



Influence of agroecology practices on rumen microbiota associated with methane emission in dairy cattle

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ABSTRACT

Moving from intensive farming to agroecology to support farm sustainability means changing feeding practices. In practical terms, this means increasing the botanical diversity and delaying mowing of the grasslands to favor fauna diversity and associated ecosystem services. However, it is unknown whether these feeding practices alter rumen microbiota and its association with methane (CH₄) emission, a potent greenhouse gas. The objective of this study was to assess CH₄ emission and rumen microbiota of several dairy breeds fed agroecology diets. Three dairy cattle breeds (Holstein Friesian, Groninger Blaarkop and Jersey) (N = 10 for each breed) were fed three grass silage-based diets that included a proportion of a control silage, an experimental silage composed of late mown grass, and an experimental silage composed of diverse botanical species. Cows were fed for 13 weeks with gradual adjustment of the proportion of each silage. Rumen fluid was sampled during the weeks that corresponded to the highest proportion of each silage in the diet. Rumen microbiota was characterized through 16 s rRNA gene amplicon sequencing for its richness and diversity, as well as its compositions according to diet type and breed. Production performances and CH₄ emission were also measured. Methane production (g/d) was similar between the control and the agroecological diets. Cows fed the experimental diets had a different rumen microbiota composition than cows fed control diet. The cows fed the agroecological diets presented reduced relative abundances of *Ruminococcaeae*, and higher relative abundances of *Chirstensenellaceae* and *Methanobrevibacter* than cows fed the control diet. Besides, the cows fed the agroecological diets presented a richer ($P < 0.01$) and more diverse ($P < 0.01$) rumen microbiota. Overall, this study highlights how feeding practices that comply with agroecology principles, and applied under practical conditions, shaped the rumen microbiota of specialized and dual-purpose cattle breeds.

Abbreviations: ANOVA, Analysis of variance; ASV, Amplicon sequence variant; CH₄, Methane; CO₂, Carbon di-oxide; CS, Control silage; DM, Dry matter; DNA, Deoxyribonucleic acid; FDR, False discovery rate; FPCM, Fat and protein corrected milk yield; GB, Groninger blaarkop; GEM, Greenfeed emission monitoring; HF, Holstein friesian; HRS, Herb rich silage; J, Jersey; LMS, Late mown silage; OST, Oral stomach tube; PCoA, Principal coordinate analysis; PERMANOVA, Permutational multivariate analysis of variance; rRNA, Ribosomal ribonucleic acid.

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1. Introduction

Innovations in cattle farming in the last decades have improved milk or meat production at the animal and farm level. However, such productivity gains rely on external inputs and human intervention (Willem Erisman et al., 2016). These methods have led to environmental damage (Haas et al., 2001; Dumont et al., 2014), such as loss of ecological diversity in farms and landscapes, as well as a decline of animal robustness (Friggens et al., 2017). Consequently, alternative farming systems such as agroecology farming are advocated to counterbalance these negative impacts.

Agroecology farming refers to the application of ecological principles in farm management to ensure both sustainability and long-term performance (Altieri, 1999; Dumont et al., 2013; Willem Erisman et al., 2016). A basic principle of agroecology is to enhance diversity within the components of a farm, such as forage or phenotypic characteristics of animals (Dumont et al., 2020). For instance, cultivating diverse botanical species in grasslands can buffer climatic disturbances on forage yield (Volaire et al., 2014; Muciño-Álvarez et al., 2021), increase the plant biomass production (Cardinale et al., 2007; Finn et al., 2013), and improve the diversity of soil organisms involved in nutrient recycling (van Groenigen et al., 2014). From an animal nutritional point of view the diversity of grassland species can result in a higher nutritional value, leading to higher milk production in cows grazing on these grasslands (Roca-Fernández et al., 2016) and a trend towards lower nitrogen excretion (Totty et al., 2013). Additionally, the diversity of botanical species may be beneficial to milk quality as cows fed with botanically diverse forages have a milk fatty acid profile richer in poly-unsaturated fatty acids compared to cows fed less diverse forages (Lourenço et al., 2008). Late mowing can also be considered as an agroecology practice as it is associated with increased plant and invertebrate diversity (Humbert et al., 2012). However, late mowing of grasslands is associated with an increase in neutral and especially acid detergents fibers and a decrease in crude protein and nitrogen content (Rinne et al., 1997; Nocera et al., 2005; Smith et al., 2022). Lastly, the diversity of phenotypic characteristics of animals can be increased by rearing different breeds. Indeed, mixing breeds makes it possible to take advantage of several trade-offs, such as a balancing performance between milk production, milk fat and protein content, and concentrate utilization (Magne et al., 2016).

The rumen microbiota of dairy cattle ensures the transformation of non-digestible feed into digestible nutrients that can be used by cows as a source of energy. However, CH₄ is produced during the fermentation of feed in the cow's rumen (Morgavi et al., 2010; Tapio et al., 2017; Mizrahi and Jami, 2018; Ramayo-Caldas et al., 2020; Ungerfeld, 2020; Bueno de Mesquita et al., 2023). In the rumen, CH₄ is only produced by *Archaea* through either an hydrogenotrophic pathway using di-hydrogen (H₂) to reduce carbon dioxide (CO₂), a methyl-based pathway that use methylated compounds as substrates but still requires hydrogen, or through a scarcer acetoclastic pathway that uses acetate as main substrate (Kurth et al., 2020). The types of pathways used by *Archaea* to produce CH₄ depend, among other things, on the products of other microorganisms such as Bacteria during feed fermentation. Yet, the composition and diversity of bacteria inhabiting the rumen of dairy cattle, and thus their degradation products, are strongly modulated by the diet (Huws et al., 2018; Newbold and Ramos-Morales, 2020) and may also depend on breed (Paz et al., 2016).

In the Netherlands, farmers tend to switch from intensive farming to agroecological farming by implementing farm management practices such as using late mown multispecies silage or rearing different cattle breeds at farm. However, the effect of these silages on the rumen microbiota of specialized and dual purposes dairy cattle breeds are not known. The objective of this study was to assess how diets based on silages from high botanical diversity and late mown grasslands modulate the rumen microbiota in the context of sustainable farming. We hypothesized that diets based on silage from diverse botanical and late mown grasslands would modulate the rumen microbiota independent of or dependent on three dairy breeds and that these modulation in rumen microbiota would influence CH₄ emissions.

2. Materials and Methods

The experiment was conducted under the Dutch law on Animal Experiments in accordance with European Union Directive 2010/63 and approved by the Central Committee of Animals Experiments (The Hague, the Netherlands, 2016. D-0066.001).

