions from anthropogenic-related sources has been identified as a key area for mitigating climate change with immediate effects[2,3]. Livestock accounts for 14.5% of anthropogenic-related GHG emissions and enteric CH_4 emissions from ruminants accounts for 5.8%[3]. Furthermore, CH_4 emissions from livestock is predicted to markedly increase due to an expected doubling in the global milk and meat demand by 2050[4].

Ruminants, the most widespread livestock species, can digest a wide variety of high fiber feedstuffs due to the distinct microbiome in their rumen. Methane is a natural by-product of gastro-enteric fermentation of high fiber plant biomass by microbial enzymatic activity in the rumen [5]. Bacteria, protozoa, and fungi in the rumen produce CO_2 and hydrogen (H_2), which are converted to CH_4, primarily by archaea known as methanogens. Approximately 99% of CH_4 emitted from cattle is released in the breath by eructation and respiration[6]. The emission of CH_4 is also a crucial pathway for maintaining H_2 balance and ruminal pH, as the optimal conditions for anaerobic fermentation by the rumen microbial community is limited to a narrow range of partial pressure of H_2 and pH [7]. Hydrogenase-expressing bacteria convert metabolic hydrogen from anaerobic fermentation into H_2 which is then converted to CH_4 via methanogenesis [7]. Furthermore, emitted CH_4 has a caloric value and represents a 2–12% net loss of a cow's gross energy intake[8,9]. Consequently, cattle and other ruminants with increased efficiency to digest high fiber feedstuffs but reduced CH_4 production could in principal benefit the global climate and concurrently improve the profitability and sustainability of cattle production.

Mitigation to decrease CH_4 production by cattle to date has been largely unsuccessful, as the available measures are temporary and not cumulative. Large international research approaches target the rumen microbial communities through feed additives (chemical or biological), feed formulations, and anti-methanogen vaccines[10]. However, rumen microbial species rapid adaptation to changes in the substrate results in resistance to treatments and CH_4 production returns to pre-treatment levels[11]. Conversely, rumen transplantation studies (transfaunation) show that the rumen bacterial community recovered to near pre-transfaunation composition after a short period of time[12]. This indicated the existence of a degree of host influence on rumen microbial composition[12]. Host genotype in cattle was reported to

explain inter-animal differences in CH_4 production[13,14] and the rumen microbial community influenced CH_4 production[15]. However, empirical evidence linking the host's genetic influence over the rumen microbial community and CH_4 production is rather limited[15].

A promising strategy is genetic selection for low CH_4 emitting cows, as it is sustainable, persistent, and cumulative over subsequent generations. Whether the host influences the rumen microbial community, and consequently CH_4 production, or the two interact to affect CH_4 production is currently unknown. If reduced CH_4 production in cows is a consequence of poor symbiosis with rumen microbes and thus fiber digestibility, there is a risk selection for reduced CH_4 production will act against the very symbiosis which has aided ruminants and rumen microbes' coexistence. Thus, the extent to which the rumen microbiome is under the host genetic influence needs elucidation. If host genetics impose a strong influence on rumen microbial composition, traits influenced by rumen microbes could be improved by using rumen microbial composition as indicator traits in selection. However, should host genetics impose a strong influence on rumen microbial composition and selection for CH_4 production proceed without cognizance of rumen microbial composition, there is a risk of unfavorable correlated responses in rumen microbial composition.

We hypothesized that: 1) the relative composition of the microbiome in the rumen is heritable i.e. controlled by host genome and 2) variation in methane emission from rumen is influenced by both the cow genome and rumen microbial content.

Results

Variation in methane emission and its heritability in lactating dairy cattle

Methane concentration in the exhalation-breath of 750 lactating Holstein dairy cows from farmer herds in Denmark was measured individually during automated machine milking for one week. Within-week methane measurements had a high repeatability coefficient of 0.70 ± 0.02 (estimate $\pm$ SE). Estimated average daily methane emission was 395.8 ± 63.5 g/d (mean $\pm$ SD), which was consistent with reports from the literature[16]. Considerable variation in estimated CH_4 emission among cows was observed. The top 10% methane emitting cows (519.28 ± 28.5 g/d) had a 41% mean difference from the low 10% emitting cows (303.8 ± 11.9 g/d) (S1 Fig). Results from linear mixed model with pedigree records indicated methane emission was moderately heritable, 0.19 ± 0.09 (heritability coefficient, $h^2 \pm$ S.E), which was consistent with previous findings in lactating Holstein cows in Denmark [13].

Rumen bacterial and archaeal community composition

We identified 3,894 bacterial operational taxonomic units (OTUs, $\geq$ 97% identity) and 189 archaeal OTUs, which were present in a minimum of 50% of the cow samples (50% threshold maximizes the variation in a binary trait i.e. presence or absence). Taxonomic classification revealed generic bacterial and archaeal composition. The predominant bacterial phylum found was *Bacteroidetes* 72.2% $\pm$ 6.5 (mean $\pm$ SD), followed by *Firmicutes* (18.3% $\pm$ 5.6) and *Tenericutes* (2.8% $\pm$ 1.0). *Absconditabacteria*, *Spirochaetes*, *Fibrobacteres*, and *Proteobacteria* each comprised less than 2%, and another 20 phyla constituted 1% of all sequence reads.

The archaeal community was dominated by two families, *Methanobacteriaceae* and *Methanomassiliicoccaceae* (35% $\pm$ 22.1) of the orders *Methanobacteriales* (64.2% $\pm$ 22.2; mean $\pm$ SD) and the recently proposed order *Methanomassiliicoccales* and class *Thermoplasmata*[17], respectively. The remaining archaeal community was comprised of 10 families, which were low in abundance, cumulatively accounting for less than 1% of all archaeal sequence reads.
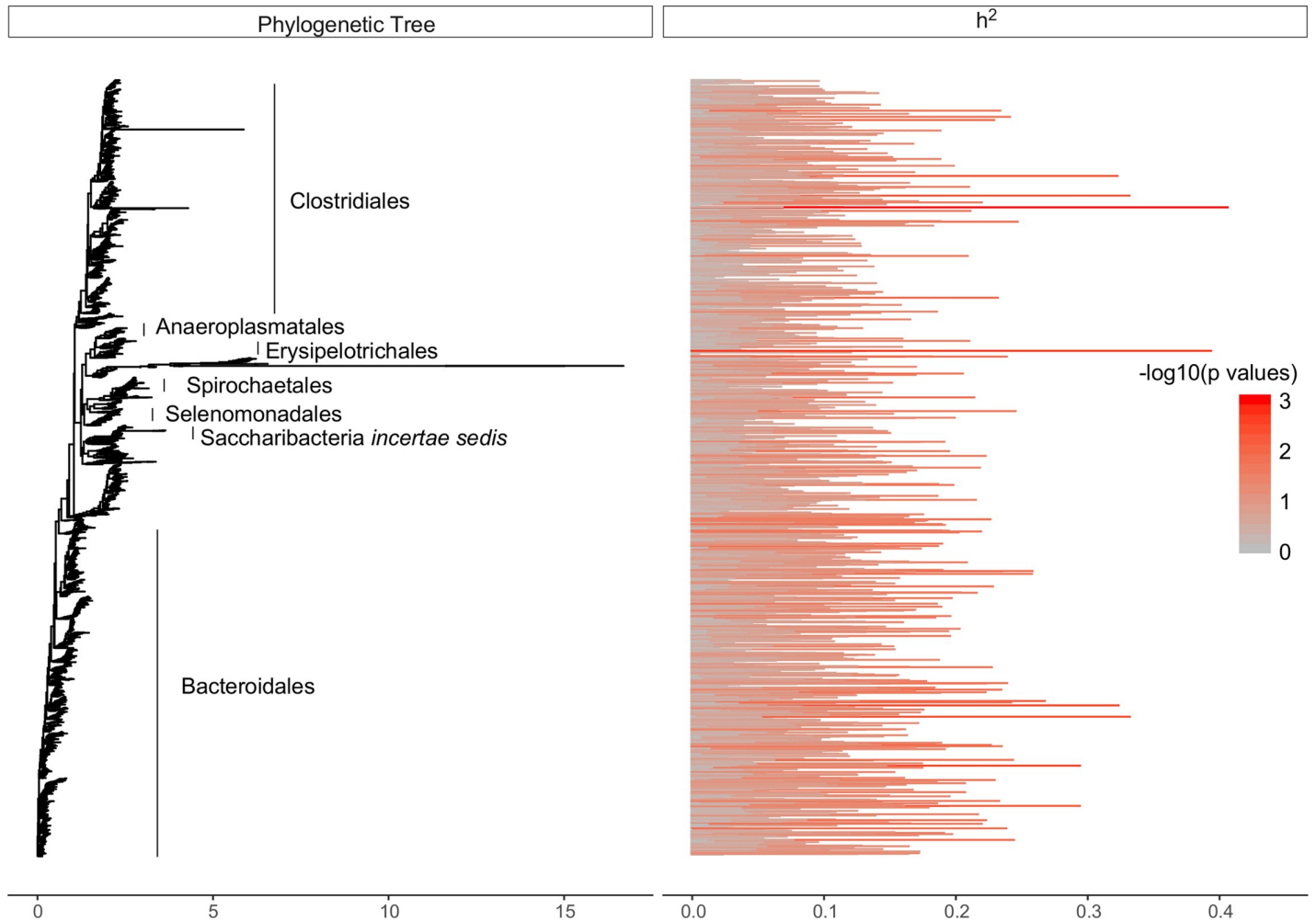
Fig 1. Phylogeny of 3,894 rumen bacterial OTU. Branch lengths represent substitution number per site calculated by FastTree2[81]. Heritability estimates (h^2) for each OTU abundance are plotted with a horizontal bar and colored by taxonomic group classification.