2.1. Experimental design

The experimental design consisted of three dairy cattle breeds (N = 30), namely Holstein Friesian (HF), Groninger Blaarkop (GB) and Jersey (J) fed composed diets of three different silage types, a control silage consisting predominantly of English ryegrass (CS), a late mown silage (LMS) and herb rich silage (HRS) over 13 weeks with adjustment of the proportion of each silage type every week (Supplemental file1: Fig. S1). To assess the effect of silage-based diets, the rumen sampling and phenotypic measures of 10 random cows per breed were made during the weeks for which a maximum of each silage type was present in the diet (CS diet, 1000 g/kg DM control silage in week 1; LMS diet, 480 g/kg DM control silage, 420 g/kg DM late mown silage and 100 g/kg herb rich silage in week 5; HRS diet, 480 g/kg control silage, 100 g/kg late mown silage and 420 g/kg herb-rich silage in week 9). Such experimental design minimized the number of animals compared to a factorial design, as recommended by the National Centre for the Replacement, Refinement and Reduction of Animals in research (Prescott and Lidster, 2017).

2.2. Animals

The experiment was carried out from October 1st 2019 to January 9th 2020 on research innovation center VIC Zegveld (Zegveld, THE NETHERLANDS). The three groups of breeds were composed of lactating dairy cows: 27 HF, 20 GB and 43 J. Cows were housed in

a free-stall barn with sawdust bedded cubicles and natural ventilation. The breeds were housed in separate groups in order to collect feed intake per breed. Cows from HF and J breed had access to a milking robot, while cows from GB breed were milked twice per day in a milking parlour. The average phenotypic measures before the experiment started are reported in Supplemental file 2: Table S1.

2.3. Diets

The diets consisted of three grass silages mixed or not (CS, LMS and HRS) and compound feed. Control silage was a conventional grass silage consisting predominantly of English ryegrass. Late mown silage was composed mainly of English ryegrass and rough meadow-grass from grassland with a delayed mowing date compared to CS. Herb rich silage was harvested from high botanical diversity grassland. The CS was harvested in May while LMS and HRS were harvested in June of 2019. The relative abundance and top cover of the botanic composition of vegetation in the grassland was assessed by visual estimation in April 2019 as described by Peratoner and Pötsch (2019) (Peratoner and Pötsch, 2019) (Supplemental file 2: Table S2). Control silage and LMS were produced from grassland mainly composed of English ryegrass (*Lolium perenne*) (61.5% and 39% respectively), while the HRS was produced from grassland composed of 35 different species, mainly soft rush (*Juncus effusus*, 28.5%), sweet vernal grass (*Anthoxanthum odoratum*, 13%), soft brome (*Bromus hordeaceus*, 10%) and meadow soft grass (*Holcus lanatus*, 9%). To keep the nutritional value of the diets constant over the weeks and to meet the nutritional needs of the cows, the grass silages were complemented with compound feed based on the Dutch protein evaluation system (Tamminga et al., 1994). The nutritional needs were determined on individual level. Briefly, the energy need for maintenance and milk production was calculated to keep the milk yield stable over the week. The energy intake was calculated based on the feed intake capacity (CVB, 2022) of the silages (i.e. estimated the maximum feed intake of these silages). The remaining energy needed was provided by compound feed intake by adjusting the compound feed supply. The average compound feed supply (in kg/cow/day) was adjusted (Supplemental file 2: Table S3) per breed and each week to meet the nutritional needs of the cows.

Cows were fed ad libitum and had free access to clean drinking water. Roughage supply was weighted every day for each group of breeds. Every morning the residuals of the previous day were weighted and recorded. The roughage supply and residuals were used to calculate the dry matter intake of each dietary group per breed per day. Roughage samples of each dietary group were collected on a weekly basis and stored at -20°C until analysis. Three weeks after the end of the trial, samples were pooled over the weeks of the trial for analysis. For the compound feed, the nutritional value was obtained from the supplier (De Samenwerking, Haastrecht, THE NETHERLANDS). Chemical composition of grass silage samples was analyzed by Eurofins Agro (Wageningen, THE NETHERLANDS) and are presented in Table 1 along the chemical composition of the total ration in Table 2. Dry matter content was analyzed by gravimetric analysis after drying for 16 h at 60°C . Crude protein, crude fiber, crude fat, crude ash, sugars, aNDF (assayed with heat stable alpha-amylase), ADF and lignin (sa-) (assayed with sulfuric solution) were analyzed by near infrared spectroscopy. Briefly, samples were analyzed by a Q-Interline FT-NIR (Q-Interline, Waddinxveen, Netherlands) ranging from 1000 to 2600 nm. The calibration models were made with locally weighted learning procedures. The NIRS method was accredited with the EN ISO/IEC 17025:2017 under registration number L122.

2.4. Emission measurements

Enteric CH_4 and CO_2 production were measured non-invasively using a GreenFeed emission monitoring system (GEM), (C-lock Inc. Rapid City, SD, USA). The GEM is an adapted feeding station that measures individual CH_4 and CO_2 production in grams per day during each visit. The GEM recorded CH_4 and CO_2 concentration by nondispersive infrared sensors, the quantitative airflow by a hot-film anemometer (flux method), temperature, head positioning (infrared sensor) and radiofrequency identification collar tags specific to each cow for individual recognition. All variables were logged at a 1 s interval and used to generate individual CH_4 and CO_2 production. The GEM measures continuously, even if there are no animals present, to correct for background emissions in the barn. The

Table 1
Chemical composition of the silage type and the compound feed.

Silage type	Control silage	Late mown silage	Herb rich silage	Compound feed 'Profijt 90' ^a
Dry matter content (g/kg)	511	496	760	868
Crude protein (g/kg DM ^b)	179	109	87	145
Crude fibre (g/kg DM)	259	338	355	135
Crude fat (g/kg DM)	40	26	16	35
Crude inorganic matter (g/kg DM)	100	64	62	81
Sugars (g/kg DM)	76	27	46	81
Neutral detergent fibre (aNDF ^c) (g/kg DM)	471	623	694	-
Acid detergent fibre (ADF ^d) (g/kg DM)	276	387	410	-
Lignin (sa) ^e (g/kg DM)	19	50	50	-
Starch (g/kg DM)	-	-	-	304

^a Composition derived from the supplier.

^b DM: dry matter.

^c aNDFom: neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash.

^d ADF: acid detergent fiber expressed inclusive of residual ash.

^e Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid.

Table 2
Chemical composition calculated for the total ration per breed and diet.

Breeds ^a	HF			GB			J		
	CS	LMS	HRS	CS	LMS	HRS	CS	LMS	HRS
Dry matter content (g/kg)	592	664	717	582	646	699	608	669	716
Crude protein (g/kg DMI ^f)	171	143	139	172	143	139	170	143	139
Crude fibre (g/kg DMI)	231	235	233	234	244	245	225	232	234
Crude fat (g/kg DMI)	39	33	32	39	33	31	39	33	32
Crude inorganic matter (g/kg DMI)	96	82	81	96	82	81	95	82	81
Sugars (g/kg DMI)	77	65	68	77	63	67	77	65	68
Neutral detergent fibre (aNDF) (g/kg DMI) ^d	364	335	333	377	365	372	343	327	334
Acid detergent fibre (ADF) (g/kg DMI) ^d	213	202	197	221	220	220	201	197	198
Lignin (sa-) (g/kg DMI) ^d	15	21	20	15	23	22	14	20	20
Starch (g/kg DMI)	69	120	128	61	103	107	83	124	127

aNDFom: neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash

ADF: acid detergent fiber expressed inclusive of residual ash

Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid

^a HF: Holstein Friesian; GB: Groninger Blaarkop; J: Jersey

^b CS: control silage diet; LMS: late mown silage diet; HRS: herb rich silage diet

^c DMI: dry matter intake

^d Minimal contents reported as aNDF, ADF and lignin (sa-) were not analyzed by the provider of compound feed

periods within a visit where the head position of the animal is correct are used for measuring gaseous emissions. Quantitative concentrations in g/d were calculated at a 1 s interval, which were then averaged per visit (minimum of 2 min).

The compound feed supply was provided in a maximum of 8 feeding periods per day with at least 3 h between each feeding period. A minimal visit time of 3 min was assured, but no longer than 5 min. Since the method of the GEM is based on short-term breath measures, measurements of seven days were averaged for each of the three diets. The measures of gaseous production of one cow were not taken into account for the estimation of the averages gaseous production as the cow visited the GEM only twice compared to an average of 32 times (11–72 visits) for the other cows.

2.5. Rumen and milk sampling

Rumen fluid samples were collected at the end of week 1, 5 and 9 of the trial corresponding to the highest inclusion of the control and experimental silages in their respective diets CS, LMS and HRS. Rumen fluid was sampled using the oral stomach tube (OST) method (Muizelaar et al., 2020). The samples of ten cows per breed, randomly selected in the first week, were collected on the last day of each of the week between 10:00 and 13:00, in the same order of breed (G, J, HF) to make the dietary and breed groups comparable in term of time influence on rumen microbiota. The first 200–500 mL of rumen fluid were discarded to minimize saliva contamination. Subsequently, 200–500 mL rumen fluid were collected again and 3 mL were sampled and frozen immediately in dry ice (−79 °C).

Daily milk production was recorded per cow. Milk samples were taken on Monday afternoon and Tuesday morning every three weeks of the trial. Samples were pooled per cow and analyzed by Fourier transform mid-infrared spectroscopy within routine milk-recording programs, performed by Qlip (Zutphen, THE NETHERLANDS). Average milk yield of the week and the percentage of protein and fat in the milk were used to calculate FPCM via the following formula:

$$FPCM \text{ (kg/cow/day)} = (0.337 + 0.16 \times \text{fat(g/100g)} + 0.06 \times \text{protein(g/100g)}) \times \text{milk yield(kg/d)}$$

2.6. DNA extraction, amplification and sequencing

Extraction of microbial DNA from rumen samples, library construction of hypervariable region V4 (from 16 S rRNA gene) and subsequent sequencing on an Illumina HiSeq platform were performed at Genotypic Technology Pvt. Ltd. (Bangalore, INDIA). Detailed method can be found in Supplemental file 6. Briefly, DNA was extracted through Qiagen DNeasy Blood and tissue Kit (Qiagen, Hilden, GERMANY) and integrity controlled by agarose gel electrophoresis. Sequencing library was prepared using the region-specific primers V4–515 F (GTGCCAGCMGCCGCGGTA) and V4–806R (GACTACHVGGGTATCTAATCC) by a two-step polymerase chain reaction (PCR) based workflow. Amplicons were then sequenced by Illumina HiSeqXTen sequencer, where it was paired-end 150 bp sequenced to generate at minimal target of 0.7 million paired-end reads.

Reads demultiplexed using bcl2fastq v2.20 software were preprocessed using QIIME2 suite v2020.8 and FASTQC v0.11.9. Reads were first checked for low quality bases (Q30) and trimmed of primers before being merged using FASTQ-join. The merged reads were denoised, dereplicated and removed from the chimera for each sample independently using the DADA2 plugin from the QIIME2 suite. The taxonomy of the resulting Amplicon Sequence Variants (ASV) was assigned using the SILVA v.138 database (Quast et al., 2012). The ASVs abundance were normalized by rarefaction without replacement to even sampling depth of 268,632 reads (Supplemental file 1: Fig. S2), resulting in 43,806 ASVs, and one sample was discarded from further analysis because its maximum library size was below the defined threshold.

2.7. Statistical analysis

All subsequent statistical analysis were performed in R v4.0.3 (R Core Team, 2020), using *Phyloseq* (McMurdie and Holmes, 2013), *metacoder* (Foster et al., 2017), *lmerTest* (Kuznetsova et al., 2017) and *emmeans* (Lenth, 2021) packages. The effect of diet, breed and their interaction on milk yield, FPCM, CH₄ production (g/cow/day), CH₄ intensity (g/kg FPCM) and CO₂ production (g/cow/day) was assessed by a two-way ANOVA (significance was indicated with a P value < 0.05) of the following linear mixed model:

$$Y_{ijn} = \mu + S_i + B_j + S_i \times B_j + C_n + \varepsilon_{ijn}$$

where Y_{ijn} is the dependent variable, μ is the overall mean, S_i is the fixed effect of the i -th diet type ($N = 3$), B_j is the fixed effect of the j -th breed ($N = 3$), $S_i \times B_j$ is the interaction term of the i -th diet type and j -th breed, C_n is the random effect of n -th cow ($N = 30$) and ε_{ijn} is the residual error of the model. Only the diet effect or the interaction effect were displayed as the breed differed in term of specialization (HF is specialized in milk yield, GB is a dual-purpose milk and meat producer and J is specialized in milk with high fat content) and it was not the objective of this study to assess performance production depending on the breed. Estimated marginal means were compared by Tukey's pairwise comparison tests when a significant effect of the independent terms or their interaction was found on the dependent variable.