Additive genetic variance estimates of rumen microbiota

OTU abundance and OTU abundance collapsed at genus and family levels were used as microbial phenotypes. The heritability thereof was estimated using a linear mixed model with pedigree records (known as 'animal models'), which partitions total variance into additive genetic and environmental variance[18]. We calculated 95% confidence intervals for OTU h^2 estimates and found for 6% of bacterial and 12% of archaeal OTUs, the estimates were significantly higher than zero ($P < 0.05$), ranging from 16–44% (Fig 1) and 18–33% (Fig 2), respectively. Due to the high number of independent tests, we calculated false discovery rate (FDR) corrected P—values for h^2 estimates with a FDR threshold of 15% (S1 Table).

Heritability of bacterial and archaeal abundance was further estimated at the genus level. In total eight bacterial genera out of 144 showed significant h^2 estimates ranging from 0.17 to 0.25 (Table 1). Only a single archaeal genus, *Methanobrevibacter*, had a h^2 estimate significantly different from zero (0.22 ± 0.09). However, *Methanosphaera* and *Methanomicrococcus* might also be under host additive genetic control with heritability estimates approaching significance thresholds (Table 1).
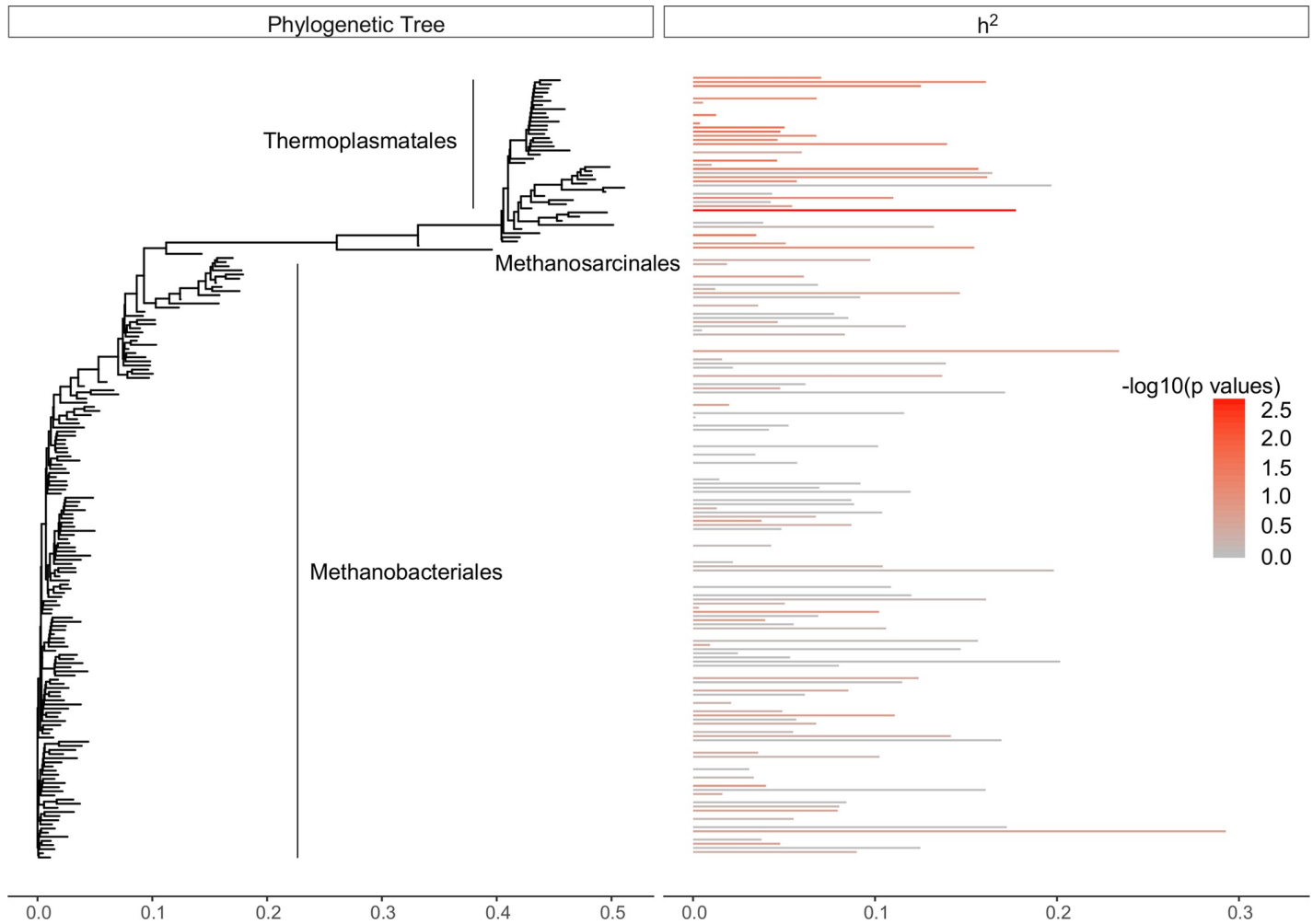
Fig 2. Phylogeny of 189 rumen archaeal OTU abundance. Branch lengths represent substitution number per site calculated by FastTree2[81]. Heritability estimates (h^2) for each OTU abundance is plotted with a horizontal bar and colored by taxonomic group classification.

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Table 1. Estimated heritability (h^2) and P-value for the relative abundances of bacterial and archaeal genera.

Genus	Relative abundance (%)	$h^2 \pm$ SE	95% Confidence Interval	P-value
Bacteria				
Paludibacter	0.01	0.25 ± 0.10	(0.05–0.45)	0.015
Unclassified *Spirochaetaceae*	0.01	0.25 ± 0.09	(0.04–0.41)	0.08
R4-45b	0.01	0.23 ± 0.09	(0.05–0.41)	0.014
F16	0.8	0.22 ± 0.09	(0.04–0.40)	0.018
Unclassified *Endomicrobia*	0.04	0.21 ± 0.09	(0.02–0.40)	0.027
Unclassified *Victivallaceae*	0.08	0.20 ± 0.09	(0.01–0.39)	0.36
Unclassified *Proteobacteria*	0.02	0.19 ± 0.09	(0.01–0.37)	0.042
Sporobacter	0.01	0.17 ± 0.08	(0.00–0.34)	0.046
Archaea				
Methanobrevibacter	55.8	0.22 ± 0.09	(0.04–0.42)	0.02
Methanosphaera	8.1	0.18 ± 0.10	(-0.00–0.36)	0.055
Methanomicrococcus	0.7	0.18 ± 0.09	(-0.02–0.38)	0.08

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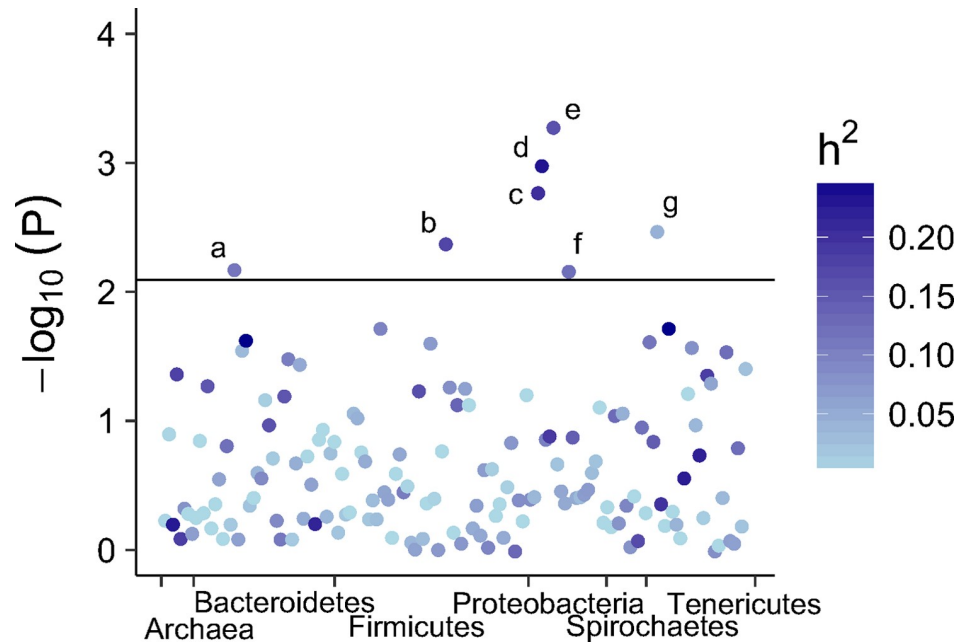
Fig 3. Manhattan plot of rumen bacterial and archaeal genera associations with methane emissions (g/day) colored by heritability (h^2) estimates. Color gradient indicates genera h^2 with light blue ($h^2 = 0$) ranging to dark blue ($h^2 = 0.30$). The y-axis is $-\log_{10}(P)$ for association tests. The horizontal line represents the Benjamini-Hochberg FDR 15% for multiple testing significance thresholds. Genera above the threshold are a) Unclassified *BS11* group; b) *Sporobacter*; c) Unclassified *Victivallaceae*; d) Unclassified *Lentisphaeria*; e) Unclassified *Alphaproteobacteria*; f) Unclassified *Rickettsiales*; and g) *Sphaerochaeta*.

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Association between microbiota abundance and methane production

Associations between relative bacterial and archaeal OTUs, genera abundance, and host CH_4 emissions were tested, while simultaneously controlling for environmental factors and familial structures common in livestock due to relatedness among study samples [19,20]. The OTU or genera log-transformed abundance present in > 50% of cows were fit as an explanatory variable in a linear mixed model for CH_4 production. Numerous significant OTUs were detected but failed to pass the threshold for multiple testing (FDR ≤ 0.15) (Supplementary Table 1). This was a hypothesis-generating analysis and not directed at specific hypothesis testing therefore we reported the significance and FDR corrected values (S1 Table). Seven genera in total were detected, which exceeded the significance threshold at FDR of 15%. The $-\log_{10}$ *P*-values are plotted in Fig 3.