The diet and breed effect on microbiota were assessed on α - and β -diversities. Diet, breed and the interaction effect on the Richness and Shannon's index were assessed as described in the previous paragraph. To assess diet and breed effect on β -diversity, rarefied ASV counts were filtered for low abundance (less than 10) and low prevalence (less than 10% of all samples). Amplicon sequence variants counts were then Hellinger-transformed to narrow the influence of the high range of ASV abundances. Beta-diversity was assessed by Principal Coordinate Analysis (PCoA) using Bray-Curtis dissimilarity metrics as similarity metric. Significance of the groups variances were assessed by permutational multivariate ANOVA (PERMANOVA) with 999 permutations. Wilcoxon's tests with false discovery rate (FDR) correction were used to assess the significance of differences of the median abundance of ASVs rarefied counts merged up to the family taxonomic rank between dietary-induced groups of cow separated along the two first axes of the PCoA. Their relative differences were expressed as base two logarithmic fold change of the median per taxonomic rank and were plotted on a taxonomic tree. *Archaea* composition was assessed at genus rank by Wilcoxon's tests between diets within breeds. All the significant threshold of P -values or adjusted P -values were set at < 0.05.

3. Results

3.1. Methane intensity and production measures

Milk yield, FPCM, fat and protein percentages in milk, CH₄ emission, CH₄ intensity, CO₂, compound feed intake, total silage intake (in kg of dry matter (DM)) and total feed intake (kg DM) are presented in Table 3. Diet had an effect on milk yield ($P < 0.001$), FPCM ($P < 0.001$), fat percentage ($P < 0.001$), protein percentage ($P < 0.001$), CH₄ intensity ($P < 0.001$), CO₂ production ($P = 0.001$). The interaction of diet and breed had an effect on milk yield ($P < 0.001$), FPCM ($P = 0.004$), fat percentage ($P = 0.01$), protein percentage ($P = 0.04$), CH₄ production ($P = 0.009$), CH₄ intensity ($P = 0.002$) and CO₂ production ($P = 0.009$).

Table 3

Milk performances and gas productions estimated marginal means of the Holstein Friesian, Groninger Blaarkop and Jersey groups fed the control silage, late mown silage and herb rich silage diets. $N = 10$ cows per group.

Breeds ^a	HF			GB			J			RSD ^c	P-values	
	Diets ^b	CS	LMS	HRS	CS	LMS	HRS	CS	LMS		HRS	diet
Milk yield (kg/d)	21.4	20.9	21.4	12.2 ^a	9.4 ^b	6.8 ^c	15.8 ^a	13.8 ^a	10.7 ^b	2.1	2.06E-07	8.00E-04
FPCM ^d (kg/d)	24.2	22.9	23.3	13.9 ^a	11.3 ^b	9.2 ^b	22.9 ^a	21.5 ^a	16.8 ^b	2.3	1.48E-07	4.20E-03
Milk fat (g/100 g)	4.60	4.59	4.54	4.85 ^b	5.26 ^b	6.42 ^a	7.39 ^b	8.05 ^b	8.43 ^a	0.7	2.16E-04	0.01
Milk protein (g/100 g)	3.55	3.78	3.78	4.07 ^b	4.30 ^{ab}	4.62 ^a	4.58 ^b	5.00 ^a	4.69 ^{ab}	0.3	7.36E-04	0.04
CH ₄ production (g/d) ^e	438 ^a	411 ^b	419 ^{ab}	346	360	345	323	347	342	23.1	0.80	9.00E-03
CH ₄ intensity (g/kg FPCM) ^e	18.2	18.1	18.4	26.1 ^c	32.8 ^b	41.3 ^a	14.1 ^b	16.3 ^{ab}	21.4 ^a	5.3	6.20E-06	2.00E-03
CO ₂ production (kg/d) ^e	12.4 ^a	11.8 ^b	11.7 ^b	10.0 ^{ab}	10.2 ^a	9.6 ^b	9.9	10.2	9.8	0.4	1.00E-03	8.60E-03
Compound feed intake (kg/d)	5.4	9.7	10.6	3.7	6.8	6.5	5.0	7.7	7.5	0.7		
Total silage intake (kg DM/d) ^f	14.0	11.7	11.3	12.4	11.4	10.6	11.1	9.9	9.0			
Total feed intake (kg DM/d) ^f	18.1	19.3	19.5	15.6	17.2	16.4	15.3	16.8	15.6			

^{a-c} Estimated Means with different letters (a,b,c) are significantly different within a breed (Tukey's test, $P < 0.05$)

^a HF: Holstein Friesian; GB: Groninger Blaarkop; J: Jersey

^b CS: control silage diet; LMS: late mown silage diet; HRS: herb rich silage diet

^c Residual standard deviation

^d Fat and Protein Corrected Milk

^e $N = 9$ for J fed HRS

^f No ANOVA was computed for Total silage intake and Total feed intake as the Total silage intake is an average of a repeated measure per group over the week and did not constitute per se replicates.

The milk yield was significantly lower in GB fed HRS diet (6.8 kg/d) compared to GB fed CS diet (12.2 kg/d) while the milk yield of GB fed LMS diets was intermediary. In J fed HRS diet, the milk yield was significantly lower (10.7 kg/d) compared to J fed CS and LMS diets (18.5 and 13.8 kg/d respectively). Fat and protein corrected milk followed the exact same pattern with values ranging from 9.2 to 13.9 kg FPCM/d in GB and from 16.8 to 22.9 kg FPCM/d in J. The fat content in milk was significantly higher in GB fed HRS diet (6.42 g/100 g) than in GB fed CS and LMS diets (4.85 and 5.26 g/100 g respectively). The fat content in milk was also significantly higher in J fed HRS diet (8.43 g/100 g) than in J fed CS and LMS diets (7.39 and 8.05 g/100 g respectively). The protein content in milk was significantly higher in GB fed HRS diet (4.62 g/100 g) than in GB fed CS diet (4.07 g/100 g) while protein content of GB fed LMS diet was intermediary. In J cows, the protein content in milk was significantly higher in cows fed LMS diet (5.00 g/100 g) than in cows fed CS diet (4.58 g/100 g) while the protein content was intermediary for cows fed HRS diet. Methane production was significantly lower in HF cows fed LMS diet (411 g/d) than in HF cows fed CS diet (438 g/d) while methane production of HF fed HRS diet was intermediary. Methane intensity was significantly higher in GB fed HRS diet (41.3 g/kg FPCM) than in GB fed CS diet (21.6 g/kg FPCM) and in GB fed LMS diet (32.8 g/kg FPCM). Methane intensity was also significantly higher in J fed HRS diet (21.4 g/kg FPCM) than in J fed CS diet (14.1 g/kg FPCM) while methane intensity was intermediary in J fed LMS diet. Carbon dioxide emissions were significantly lower in HF fed HRS diet (11.7 kg/d) than in HF fed CS diet (12.4 kg/d) while in HF fed LMS were intermediary.

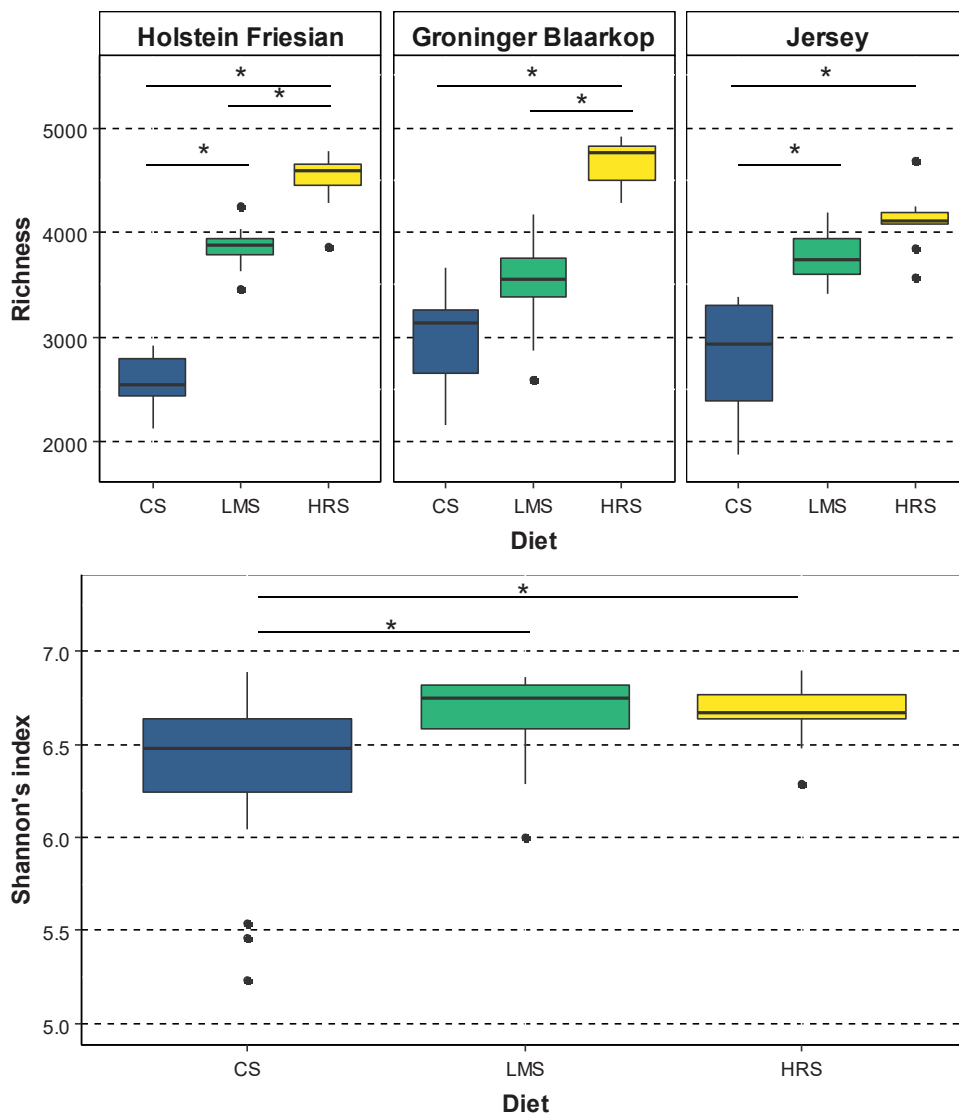


Fig. 1. Influence of agroecological diets and breeds on α -diversity metrics of rumen microbiota. A) Richness and B) Shannon's index of rumen microbiota from Holstein Friesian, Groninger Blaarkop or Jersey cows fed either control silage (CS, dark blue), late mown silage (LMS, green) and herb rich silage (HRS, yellow) diets. Lines with an asterisk indicate significance differences between groups (Tukey's test, $P < 0.05$).

3.2. Rumen microbiota diversities

Rumen microbiota α -diversity was assessed on rarefied ASV counts to ensure comparable results between the groups. The diet, breed and their interaction effects on Richness and diversity, represented by Shannon's index, are presented in Fig. 1. Richness (Fig. 1a) was significantly affected by diet ($P < 0.01$) and by the interaction between diet and breed ($P < 0.01$) but not by breed. Richness of HF and J cows fed LMS diet was significantly higher (mean \pm sd: 3865 ± 217 and 3758 ± 244 respectively) compared to HF and J cows fed CS diet cows (2568 ± 239 and 2796 ± 567 respectively) ($P < 0.01$ in each case). The Richness of HF, GB and J cows fed the HRS were significantly higher (4488 ± 276 ; 4667 ± 229 ; 4107 ± 303 respectively) compared to HF, GB and J cows fed the CS diet (2568 ± 239 ; 3019 ± 506 ; 2796 ± 566 respectively) ($P < 0.01$ in each case).

Shannon's index was significantly affected by diet ($P < 0.01$), but not by breed or the interaction of diet and breed. Consequently, only the diet effect on Shannon's index is presented in Fig. 1b. Shannon's index of cows fed LMS and HRS diet were significantly higher (6.67 ± 0.20 and 6.68 ± 0.13 respectively) compared to cows fed CS diet (6.36 ± 0.08) ($P < 0.01$ in each case).

Rumen microbiota β -diversity was assessed through PCoA based on Bray-Curtis overabundant fraction similarity index presented in Fig. 2. The two first axes of the PCoA explained 44.6% of the total variation in similarity. Diet explained 25% ($P = 0.001$) and breed only 5.3% ($P = 0.003$). Two groups of cows were separated along the first axis, one group mainly composed of HF and J cows fed the CS diet, and another group composed of the remaining cows, HF, GB and J cows fed LMS and HRS diets as well as GB cows fed CS diet. In the second group, the HF, GB and J cows fed LMS diet were separated on the second axis from the HF and J cows fed HRS diet. Cows of the GB breed fed CS diet tended to be grouped between cows separated along the second axis and described previously. Cows of the GB breed fed HRS diet were spread among the extremities of the second axis.