Microbial community structure

Traditionally, dimension reduction techniques such as principal coordinates analysis (PCoA) are used to summarize community composition differences between individuals (Beta diversity) into clusters, which are further examined for associated biological or explanatory variables. Differences in bacterial and archaeal community structures were estimated for the entire sample population at OTU level using the Bray-Curtis[21] dissimilarity metric (PCoA, Fig 4A and 4B). Briefly, the Bray-Curtis dissimilarity is the sum of minimum counts of shared species in two animals divided by the sum of counts of all species in each animal, where 0 indicates the same composition and 1 indicates no shared composition. Analysis revealed clustering of cows into 'ruminotypes' for both bacterial and archaeal community composition, which both associated significantly with high and low CH_4 emitters at opposing polar regions (Mann-Whitney
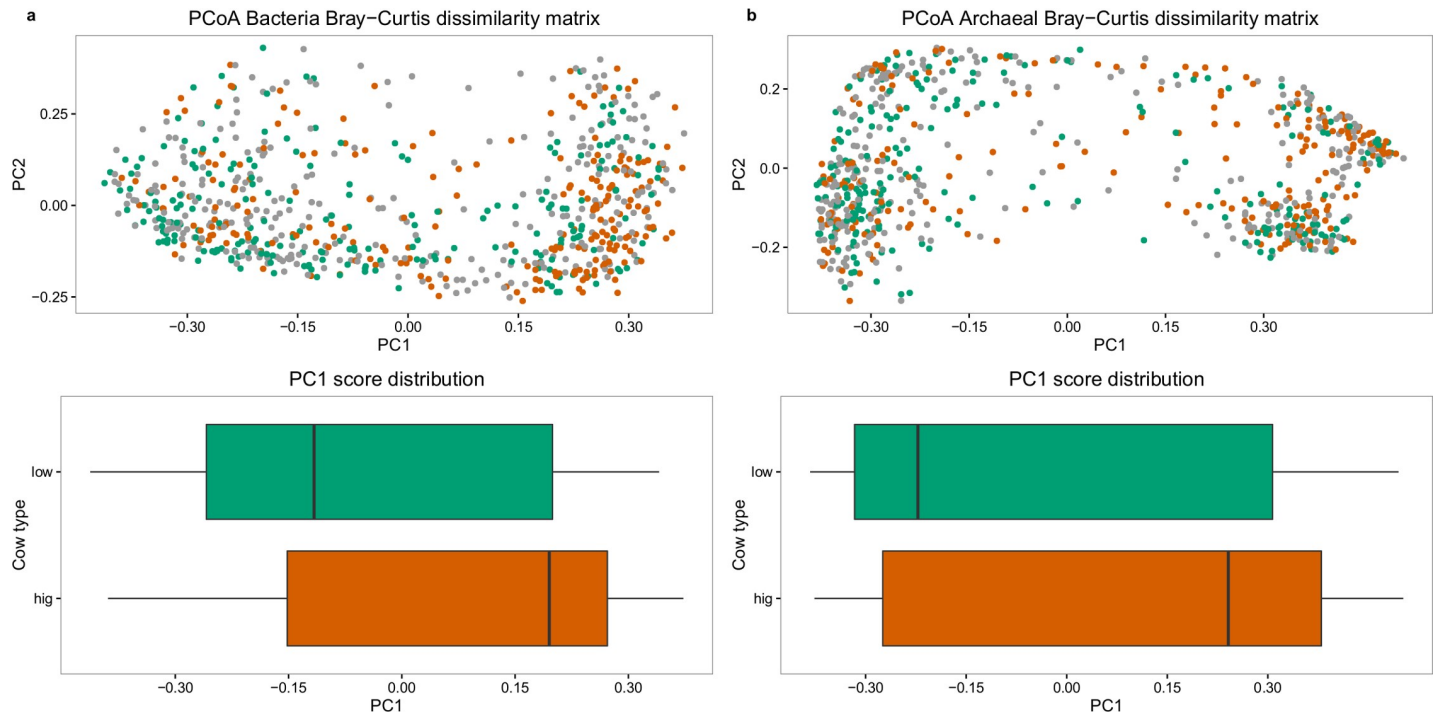
Fig 4. Principal coordinate analysis (PCoA) of rumen bacterial community diversity (a) and archaeal community diversity (b) based on 16S rRNA amplicon sequencing contrasting 10% highest methane emitters (orange), 10% lowest methane emitters (blue), and 80% intermediate emitters (grey). Distribution of high and low emitters along PCo1 showed significant differences ($P < 0.001$) for both figures.

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test, $P < 0.001$) but failed to cluster distinctly from the intermediate CH_4 emitters. Analysis of community structures using ANOVA revealed bacterial PCo1 was partly explained by non-genetic factors: parity (i.e. lactation number) (3.6%), sequencing batch (2%) and lactation stage (1%). A genetic analysis controlling for these factors showed PCo1 was likely heritable (0.20 ± 0.10) and thus influenced by the host additive genetics. Bacterial PCo2 was partly explained by the herd of origin ($< 1\%$) and parity ($< 1\%$) and was not heritable (0.02 ± 0.05). Similar findings were observed for archaea, with the variation in PCo1 partly explained by herd ($< 1\%$), parity (19.9%), sequencing batch (5%) and lactation stage ($< 1\%$). The genetic analysis controlling for these factors exhibited moderate heritability (0.39 ± 0.05). Archaeal PCo2 variation was partly explained by herd ($< 1\%$) and parity ($< 1\%$), which were likely not heritable (0.05 ± 0.05).

Variation in methane emission attributed to cows' additive genetics and rumen microbiome

The relative proportion of variation in CH_4 emissions due to rumen microbial composition and host additive genetic components was estimated individually and jointly using linear mixed models. Likelihood ratio tests revealed that fitting either random effect of rumen microbial composition or individual cow's polygenic component fitted the data significantly better than the null model i.e. including only fixed effects ($P < 0.001$). The model fitting both random effects (microbial composition and polygenic component) was significantly better ($P < 0.001$) than models including only one random effect. The proportion of variance in CH_4 production explained by the microbiome, here defined as microbiability (m^2), was calculated in analogy to the heritability (h^2)[22,23]. The contrast between the two intra-class correlation

coefficients h^2 and m^2 with their respective standard errors for all models are depicted in Fig 5. The m^2 of CH_4 emission estimated individually was 0.15 ± 0.08 (estimate ± S.E) and the h^2 estimated individually was 0.19 ± 0.09. Simultaneous estimates of both effects indicated slightly lower microbiability (0.13 ± 0.08), whereas h^2 exhibited a corresponding increase (0.21 ± 0.09) as compared to the preceding models fitting only one of the random effects. The combined microbial abundance and additive genetic effects were responsible for ~ 34% of the total phenotypic variation in CH_4 emissions.

Discussion

Additive genetic variation in methane emissions and bacterial and archaeal taxa abundance

The results of this study show that estimated CH_4 emissions from a dairy cow were partially under the influence of host (cow's) additive genetics, which explained 19% of the total variation. Of the rumen bacterial OTUs, a modest ~ 6% were associated with host additive genetics exhibiting significant heritability estimates (16–44%) (Fig 1). Similarly, only ~ 12% of archaeal OTU abundance was influenced by host additive genetics, with heritability estimates ranging from 18–33% (Fig 2). However, bacterial and archaeal heritability estimates failed to pass the threshold for multiple testing. Our test was conservative as a large number of taxa were analyzed with many OTUs having little or no influence by the host genome. Studies with larger sample sizes would give more reliable estimates of the heritabilities, especially for lower heritable OTUs. The h^2 estimates observed in this study were consistent with findings of intestinal microbiota in mice[24,25] and humans[26,27] and confirm that the majority of variation in rumen microbial abundance is due to factors other than host additive genetics [28]. Interestingly, the patterns of h^2 with phylogeny differed between the bacteria and the archaea (Fig 1 and Fig 2). Heritable OTUs were distributed throughout the bacterial microbiome whereas archaea showed increased heritability within the *Thermoplasmatales*. This highlights the value of collating phylogeny with heritability estimates to focus research into possible mechanisms which predispose differential relative abundance of certain taxa across genetically related cows. The method employed to sample rumen contents is high-throughput and less invasive
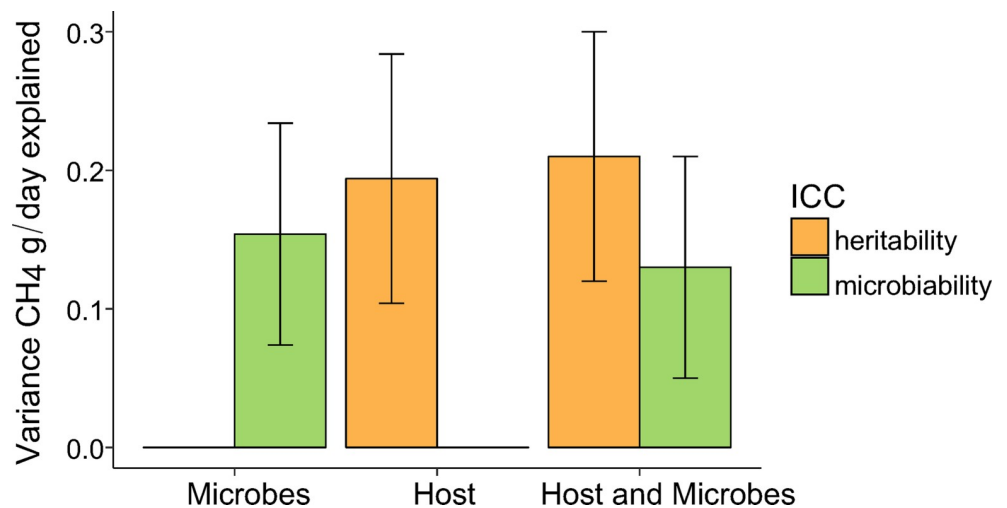

Fig 5. Proportion of variance in CH4 explained by different sources (Intra-class correlation coefficients, ICC) due to additive genetic effects (heritability) and rumen microbe content (microbiability), with respective standard errors when fit separately or jointly.

than surgical procedures, making it better suited to sampling large numbers of cows under commercial farm conditions. Large sample size is critical in genetic evaluations. However, it is important to note that the floral rumen scoop is inserted into an undefined portion of the rumen and likely samples the liquid phase. Recognizing that rumen microbial communities differ between liquid, solid and epimural phases[29], studies testing the repeatability and representativeness of sampling are needed.