3.3. Dietary-induced modification of rumen microbiota composition

The Wilcoxon tests were conducted on dietary groups of cows separated along the first and second axis of the PCoA (for axis 1: cows fed CS diet – cows fed LMS diet and for axis 2: cows fed LMS diet – cows fed HRS diet). The composition of microbiota was dominated by the *Firmicutes* and *Bacteroidota* phyla that represented more than 75% of the composition followed by the *Proteobacteria* phylum (Supplemental file 1: Fig. S3). Comparisons between the representative groups are presented for these three phyla up to the family taxonomic rank in Fig. 3, Supplemental file 3 and Figs. 4 and 5, Supplemental file 4 respectively. Comparison between cows fed CS diet and cows fed HRS diet is presented in Supplemental file 1: Fig. S4 and Supplemental file 5 and is similar to cow fed CS diet and cows fed LMS diet as the cows fed the experimental diets were separated from the control diet on the same first axis of the PCoA.

The ASVs were merged into 820 identified taxonomic ranks for the groups comparison. The differential median abundance of 547 of these 820 taxonomic levels were significantly different between cows fed CS diet compared to cows fed LMS diet (Fig. 3 and Supplemental file 3). Amongst the most abundant taxonomic levels, the *Ruminococcaceae* and *Succinivibrionaceae* families were significantly less abundant in cows fed LMS diet (median: 2.2% and 0.5% respectively) than in cows fed CS diet (5.2% and 1.9%) ($P < 0.001$ and $P < 0.001$ respectively). On the opposite, the *Christensenellaceae* were significantly more abundant in cows fed LMS diet (4.5%) than cows fed CS diet (2.1%) ($P < 0.001$). Among the highest fold changes, the *Bacillaceae* family was more abundant in

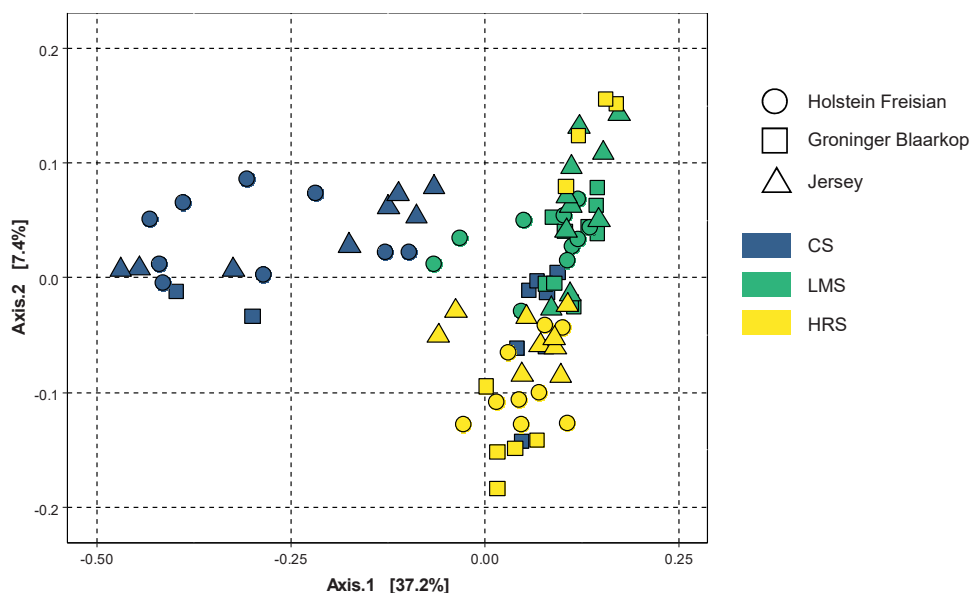


Fig. 2. Principal Coordinate Analysis of rumen fluid samples to assess the influence of agroecological diets and breeds on rumen microbiota composition assessed by Bray-Curtis dissimilarity metric. Holstein Friesian (circle), Groninger Blaarkop (square) or Jersey (triangle) fed control silage (CS, dark blue), late mown silage (LMS, green) and herb rich silage (HRS, yellow).