Associating rumen microbial taxa abundance with CH_4 emissions

We utilized linear mixed model analysis to test for associations between bacterial and archaeal OTUs, genera and families with estimated CH_4 emissions, while concurrently accounting for effects such as parity, lactation stage, herd of origin and familial structure from the pedigree. Several bacterial genera associated with CH_4 emission were detected. Out of these, four were found either to be affected by methane inhibitors or related to H_2 production and other methanogenesis substrates. Three were moderately heritable (0.17–0.25) (S1 Table). One of the identified bacteria, *Sporobacter*, with a mean relative abundance of 0.01% (*Ruminococcaceae*, *Clostridiales*, *Firmicutes*), belongs to a group with only a single cultured representative, *Sporobacter termitidis*, isolated from the intestine of wood-feeding termites (*Nasutitemes lujae*), also known for producing large amounts of CH_4. However, when this isolate was co-cultured with an archaea species, *Methanospirillum hungatei*, CH_4 was not produced. *S. termitidis* was found to generate acetate and methylsulfides, but not H_2 or CO_2, therefore interspecies H_2 transfer did not occur and facilitate CH_4 production[30]. The recent discovery and proposed archaeal order *Methanomassiliicoccales* species found to utilize methylsulfides and H_2 in methanogenesis[31], provides a possible mechanism for methylsulfide producers to contribute to CH_4 production when H_2 producers are present. *Methanomassilicoccales* was prevalent in our samples (mean relative abundance 35%); therefore, *Sporobacter* could potentially be contributing to CH_4 production via a similar pathway.

We also detected *Sphaerochaeta* with a mean relative abundance of 0.01%, associated with estimated CH_4 production. Genomes from cultured *Sphaerochaeta* isolates revealed acetate, formate, ethanol, H_2, and CO_2 were potential fermentation end products[32], many of which are methanogenic archaea substrates[33]. Furthermore, seed extracts from *Perilla frutescens* (*Lamiaceae*), a medicinal herb, decreased CH_4 production *in vitro* from rumen samples of lactating dairy cows and decreased *Sphaerochaeta* abundance[34]. Interestingly, Caro-Quintaro et al.[32] reported up to 40% of the genes from *Spaerochaeta* species were exchanged with members of *Clostridiales* (*Firmicutes*) and this inter-order-species horizontal gene transfer was most extensive in mesophilic anaerobic bacteria, such as the conditions found in termite and ruminant guts[35]. Here 16S rRNA gene sequencing is used as a proxy for metabolic activity but cannot account for inter-order-species horizontal gene transfer. Therefore, full metagenome sequence may have an advantage over the 16S rRNA gene to describe rumen microbial contents.

One bacterial genus detected in the present study, which is positively associated with estimated CH_4 production, is classified in the yet uncultured BS11 gut group of the *Bacteroidales* (mean relative abundance 1.4%). The relative abundance of the BS11 group reportedly decreased concomitantly with CH_4 production by dietary methanogenic inhibitors, such as *P. frutescens* seed extract, mentioned previously[34], monesin and essential oil supplementation in dairy cattle[36,37], and bromochloromethane in Japanese goats[38]. Thus, supporting our finding of a positive association between BS11 and CH_4 production. Solden et al.[39] employed metagenomics sequencing and shotgun proteomics approaches to phylogenetically and metabolically resolve the BS11 gut group. They resolved two genera within the group and

both exhibited multiple pathways to ferment hemicellulose, a capability previously unknown for BS11. The resulting fermentation end products included acetate, butyrate, propionate, CO_2, H_2[39] the latter two being methanogenesis substrates. Genes encoding 'fucose sensing' pathways were found for only one of the proposed BS11 genera, offering a possible mechanism for interaction between genes in the BS11 group and the host[15]. However, further studies are needed to elucidate the links between CH_4 inhibitors, host genes and CH_4 production.

Due to the absence of cultured rumen bacteria isolates, an understanding of the metabolic function in many bacterial genera remains in its infancy. However, from the isolates discussed above, results suggested CH_4 emissions depend on abundance of bacterial taxa that produce substrates for methanogenesis, such as H_2. Remarkably, associations between archaeal relative abundance and estimated CH_4 production were not detected in the present study, despite the knowledge that archaea are directly responsible for CH_4 production. A meta-transcriptome study in sheep found archaeal transcription pathways and not simply abundance, contributed to inter-animal differences in CH_4 production[40]. This study was congruent with conclusions reached in two recent reviews, which examined results from dairy cattle and other ruminant studies employing 16S rRNA[41] and 'meta-omics' approaches[42], where bacteria abundance produced and utilized H_2 or stabilized pH, which affected CH_4 emissions and feed efficiency and archaeal activity matched substrate availability.

Associating rumen community structure with methane emissions

The combined effects of the bacterial and archaeal community structure (beta diversity) on estimated CH_4 emissions were investigated by conducting PCoA on the archaeal and bacterial communities, which revealed 2–3 clusters for archaea (Fig 4A) and two clusters for bacteria (Fig 4B). Beta diversity is a non-parametric distance measure used in microbiology and ecology to assess the differences between environments or samples (in this case cows) as opposed to alpha diversity which takes into account the diversity within cows. Clusters of a similar nature were first reported in intestinal bacterial community types in humans[43,44], chimpanzees[45], mice[46] and pigs[47], referred to as "enterotypes", and found associated with specific host phenotypes. This concept was extended to sheep rumen bacterial communities and referred to as "ruminotypes"[48]. The ruminotypes observed herein followed a continuous gradient and did not form discrete clusters, which is consistent with the latest findings in microbiome stratification. [49]. Importantly, we found that animal and farm factors like herd of origin, parity and lactation stage, as well as technical factors, i.e. sequencing batch, contributed to the observed variation and stratification in ruminotypes. Similar findings were reported in rumen bacterial richness at different lactation stages and over different parities [50], suggesting later parities (higher parity cows are older) decreased bacterial richness and increased production[51]. We detected a moderate heritable genetic component acting along PCo1 axis, with h^2 of 20% for bacterial and 39% for archaea, when controlling for lactation stage and parity, demonstrating the first evidence of host additive genetic influence on rumen bacterial and archaeal community structure (beta diversity). All the above-mentioned factors contribute to microbiome structure and associations with host phenotypes.

An association was detected between the highest and lowest CH_4 emitters and bacterial and archaeal ruminotypes along PCo1, however, ruminotype cluster memberships were not exclusive to high and low emitters. This suggested ruminal bacterial and archaeal community structure provided a modest contribution to CH_4 emission. Kittlemann et al.[48] surveyed microbial community composition in multiple sheep cohorts with low and high CH_4 yield (methane emission per kg dry matter intake, CH4/DMI). A ruminotype "S" associated with low CH_4 yield and enriched with *Sharpea azabuensis* was reported. A follow up study in sheep

also found low CH_4 yielding sheep to be associated with ruminotype "S", enriched with *Sharpea* spp. It was hypothesized a smaller rumen size and higher turnover rate promoted faster growing bacteria, such as *Sharpea*, which favor hetero-fermentative growth on soluble sugars, resulting in lower H_2 production and subsequently decreased CH_4 formation by hydrogenotrophic methanogens[52]. Smuts et al.[53] reported passage rate (and consequently turnover rate) in sheep was heritable, indicating a possible mechanism for host genetics to influence ruminotypes. Methane emission phenotypes differed between the sheep and the present study. Kittlemann et al.[48] assessed the amount of CH_4 production per unit of DMI but not CH_4 production directly. DMI measurements are not currently recorded on dairy cattle under commercial farms due to the high costs and therefore, CH_4 emissions in the present study could not be corrected for feed intake. In light of the differences in phenotype definitions and similarities in ruminotypes between studies, it would be of interest in future work to obtain DMI records on cows and test if the ruminotypes observed show an increased relationship with CH_4 yield. The heritability estimates for PCo1 and PCo2 indicates these measures could potentially be used as indicator traits in genetic selection should they be highly correlated to a trait of interest, however PCo1 and PCo2 (beta diversity) does not account for the total rumen microbial variation within and between individuals.

The method employed to measure CH_4 production in the present study is high throughput and non-invasive, making it practically viable for measuring large numbers of animals under commercial farm conditions. However, the cost trade off of this method is that it makes use of milk yield and body weight in the estimation of CH_4 production. Validation of this method with the 'gold standard method' (climate respiration chambers) has yielded highly correlated (r = 0.8–0.89) and concordant (concordance correlation coefficient = 0.84) results in dairy cattle [54,55]. However, the effects of body weight and milk yield on estimation of CH_4 cannot be discounted and further research into the relationships between these variables and the rumen microbiome would be of value.

Quantifying variation in CH_4 due to cow additive genetic effects and rumen microbiome

In this study, we quantified the combined effects of all rumen bacterial and archaeal OTUs simultaneously on estimated host CH_4 emissions using a microbial relationship matrix among cows. This is a parametric approach similar to assessing both alpha and beta diversity, as total rumen microbial variation within and between individuals is taken into account simultaneously. We expressed the combined effects as the variance ratio due to microbial composition to the total variance in estimated CH_4 emissions (m^2, microbiability), an analogy to h^2. Estimated CH_4 emissions had 15% m^2, indicating the combined rumen bacteria and archaea abundance of dairy cattle was associated with a considerable amount of variation in estimated CH_4 emissions among animals. Ross et al.[56] first proposed the generation of metagenomic relationship matrices in dairy cattle and reported a CH_4 emission prediction accuracy of 0.47, explaining 22% of the total variation in CH_4 production [57]. However, Ross et al. [57] did not have sufficient data to estimate h^2 or microbiability (m^2) in CH_4 production. A study with 207 pigs employing 16S rRNA sequencing of gut microbes, found eight of the 49 bacterial genera to be heritable and estimated m^2 and h^2 for feed intake (m^2 = 0.16, h^2 = 0.11), daily gain (m^2 = 0.28, h^2 = 0.42) and feed conversion ratio (m^2 = 0.21, h^2 = 0.19) [23]. Only daily gain had higher h^2 compared with m^2. These findings suggest agreement with holobiont theory, where variation in the genome and microbiome can cause variation in some complex traits, on which artificial, natural selection and genetic drift can act [58,59]. However, the aforementioned study did not have adequate numbers of animals to estimate m^2 and h^2 simultaneously to

assess the relative interactions between additive genetics and the microbiome. Thus, it was unable to assess if host additive genetics co-influences the microbiome and variation in phenotypes.

In contrast, we estimated m^2 and h^2 concurrently to examine the shared information between the two effects. Microbiability of estimated CH_4 production decreased by two percentage points to 13% and h^2 exhibited a corresponding increase from 19 to 21%. This result indicated host genetic effects do interact with the microbial community composition but are not the primary mechanism for host genetic effects on estimated CH_4 emissions. A possible explanation for the negligible amount of shared influence between the two relationship matrices might be the small percentage of heritable bacterial and archaeal OTUs. This implies that the rumen bacterial and archaeal communities affected estimated host CH_4 emissions independently and host genetics influenced a small portion of these bacteria and archaea. The combined host additive genetics and rumen microbial community composition explained ~ 34% of the total variance in estimated CH_4 emissions in dairy cattle. Thus, breeding for low CH_4 production can be expected to result in limited correlated genetic responses to shape the rumen microbiome and breeding can likely proceed without taking cognizance of the rumen microbiome for this trait. However, larger studies estimating genetic correlations between rumen microbiota and CH_4 emissions and better functional annotation of rumen microbiota are needed to confirm this.

Microbiability estimates can be used as a tool for quantifying the cumulative effects of microbial abundance on phenotypes, e.g. complex diseases and quantitative traits. However, further research is required to elucidate the biological mechanisms shaping microbiability. For example, animal factors known to affect CH_4 production and rumen microbial populations, such as passage rates or individual differences in feed intake might influence microbiability estimates. Human intestinal microbiome studies find that numerous disease phenotypes are associated with microbial richness, species abundance, and microbial community structure [60,61]. Subsequent work using stool consistency and opaque markers as proxies for colonic transit time found all three metrics and disease phenotypes are partially confounded with colonic transit time[62,63]. Similarly, in sheep studies, low CH_4 yielding sheep are associated with lower retention time and smaller rumens[64], relationships with specific rumen microbial clusters[48] and different bacterial and archaeal species[52]. Therefore, studies are needed to determine if microbial differences among subjects associated with phenotypic differences are causative or are consequences of unknown extraneous factors. It is also necessary to clarify the mechanisms which allow rumen microbes to be passed on to successive generations, to assess the efficacy of perturbations of the rumen microbiome such as probiotics and rumen transplants aimed at desired changes to the rumen microbiome and associated changes in phenotypes[65]. Regardless of the underlying biology, quantifying the relative contribution of rumen microbes and additive genetics to complex phenotypes helps characterize whether the host genome and microbiome are acting jointly as a holobiont and highlights the merits of targeting microorganisms to achieve a specific change in a phenotype or selective breeding. Furthermore, providing additional information, such as relative abundance of rumen fungi and protozoa, or 'meta-omics', including meta-transcriptomics or meta-proteomics data can be readily adopted and incorporated into this methodology, offering insights into economically important livestock and disease traits in humans.

Conclusions

Methane production by dairy cows is not only influenced by factors such as feed intake and composition among others, but also the cow's individual genetic composition and rumen

microbial composition. Each cow's additive genetic effects influence a modest amount of variation in the abundance of a small percentage of rumen bacterial and archaeal taxa, and thereby contribute to variation in rumen microbiome composition and function. We detected associations between CH_4 emissions and rumen bacteria abundance, which are known to produce methanogenesis substrates, suggesting bacteria driven CH_4 production pathways. Although we detected a heritable component to ruminotypes, the association to CH_4 production was weak. Concurrently, host additive genetic effects and rumen microbes contributed to inter-animal differences in CH_4 production, however negligible interaction was observed between microbiability and heritability. Consequently, cow additive genetic effects on CH_4 emissions were largely unmodulated by cow additive genetic effects on rumen bacteria and archaea abundance. Strategies to reduce CH_4 emissions in ruminants can be optimized by a multifaceted approach, for instance, selective breeding to unlock host's genetic potential and strategies which may effect desired changes in the rumen microbiota like rumen transplantation, and probiotics.

Materials and methods

Experimental design

Methane emissions from 750 lactating Holstein cows in five commercial herds were recorded using a portable Fourier Transform Infrared unit (FTIR; Gasmet DX-4000, Gasmet Technologies, Helsinki, Finland)[13,66] and one research herd using a permanently installed non-dispersive infrared (NDIR; Guardian NG/Gascard Edinburgh Instruments Ltd., Livingston, UK) [67]. Briefly, the FTIR and NDIR equipment were installed within the feed bins of automated milking systems (AMS) in each commercial herd with the FTIR for seven consecutive days and the NDIR were permanently placed in the research herd. The FTIR and NDIR device inlets were installed in the AMS feed bins and methane (CH_4) and carbon dioxide (CO_2) gas concentrations (ppm) sampled continuously every 5 s and 1 s, respectively[66,67]. Cows were milked individually in the AMS and milked on average (18.2 ± 3.4) times during the seven-day period, for durations ranging from five minutes to 12.2 minutes. Mean CH_4 and CO_2 gas concentrations were corrected for environmental factors, including diurnal variation and day to day differences using a linear mixed model following Difford et al.[67] to approximate daily averages. Measurement stability was assessed by model repeatability and used as data quality control. 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 given during milking. Although all commercial herds employed a standardized TMR recipe, ingredient-specific differences among farms were expected to contribute to differences in TMR dietary values over herds.

Weekly mean values for milk yield and body weight were combined with weekly gas concentrations, as described in Lassen et al. [66] and applied to predict cow heat production[68]. During each week of CH_4 and CO_2 recording at different herds, milk samples were collected to estimate milk fat and protein percentages. Cow fat and protein corrected milk yield (FPCM) was estimated following the national recording scheme (RYK, Skejby, Denmark)[69]. Methane production (L/day) was estimated using the CH_4 to CO_2 ratio and predicted CO_2 emission[70] from the conversion of cow heat production units to CO_2 production, following Madsen et al. [71] and then converted to (g/d) using CH_4 density at standard temperature and pressure.

Holstein cow pedigree records were traced in the Danish national database (NAV, Skejby, Denmark) as far back as 1926 to construct a pedigree-based relationship matrix for the quantitative genetic analysis.

Sampling rumen liquid fraction

Immediately following the CH_4 recording period, rumen content samples were drawn from individual cows by oral insertion of the probe "Flora Rumen Scoop" [72]. Approximately 40 mL of the liquid fraction containing particulate matter was drawn from the rumen using this method. Trained technicians conducted the sampling to ensure correct probe insertion into the rumen following a previously established protocol [72], recognizing that the location of the flora rumen scoop may differ somewhat from sampling to sampling. The entire "Flora Rumen Scoop" was rinsed vigorously between animal sampling to minimize cross-contamination. Samples were labeled, immediately placed on ice, and transferred to the laboratory within two hours for further processing. Each 40 mL sample was mixed vigorously, a subsample of 1.2 mL rumen fluid was collected, and transferred to a 1.5 mL vial, then snap frozen in liquid nitrogen, before storing at -80°C, until shipped on dry ice to a commercial sequencing company (GATC Biotech, Constance, Germany) for analysis.

DNA extraction, bacterial and archaeal 16S rRNA gene amplification, and sequencing

DNA extraction, sequencing library construction and sequencing were conducted by GATC Biotech (Constance, Germany). Rumen samples were defrosted at 4°C overnight and vortexed until homogenous. A representative sample (500 μl) containing rumen liquid and solids was used for DNA isolation using the Qiagen QIAamp stool kit (Valencia, United States of America) following the manufacturer's instructions, modified for the larger sample size[73].

Two primer sets were used to create 16S rRNA libraries, one set for all bacteria and one set for all archaea. Universal bacterial 16S rRNA gene primers (covering the V1-V3 variable regions) 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 534R: 5'-ATTACCGCGGCTGCTG G-3' were used to generate the bacterial amplicon libraries (expected amplicon size 508 bp) [74]. Universal archaeal 16S rRNA gene primers (covering the V4-V6 variable regions) S-D-Arch-0519-a-S-15 5'-CAGCMGCCGCGGTAA-3' and S-D-Arch-1041-a-A-18 5'-GGCC ATGCACCWCCTCTC-3' were used to generate the archaeal amplicon libraries (expected amplicon size 542 bp)[75]. Following protocols standardized by GATC Biotech, PCR amplifications were conducted with GoTaq Green polymerase (Promega, Madison, USA) with 30 PCR cycles and a 60°C annealing temperature for the archaeal amplicon libraries and 25 PCR cycles with a 60°C annealing temperature for the bacterial amplicon libraries. The 16S rRNA amplicons were purified using the Axyprep Fragment Select bead purification system (Axygen Biosciences, New York, USA), according to the manufacturer's instructions. The size and purity of the PCR product was verified on a Fragment Analyzer using a High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies, Ankeny, USA). Multiplex indices and Illumina overhang adapters were added to both amplicon libraries in a second PCR amplification round (six cycles), followed by Fragment Analyzer analysis to confirm the correct size of the amplicons (Advanced Analytical Technologies, Ankeny, USA). Ninety-six libraries were pooled in equimolar concentrations and sequenced with an Illumina sequencing instrument using the 300 bp paired-end read mode, according to the manufacturer's specifications. Approximately half the samples were run using the illumina MiSeq platform and half with the HiSeq platform. The 300 bp paired end protocol was adapted to HiSeq by GATC Biotech. The specific samples entered into sequencing batches within each sequencing platform were recorded for subsequent significance testing to examine possible differences between sequencing batches and sequencing platforms in statistical analyses.