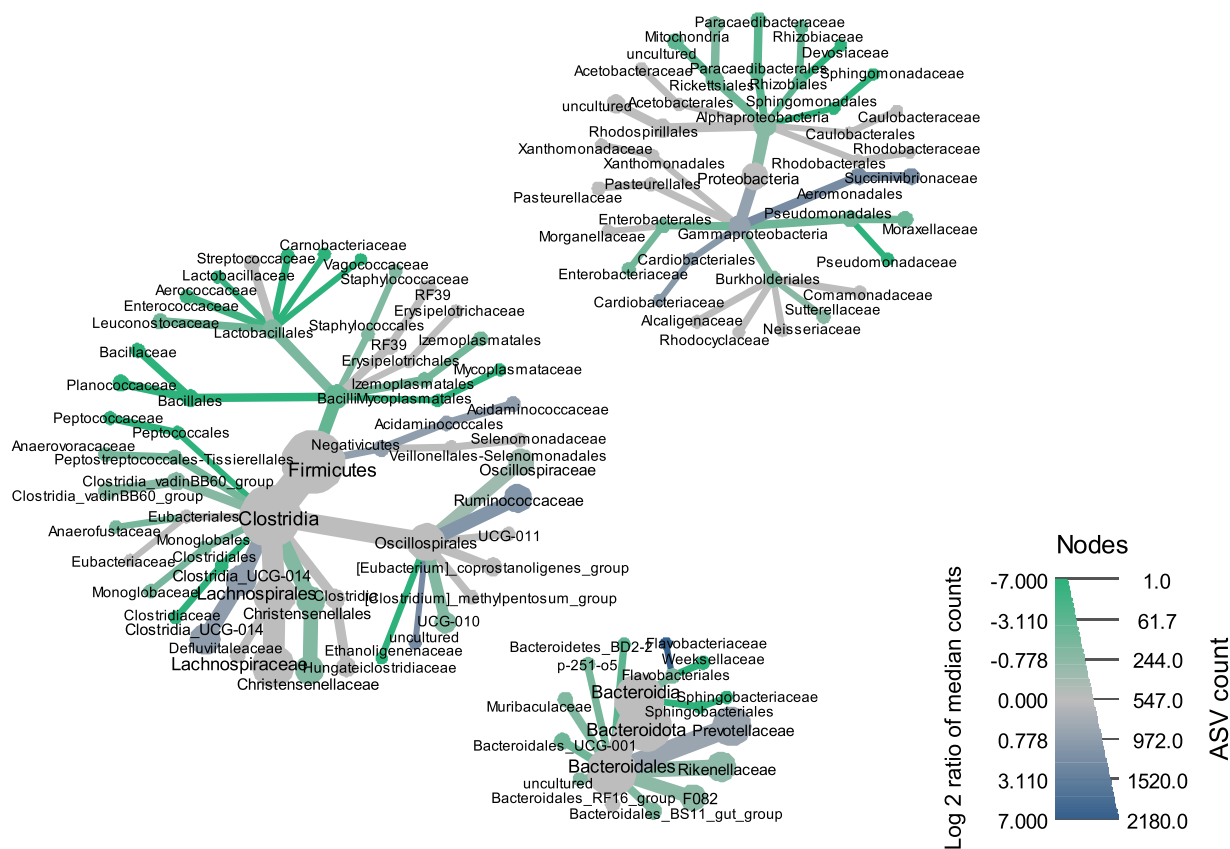


Fig. 3. Comparison of taxa abundance up to Family rank within the three highest abundant Phyla between cows fed control silage (CS) and late mown silage (LMS) diets. The color scale represents the log₂ ratio per taxa of the medians of individuals fed LMS diet on individuals fed CS diet. Green and blue colors indicate overabundance of ASV in LMS and CS individuals respectively. The node size represents the ASVs counts in each taxa. Node are colored only when the difference of median was statistically significant (Wilcoxon test, False discovery rate, $P\text{-adj} < 0.05$).

both cows fed LMS and HRS diet (0.2% and 0.02% respectively) than in cows fed CS diet (0.002%) ($P < 0.001$). The differential median abundance of 347 of these 820 taxonomic ranks were significantly different between cows fed LMS diet compared to cows fed HRS diet (Fig. 4 and Supplemental file 4). Amongst the most abundant taxonomic levels, the *Proteobacteria* phyla was significantly more abundant in cows fed HRS (3.5%) diet than in cows fed LMS diet (2.2%) ($P = 0.03$).

3.4. Archaea composition

The *Archaea*, that represented in average 2.3% of the total *Bacteria* and *Archaea*, were composed of more than 90% of the *Methanobrevibacter* genus (Supplemental file 1: Fig. S5). The relative abundance of *Methanobrevibacter* was significantly higher in HF cows fed LMS (1.8%) and HRS diet (2.8%) compared to CS diet (0.4%) ($P < 0.001$ and $P < 0.001$ respectively). Same pattern was observed in J cows (2.6%, 2.3% and 0.4% respectively, $P < 0.001$ and $P < 0.001$). Only the GB cows fed the HRS diet tended to present higher relative abundance of *Methanobrevibacter* (2.8%) compared to GB cows fed CS diet (1.9%) ($P = 0.09$).

4. Discussion

This study was part of a trial designed to assess the effects of agroecological practices, namely the use of high botanical diversity and late mown silages in diets of dairy cows of diverse breeds, on enteric CH₄ emissions under practical conditions. Rumen fluid was sampled to assess how these practices modified the rumen microbiota and whether this could be linked to CH₄ emission.

Two limitations were faced due to the study design. First, it was not possible to correct the diversity and composition of the microbiota for the confounding variable of time in the data collected in this study as the same cows were sampled throughout the duration of the study. It is known that there is a potential time effect on rumen microbiota as observed by others (Vaidya et al., 2020). It was however assumed that the effect of time would be minor compared to the diet and/or breed effect as long as the feed intake was similar over the weeks. Similarly, it was assumed that the observed modifications would be stable over the time if the same diet compositions were fed over a longer period. This assumption was based on the fact that the modified taxa corresponded to autochthonous taxa that already occupy an ecological niche, which has been argued as stability factor (Weimer, 2015). The second limitation

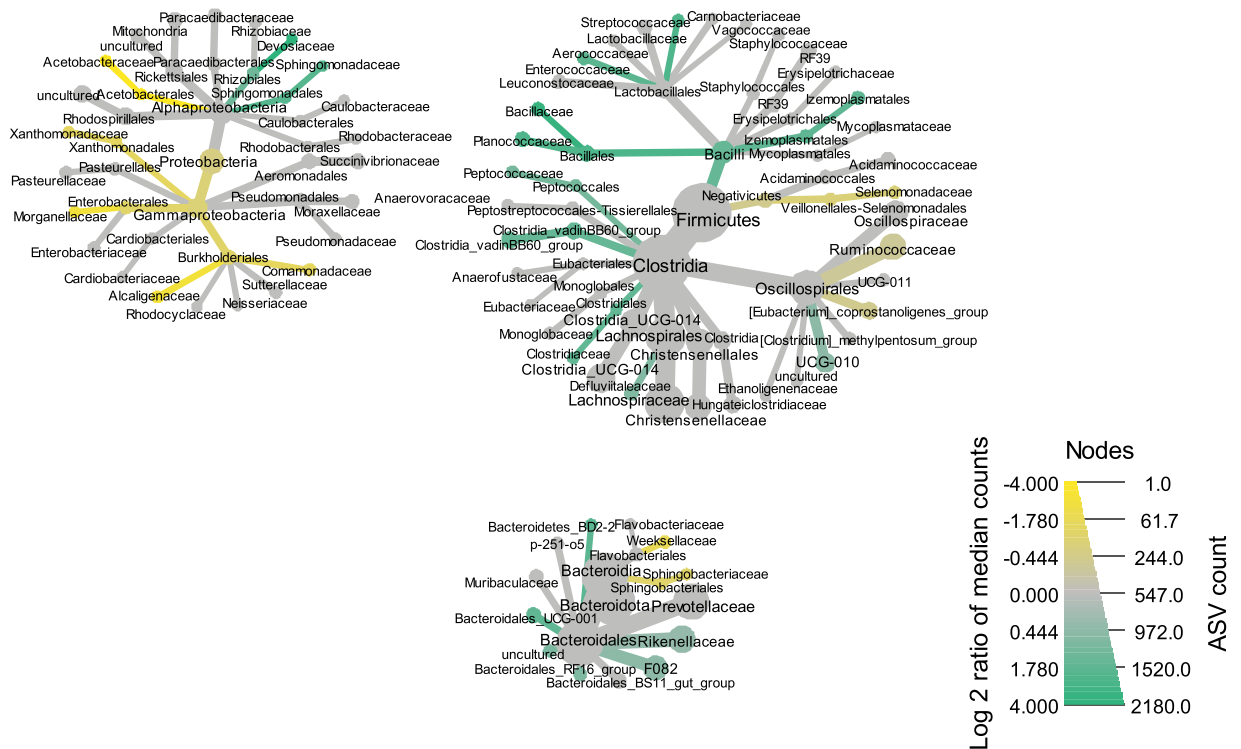


Fig. 4. Comparison of taxa abundance up to Family rank within the three highest abundant Phyla between cows fed late mown silage (LMS) and herb rich silage (HRS) diets. The color scale represents the log₂ ratio per taxa of the medians of individuals fed HRS diet on individuals fed LMS diet. Green and yellow colors indicate overabundance of ASV in LMS and HRS individuals respectively. The node size represents the ASVs counts in each taxa. Node are colored only when the difference of median was statistically significant (Wilcoxon test, False discovery rate, P -adj < 0.05).