Bacterial and archaeal 16S rRNA gene sequence processing and OTU table construction

Bacterial and archaeal sequence reads underwent quality control, processing and were clustered into operational taxonomic units (OTUs) using the LotuS pipeline[76] with the following options: Sequence truncation length and minimum sequence length after barcode and primer removal was 230 bp. Minimum average sequence quality score was 27, the maximum number of ambiguous bases was 0, maximum homonucleotide run was set to 8. Sequences were filtered away if any of the 50 bp segments in a sequence had average scores below 25 or if the expected number of errors exceeded 2.5 in the binomial error model. The low-quality sequence ends were trimmed by applying a sliding window quality filter with a width of 20 bp and a minimum average quality score within the window of 25. Sequences were truncated if the probabilistic accumulated error exceeded 0.75. The reads were de-replicated and sequences with a minimum of 10 replicates were retained for OTU clustering within the Lotus pipeline. Sequence pairs were merged with Flash[77] and clustered into OTUs based on sequence similarity (97%) with UPARSE[78] and chimeric sequences removed with UCHIME reference-based chimera detection[79]. Representative sequences from each OTU were aligned with ClustalO[80] and a phylogenetic tree built with FastTree2[81]. Representative sequences, the OTU table, and phylogenetic trees were transferred to QIIME (version 1.9.0)[82], where further analyses were performed. Taxonomy was assigned to each OTU using the RDP classifier with a confidence level of 0.8[83] using greengenes (gg_13_8_otus) as the reference database. Unclassified OTUs and OTUs classified to non-target kingdoms were filtered from the OTU tables, i.e. only OTUs classified as k_Bacteria were maintained for the bacterial primer set and similarly OTUs classified as k_Archaea maintained for the archaeal primer set. Finally, samples with < 50,000 sequences were removed and OTUs containing < 10 sequences were filtered out of the OTU table.

Statistical models

Additive genetic variance estimation.   The linear mixed model utilized to estimate additive genetic variance is as follows:

$$y_{ijkl} = \mu + h_j + p_k + b_1(dim_l) + b_2(e^{-0.065 \ x \ diml}) + a_i + e_{ijkl} \tag{1}$$

where y_{ijklm} is the observed phenotype, e.g. methane emission in grams/day; μ is the model intercept; h_j is the herd fixed effect (j = 6 levels); p_k is the parity fixed effect (k = 4 levels); b_1 is days in milk fixed regression coefficient (dim l = 1–350); and b_2 is the Wilmink term fixed regression coefficient generated on dim to account for non-linearity in early lactation [84]. Term a_i is individual animal random additive genetic effects $\sim$ NID(0, $\mathbf{A}\sigma^2_a$), where σ^2_a is the additive genetic variance and $\mathbf{A}$ is the pedigree derived numerator relationship matrix (i = 750 animals); and e_{ijkl} is the random residual $\sim$ NID(0, σ^2_e), where σ^2_e is the error variance. The additive host genetic effects on relative rumen bacterial and archaeal abundance was estimated applying the same general equation as model 1 above, with the addition of the sequencing batch fixed effects nested within the sequencing platforms (11 levels). The analyses were performed using the DMU software[85].

Rumen microbial variance estimation.   The relationship among cows based on their similarity in rumen microbiome composition was estimated by constructing a microbial relationship matrix (**M**) inspired by Ross et al.[56], where a metagenomic relationship matrix was created from a vector of aligned rumen microbial contig sequences. The matrix was computed as a variance-covariance matrix from rumen bacterial and archaeal abundance as follows:

$$M = \frac{XX'}{n} \tag{2}$$

where $\mathbf{X}$ is the matrix of natural log transformed bacterial and archaeal relative abundance for all animals and n is the number of bacterial and archaeal OTUs within the population. Matrix $\mathbf{X}$ is derived from OTU tables after filtering out OTUs, which were absent from more than 50% of the samples and were homogeneous. The matrix $\mathbf{X}$ was subsequently scaled and centered within sequencing instrument (Miseq or Hiseq) to account for differences between instruments and recombined into a single matrix prior to the calculation of $\mathbf{M}$.

The variance explained by microbial composition was estimated employing models similar to Eq 1, where the random effect of m_i was fit separately and jointly with a_i, i.e. random additive genetic effects. Term m_i is the rumen microbial effect for the ith animal $\sim$ NID(0,$\mathbf{M}\sigma^2_m$), where σ^2_m is the rumen microbial variance and $\mathbf{M}$ is the microbial relationship matrix, described in (2), i = 750 animals.

Association between rumen bacterial and archaeal OTU's and host methane emission. The association between the relative abundance of each bacterial and archaeal OTU abundance with host methane production was conducted using linear mixed model analyses as proposed by Yu et al.[20], with the exception that OTU effects were estimated in place of allele substitution effects for genetic variants, as performed in genetic association analysis. The significance threshold was calculated using a Benjamini Hochberg false discovery rate correction for multiple testing. There were 189 archaeal and 3894 bacterial OTUs tested, and the microbiome wide significant threshold at FDR of 15% was in $-\log_{10}(P)$ scale 2.17.

Microbial community analysis. A principal coordinate analysis (PoCA) was conducted to investigate similarities or dissimilarities using a distance matrix from the archaeal and bacterial rumen community composition. The Bray-Curtis coefficient was employed separately for the archaeal and bacterial OTU tables to create sample-summary matrices, which were further explored using non-metric multidimensional scaling (NMDS)[21]. The effects of environmental and genetic parameter effects on community structures were evaluated using the following model:

$$y_{ijkl} = \mu + sb_j + p_k + b_1(dim_l) + b_2(e^{-0.065 \ x \ diml}) + a_i + e_{ijkl} \tag{3}$$

where y_{ijklm} is the observed phenotype, e.g. PCoA 1 and PCoA2 for bacteria or archaea; μ is the model intercept; sb_j is the sequencing batch run fixed effect nested within the sequencing platform (j = 11 levels); p_k, b_1, dim_l, b_2, a_i, and e_{ijkl} are as described in Eq (1). Additive genetic effects of host could not be detected from the bacterial and archaeal community structures for PCoA 2 and only herd environmental effects were significant. The distribution of *a priori* defined high and low emitters along PCoA1 was tested for bacterial and archaeal community structures, respectively by means of Mann-Whitney tests.

Ethics statement

All handling of animals were conducted according to 'Metagenomics in Dairy Cows' protocol. The protocol and study were approved by The Animal Experiments Inspectorate, Danish Veterinary and Food Administration, Ministry of Environment and Food of Denmark (Approval number 2016-15-0201-00959).

Supporting information

S1 Fig. Methane (CH$_4$) (g/day) measurements from 750 Holstein cows sorted by phenotypes corrected for environmental effects. The 10% highest CH$_4$ emitters (red), 10% lowest CH$_4$ emitters (green), and medium CH$_4$ emitters (grey). *P*-value indicates significant differences between high and low CH$_4$ emitters.
(TIFF)

S2 Fig. Methane (CH$_4$) (g/day) measurements from 750 Holstein cows against Energy Corrected Milk yield (ECM) (kg). The 10% highest CH$_4$ emitters (red), 10% lowest CH$_4$ emitters (green), and medium CH$_4$ emitters (grey).
(TIFF)

S3 Fig. Methane (CH$_4$) (g/day) measurements from 750 Holstein cows against Body Weight (kg). The 10% highest CH$_4$ emitters (red), 10% lowest CH$_4$ emitters (green), and medium CH$_4$ emitters (grey).
(TIFF)

S1 Table. Heritability estimates and effect of abundance of rumen archaea and bacteria at taxonomic levels level on Methane (CH4 g/day).
(XLSX)

S2 Table. Bacterial OTU table of counts with prevalence in more than 50% of samples (Bac_OTU).
(TXT)

S3 Table. Archaeal OTU table of counts with prevalence in more than 50% of samples (Arc_OTU).
(TXT)

S4 Table. Individual cow phenotypes and metadata.
(TXT)

Acknowledgments

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References

1. Holmes CD, Prather MJ, Søvde OA, Myhre G. Future methane, hydroxyl, and their uncertainties: Key climate and emission parameters for future predictions. Atmos Chem Phys. 2013; 13(1):285–302.

2. IPCC. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Core Writing Team, R.K. Pachauri and L.A. Meyer. 2014. 151 p.

3. Gerber P., Steinfeld H, Henderson B, Mottet A, Opio C, Dijkman J, et al. Tackling climate change through livestock–A global assessment of emissions and mitigation opportunities. Vol. 14, Food and Agriculture Organization of the United Nations (FAO), Rome. 2013.

4. Alexandratos N, Bruinsma J, Boedeker G, Schmidhuber J, Broca S, Shetty P, et al. World agriculture: towards 2030/2050 Prospects for food, nutrition, agriculture and major commodity groups. 2006.

5. Hill J, McSweeney C, Wright A-DG, Bishop-Hurley G, Kalantar-zadeh K. Measuring methane production from ruminants. Trends Biotechnol [Internet]. Elsevier Ltd; 2016; 34(1):26–35. Available from: http://linkinghub.elsevier.com/retrieve/pii/S016677991500222X https://doi.org/10.1016/j.tibtech.2015.10.004 PMID: 26603286

6. Janssen PH, Kirs M. Structure of the archaeal community of the rumen. Appl Environ Microbiol. 2008; 74(12):3619–25. https://doi.org/10.1128/AEM.02812-07 PMID: 18424540

7. McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. Aust J Exp Agric. 2008; 48(1–2):7–13.

8. Johnson KA, Johnson DE. Methane emissions from cattle Methane Emissions from Cattle. Anim Sci J. 1995; 73:2483–92.

9. Yan T, Mayne CS, Gordon FG, Porter MG, Agnew RE, Patterson DC, et al. Mitigation of enteric methane emissions through improving efficiency of energy utilization and productivity in lactating dairy cows. J Dairy Sci [Internet]. Elsevier; 2010; 93(6):2630–8. Available from: https://doi.org/10.3168/jds.2009-2929 PMID: 20494172

10. Moss AR, Jouany J-P, Newbold J. Methane production by ruminants: its contribution to global warming (Review article). Ann Zootech [Internet]. 2000; 49(3):231–53. Available from: http://hal.archives-ouvertes.fr/docs/00/88/98/94/PDF/hal-00889894.pdf

11. Hristov AN, Oh J, Firkins JL, Dijkstra J, Kebreab E, Waghorn G, et al. SPECIAL TOPICS-Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J Anim Sci. 2013; 91(11):5045–69. https://doi.org/10.2527/jas.2013-6583 PMID: 24045497

12. Weimer PJ, Stevenson DM, Mantovani HC, Man SLC. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. J Dairy Sci [Internet]. Elsevier; 2010; 93(12):5902–12. Available from: https://doi.org/10.3168/jds.2010-3500 PMID: 21094763

13. Lassen J, Løvendahl P. Heritability estimates for enteric methane emissions from Holstein cattle measured using noninvasive methods. J Dairy Sci [Internet]. Elsevier; 2016; 99(3):1959–67. Available from: https://doi.org/10.3168/jds.2015-10012 PMID: 26805978

14. Donoghue KA, Bird-Gardiner T, Arthur PF, Herd RM, Hegarty RF. Genetic and phenotypic variance and covariance components for methane emission and postweaning traits in Angus cattle. J Anim Sci. 2016; 94(4):1438–45. https://doi.org/10.2527/jas.2015-0065 PMID: 27136003

15. Roehe R, Dewhurst R, Duthie C-A, Rooke J, McKain N, Ross D, et al. Bovine Host Genetic Variation Influences Rumen Microbial Methane Production with Best Selection Criterion for Low Methane Emitting and Efficiently Feed Converting Hosts based on Metagenomic Gene Abundance. PLoS Genet. 2016; 12(2):1–28.

16. Lassen J, Poulsen NA, Larsen MK, Buitenhuis AJ. Genetic and genomic relationship between methane production measured in breath and fatty acid content in milk samples from Danish Holsteins. Anim Prod Sci. 2016; 56(3):298–303.

17. Iino T, Tamaki H, Tamazawa S, Ueno Y, Ohkuma M, Suzuki K, et al. Candidatus Methanogranum caenicola: a Novel Methanogen from the Anaerobic Digested Sludge, and Proposal of Methanomassiliicoccaceae fam. nov. and Methanomassiliicoccales ord. nov., for a Methanogenic Lineage of the Class Thermoplasmata. Microbes Environ [Internet]. 2013; 28(2):244–50. Available from: http://jlc.jst.go.jp/DN/JST.JSTAGE/jsme2/ME12189?lang=en&from=CrossRef&type=abstract https://doi.org/10.1264/jsme2.ME12189 PMID: 23524372

18. Henderson CR. Best linear unbiased estimation and prediction under a selection model published by: international biometric society stable. Biometrics. 1975; 31(2):423–47. PMID: 1174616

19. Tsilimigras MCB, Fodor AA. Compositional Data Analysis of the Microbiome: Fundamentals, Tools, and Challenges. Ann Epidemiol [Internet]. Elsevier Inc; 2016; 26(5):330–5. Available from: http://www.sciencedirect.com/science/article/pii/S1047279716300722 https://doi.org/10.1016/j.annepidem.2016.03.002 PMID: 27255738

20. Yu J, Pressoir G, Briggs WH, Vroh Bi I, Yamasaki M, Doebley JF, et al. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat Genet. 2006; 38(2):203–8. https://doi.org/10.1038/ng1702 PMID: 16380716

21. Bray JR, Curtis JT. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecol Monogr. 1957 Feb; 27(4):325–49.

22. Difford GF, Lassen J, Løvendahl P. Genes and microbes, the next step in dairy cattle breeding. In: EAAP—67th Annual Meeting. 2016.

23. Camarinha-silva A, Maushammer M, Wellmann R, Vital M, Preuss S. Host genome influence on gut microbial composition and microbial prediction of complex traits in pigs. Genetics. 2017; 206 (July):1637–44. https://doi.org/10.1534/genetics.117.200782 PMID: 28468904

24. Org E, Parks BW, Joo JWJ, Emert B, Schwartzman W, Kang EY, et al. Genetic and environmental control of host-gut microbiota interactions. Genome Res. 2015; 25(10):1558–69. https://doi.org/10.1101/gr.194118.115 PMID: 26260972

25. Leamy LJ, Kelly S a, Nietfeldt J, Legge RM, Ma F, Hua K, et al. Host genetics and diet, but not immuno-globulin A expression, converge to shape compositional features of the gut microbiome in an advanced intercross population of mice. Genome Biol [Internet]. 2014; 15(12):552. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4290092&tool=pmcentrez&rendertype=abstract https://doi.org/10.1186/s13059-014-0552-6 PMID: 25516416

26. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell [Internet]. Elsevier Inc.; 2014; 159(4):789–99. Available from: https://doi.org/10.1016/j.cell.2014.09.053 PMID: 25417156

27. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic Determinants of the Gut Microbiome in UK Twins. Cell Host Microbe [Internet]. Elsevier Inc.; 2016; 19(5):731–43. Available from: https://doi.org/10.1016/j.chom.2016.04.017 PMID: 27173935

28. Rothschild D, Weissbrod O, Barkan E, Korem T, Zeevi D, Costea PI, et al. Environmental factors dominate over host genetics in shaping human gut microbiota composition. bioRxiv [Internet]. Nature Publishing Group; 2017; 555(7695):150540. Available from: https://www.biorxiv.org/content/early/2017/06/16/150540

29. Kong Y, Teather R, Forster R. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. FEMS Microbiol Ecol. 2010; 74(3):612–22. https://doi.org/10.1111/j.1574-6941.2010.00977.x PMID: 21044097

30. Grech-Mora I, Fardeau M-L, Patek BKC, Ollivier B, Rimbault A, Prensier G, et al. Isolation and Characterization of Sporobacter termitidis gen. nov., sp. nov., from the Digestive Tract of the Wood-Feeding Termite Nasutitermes lujae. Int J Syst Bacteriol. 1996; 46(2):512–8.

31. Borrel G, Parisot N, Harris HM, Peyretaillade E, Gaci N, Tottey W, et al. Comparative genomics highlights the unique biology of Methanomassiliicoccales, a Thermoplasmatales-related seventh order of methanogenic archaea that encodes pyrrolysine. BMC Genomics [Internet]. 2014; 15(1):679. Available from: http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-15-679

32. Caro-Quintero A, Ritalahti KM, Cusick KD, Löffler FE, Konstantinidis KT. The chimeric genome of sphaerochaeta: Nonspiral spirochetes that break with the prevalent dogma in spirochete biology. MBio. 2012; 3(3):1–9.

33. Knapp JR, Laur GL, Vadas P a, Weiss WP, Tricarico JM. Invited review: Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. J Dairy Sci [Internet]. Elsevier; 2014; 97(6):3231–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24746124 https://doi.org/10.3168/jds.2013-7234 PMID: 24746124

34. Wang J, Liu M, Wu Y, Wang L, Liu J, Jiang L, et al. Medicinal herbs as a potential strategy to decrease methane production by rumen microbiota: a systematic evaluation with a focus on Perilla frutescens seed extract. Appl Microbiol Biotechnol [Internet]. Applied Microbiology and Biotechnology; 2016; 100 (22):9757–71. Available from: https://doi.org/10.1007/s00253-016-7830-z PMID: 27660180

35. Caro-Quintero A, Konstantinidis KT. Inter-phylum HGT has shaped the metabolism of many mesophilic and anaerobic bacteria. ISME J [Internet]. Nature Publishing Group; 2014; 9(4):958–67. Available from: http://www.nature.com/doifinder/10.1038/ismej.2014.193

36. Boadi D, Benchaar C, Chiquette J, Massé D. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can J Anim Sci [Internet]. 2004; 84(3):319–35. Available from: http://pubs.aic.ca/doi/abs/10.4141/A03-109

37. Schären M, Drong C, Kiri K, Riede S, Gardener M, Meyer U, et al. Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. J Dairy Sci [Internet]. American Dairy Science Association; 2017; 100(4):2765–83. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030217301030 https://doi.org/10.3168/jds.2016-11994 PMID: 28161182

38. Denman SE, Fernandez GM, Shinkai T, Mitsumori M, McSweeney CS. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front Microbiol. 2015; 6(OCT):1–12.

39. Solden LM, Hoyt DW, Collins WB, Plank JE, Daly RA, Hildebrand E, et al. New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. ISME J [Internet]. Nature Publishing Group; 2016; 11(3):691–703. Available from: http://www.nature.com/doifinder/10.1038/ismej.2016.150 https://doi.org/10.1038/ismej.2016.150 PMID: 27959345

40. Shi W, Moon CD, Leahy SC, Kang D, Froula J, Kittelmann S, et al. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome Res. 2014; 24(9):1517–25. https://doi.org/10.1101/gr.168245.113 PMID: 24907284

41. Tapio I, Snelling TJ, Strozzi F, Wallace RJ. The ruminal microbiome associated with methane emissions from ruminant livestock. J Anim Sci Biotechnol [Internet]. Journal of Animal Science and Biotechnology; 2017; 8(7):2–11. Available from: https://jasbsci.biomedcentral.com/articles/10.1186/s40104-017-0141-0#CR56

42. Wallace RJ, Snelling TJ, McCartney CA, Tapio I, Strozzi F. Application of meta-omics techniques to understand greenhouse gas emissions originating from ruminal metabolism. Genet Sel Evol [Internet]. BioMed Central; 2017; 49(1):9. Available from: http://gsejournal.biomedcentral.com/articles/10.1186/s12711-017-0285-6 https://doi.org/10.1186/s12711-017-0285-6 PMID: 28093073

43. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science (80-). 2011; 334(October):105–9.

44. Arumugam M, Raes J, Pelletier E, Paslier D Le, Batto J Bertalan M, et al. Enterotypes of the human gut microbiome. Nature. 2013; 473(7346):174–80.

45. Moeller AH, Degnan PH, Pusey AE, Wilson ML, Hahn BH, Ochman H. Chimpanzees and humans harbour compositionally similar gut enterotypes. Nat Commun [Internet]. Nature Publishing Group; 2012; 3:1179. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23149725%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3520023 https://doi.org/10.1038/ncomms2159 PMID: 23149725

46. Hildebrand F, Nguyen TLA, Brinkman B, Yunta, Roberto Garcia Cauwe B, Vandenabeele P, Liston A, et al. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol [Internet]. 2013; 14(1):R4. Available from: http://genomebiology.biomedcentral.com/articles/10.1186/gb-2013-14-1-r4 https://doi.org/10.1186/gb-2013-14-1-r4 PMID: 23347395

47. Ramayo-Caldas Y, Mach N, Lepage P, Levenez F, Denis C, Lemonnier G, et al. Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. ISME J [Internet]. 2016; 10(12):2973–7. Available from: http://www.nature.com/doifinder/10.1038/ismej.2016.77 https://doi.org/10.1038/ismej.2016.77 PMID: 27177190

48. Kittelmann S, Pinares-Patiño CS, Seedorf H, Kirk MR, Ganesh S, McEwan JC, et al. Two different bacterial community types are linked with the low-methane emission trait in sheep. PLoS One. 2014; 9(7):1–9.

49. Knights D, Ward TL, McKinlay CE, Miller H, Gonzalez A, McDonald D, et al. Rethinking "Enterotypes." Cell Host Microbe. Elsevier Inc.; 2014 Oct; 16(4):433–7. https://doi.org/10.1016/j.chom.2014.09.013 PMID: 25299329

50. Jewell KA, McCormick CA, Odt CL, Weimer PJ, Suen G. Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. Appl Environ Microbiol [Internet]. 2015; 81(14):4697–710. Available from: http://aem.asm.org/content/81/14/4697.short https://doi.org/10.1128/AEM.00720-15 PMID: 25934629

51. Lima FS, Oikonomou G, Lima SF, Bicalho MLS, Ganda EK, de Oliveira Filho JC, et al. Prepartum and Postpartum Rumen Fluid Microbiomes: Characterization and Correlation with Production Traits in Dairy Cows. Appl Environ Microbiol [Internet]. 2015; 81(4):1327–37. Available from: http://aem.asm.org/lookup/doi/10.1128/AEM.03138-14 https://doi.org/10.1128/AEM.03138-14 PMID: 25501481

52. Kamke J, Kittelmann S, Soni P, Li Y, Tavendale M, Ganesh S, et al. Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. Microbiome [Internet]. Microbiome; 2016; 4(1):56. Available

from: http://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-016-0201-2 https://doi.org/10.1186/s40168-016-0201-2 PMID: 27760570

53. Smuts M, Meissner HH, Cronjé PB. Retention time of digesta in the rumen: its repeatability and relationship with wool production of Merino rams. J Anim Sci. 1995; 73(1):206–10. PMID: 7601735

54. Negussie E, Lehtinen J, Mäntysaari P, Bayat AR, Liinamo A-E, Mäntysaari EA, et al. Non-invasive individual methane measurement in dairy cows. Animal [Internet]. 2016; 4:1–10. Available from: http://www.journals.cambridge.org/abstract_S1751731116002718

55. Garnsworthy PC, Craigon J, Hernandez-Medrano JH, Saunders N. On-farm methane measurements during milking correlate with total methane production by individual dairy cows. J Dairy Sci [Internet]. Elsevier; 2012; 95(6):3166–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22612952 https://doi.org/10.3168/jds.2011-4605 PMID: 22612952

56. Ross EM, Moate PJ, Bath CR, Davidson SE, Sawbridge TI, Guthridge KM, et al. High throughput whole rumen metagenome profiling using untargeted massively parallel sequencing. BMC Genet [Internet]. BMC Genetics; 2012; 13(1):53. Available from: BMC Genetics

57. Ross EM, Moate PJ, Marett LC, Cocks BG, Hayes BJ. Metagenomic predictions: from microbiome to complex health and environmental phenotypes in humans and cattle. PLoS One [Internet]. 2013; 8(9): e73056. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3762846&tool=pmcentrez&rendertype=abstract https://doi.org/10.1371/journal.pone.0073056 PMID: 24023808

58. Bordenstein SR, Theis KR. Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. PLoS Biol. 2015; 13(8):1–23.

59. Zilber-Rosenberg I, Rosenberg E. Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. FEMS Microbiol Rev. 2008; 32(5):723–35. https://doi.org/10.1111/j.1574-6976.2008.00123.x PMID: 18549407

60. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature [Internet]. 2013; 500(7464):541–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23985870 https://doi.org/10.1038/nature12506 PMID: 23985870

61. Falcony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, et al. Population-level analysis of gut microbiome variation. Science (80-). 2016; 352(6285):560–4. https://doi.org/10.1126/science.aad3503 PMID: 27126039

62. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut [Internet]. 2016; 65(1):57–62. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4717365&tool=pmcentrez&rendertype=abstract https://doi.org/10.1136/gutjnl-2015-309618 PMID: 26069274

63. Roager HM, Hansen LBS, Bahl MI, Frandsen HL, Carvalho V, Gøbel RJ, et al. Colonic transit time is related to bacterial metabolism and mucosal turnover in the gut. Nat Microbiol [Internet]. Nature Publishing Group; 2016; 1(9):16093. Available from: http://www.nature.com/articles/nmicrobiol201693 https://doi.org/10.1038/nmicrobiol.2016.93 PMID: 27562254

64. Goopy JP, Donaldson A, Hegarty R, Vercoe PE, Haynes F, Barnett M, et al. Low-methane yield sheep have smaller rumens and shorter rumen retention time. Br J Nutr. 2014; 111(4):578–85. https://doi.org/10.1017/S0007114513002936 PMID: 24103253

65. Bickhart DM, Weimer PJ. Host–rumen microbe interactions may be leveraged to improve the productivity of dairy cows. J Dairy Sci [Internet]. American Dairy Science Association; 2017;1–10. Available from: http://linkinghub.elsevier.com/retrieve/pii/S002203021730958X

66. Lassen J, Løvendahl P, Madsen J. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. J Dairy Sci [Internet]. Elsevier; 2012; 95(2):890–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22281353 https://doi.org/10.3168/jds.2011-4544 PMID: 22281353

67. Difford GF, Lassen J, Løvendahl P. Interchangeability between methane measurements in dairy cows assessed by comparing precision and agreement of two non-invasive infrared methods. Comput Electron Agric [Internet]. Elsevier B.V.; 2016; 124:220–6. Available from: http://dx.doi.org/10.1016/j.compag.2016.04.010

68. CIGR. 4th Report of working group on climatization of animal houses: Heat and moisture production at animal and house levels [Internet]. Pedersen S, Sällvik K, editors. 4th Report of Work Group on Climatization of animal houses. Research Centre Bygholm. DIAS, Horsens, Denmark; 2002. 46 p. Available from: http://www.cigr.org/documents/CIGR_4TH_WORK_GR.pdf

69. Sjaunja LO, Baevre L, Junkkarinen L, Pedersen J, Setala J. A Nordic proposal for an energy corrected Milk (ECM) formula EAAP Publication 50: Performance Recording of Animals. Wageningen, the Netherlands: State of the Art 1990, Centre for Agricultural Publishing and Documentation (PUDOC); 1991. 156–157 p.

70. Pedersen S, Blanes-Vidal V, Joergensen H, Chwalibog A, Haeussermann A, Heetkamp MJW, et al. Carbon Dioxide Production in Animal Houses: A literature review. Agric Eng Int CIGR Ejournal. 2008;X (BC 08 008):1–19.

71. Madsen J, Bjerg BS, Hvelplund T, Weisbjerg MR, Lund P. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest Sci [Internet]. Elsevier B.V.; 2010; 129(1–3):223–7. Available from: http://dx.doi.org/10.1016/j.livsci.2010.01.001

72. Geishauser T, Linhart N, Neidl A, Reimann A. Factors associated with ruminal pH at herd level. J Dairy Sci [Internet]. Elsevier; 2012; 95(8):4556–67. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030212004456 https://doi.org/10.3168/jds.2012-5380 PMID: 22818470

73. Johnson DJ, Martin LR, Roberts K a. STR-typing of human DNA from human fecal matter using the QIAGEN QIAamp stool mini kit. J Forensic Sci. 2005; 50(4):802–8. PMID: 16078481

74. Ward D V., Gevers D, Giannoukos G, Earl AM, Meth?? BA, Sodergren E, et al. Evaluation of 16s rDNA-based community profiling for human microbiome research. PLoS One. 2012; 7(6).

75. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013; 41(1):1–11. https://doi.org/10.1093/nar/gks1039

76. Hildebrand F, Tadeo R, Voigt AY, Bork P, Raes J. LotuS: an efficient and user-friendly OTU processing pipeline. Microbiome [Internet]. 2014; 2(1):30. Available from: http://www.microbiomejournal.com/content/2/1/30/abstract%5Cnhttp://www.microbiomejournal.com/content/2/1/30%5Cnhttp://www.microbiomejournal.com/content/pdf/2049-2618-2-30.pdf https://doi.org/10.1186/2049-2618-2-30 PMID: 27367037

77. Magoč T, Salzberg SL. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011; 27(21):2957–63. https://doi.org/10.1093/bioinformatics/btr507 PMID: 21903629

78. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods [Internet]. 2013; 10(10):996–8. Available from: https://doi.org/10.1038/nmeth.2604 PMID: 23955772

79. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27(16):2194–200. https://doi.org/10.1093/bioinformatics/btr381 PMID: 21700674

80. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol [Internet]. 2011; 7(1):539. Available from: http://msb.embopress.org/content/7/1/539.abstract

81. Price MN, Dehal PS, Arkin AP. FastTree 2—Approximately maximum-likelihood trees for large alignments. PLoS One. 2010; 5(3).

82. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat Publ Gr [Internet]. Nature Publishing Group; 2010; 7(5):335–6. Available from: http://dx.doi.org/10.1038/nmeth0510-335

83. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007; 73(16):5261–7. https://doi.org/10.1128/AEM.00062-07 PMID: 17586664

84. Wilmink JBM. Adjustment of test-day milk, fat and protein yield for age, season and stage of lactation. Livest Prod Sci. 1987; 16(4):335–48.

85. Madsen P, Jensen J, Labouriau R, Christensen OF, G. S. DMU—A Package for Analyzing Multivariate Mixed Models in quantitative Genetics and Genomics. In: 10th World Congress on Genetics Applied to Livestock Production. 2010. p. 2–4.