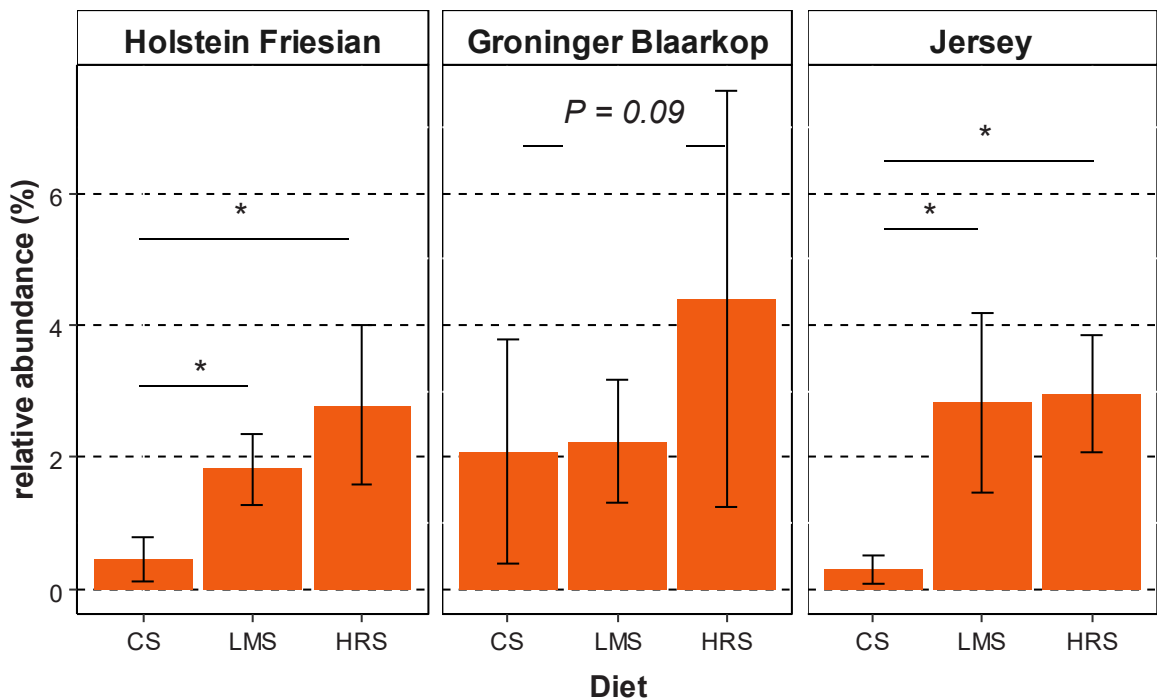


Fig. 5. Relative abundance of the *Methanobrevibacter* Genus in Holstein Friesian, Groninger Blaarkop and Jersey cows fed control silage (CS), late mown silage (LMS) and herb rich silage (HRS) diets, Wilcoxon test within breed ($P < 0.05$).

was the difference between the dietary treatments in silage to compound feed ratio. The experiment was conducted under practical conditions and therefore it was preferable to keep the milk production stable in order to be economically sustainable for the farmers. Due to the lower nutritional value of the agroecological silages, a higher proportion of compound feed was provided. We considered this point in the discussion section and how it might have affected the microbiota.

Methane production was not significantly modified by the agroecology diets, although the interaction between diet and breed led to a slightly reduced methane production in HF fed LMS diet. Methane intensity was significantly increased by the agroecology diets, especially in GB and J cows, but this is mostly due to differences in FPCM yield. It is surprising that methane production was not decreased in cows fed the agroecology diets as they received, and ate, more compound feed than cows fed the control diet. Indeed, it has been shown that compound feeds accelerate the passage rate in the rumen and reduce CH₄ emission by promoting microbiota that produce less H₂ (Kamke et al., 2016; Shabat et al., 2016). This suggests that the silage type in the diets either slowed the passage rate and/or promoted rumen microbiota associated with CH₄ emission as the feed intake, the main known driver of CH₄ emissions in dairy cows (Beauchemin et al., 2009), was similar between the dietary groups.

The composition of the rumen microbiota was strongly influenced by the diets as shown by the tight grouping of individuals fed the same diets on the PCA. The ratio of compound feed to roughage could not be excluded as a cause of change in rumen microbiota composition. However, previous studies showed that only a highly unbalanced shift in this ratio from 80:20–20:80 (Fernando et al., 2010; Zhang et al., 2017) can induce a modification of rumen microbiota composition such as the one we observed. In our case, the minimum to maximum ratios ranged from an average of 30:70 for cows fed CS diet to 50:50 for cows fed LMS diet. It suggests that this narrow range of maximum to minimum ratios had little effect on the composition of the rumen microbiota. Instead, the separation of LMS and HRS fed cows on the same side of the first axis of the PCA suggests that the increased proportion of agroecological silages in these diets compared to CS diet was the major source influencing the rumen microbiota. Second was the type of silage as cows fed LMS and HRS diet were grouped on either side of the second axis.

The observed modification in microbiota composition of cows fed agroecological diets compared to cows fed the CS diet can be linked to the diet composition. For instance, *Succinivibrionaceae* and *Acidaminococcaceae* families have been associated with non-fiber carbohydrate to produce succinate and ferment it to produce propionate (Deusch et al., 2017). The reduced abundance of these families in cows fed LMS and HRS diets can be explained by the higher content of NDF in these diets as these fibers contain less non-fiber carbohydrates (Villalba et al., 2021). The reduced abundance of the *Ruminococcaceae* family in LMS and HRS fed cows can be explained by the higher lignin content in these silages (Table 1). The *Ruminococcaceae* family has a well-known cellulolytic activity but in the LMS and HRS diets, the higher content of lignin probably reduced the access of microbiota to cellulose (Weimer, 2015). Thus, it can be hypothesized that the lignin content in LMS and HRS diets did not favor the growth of *Ruminococcaceae* in the rumen.

The modification in rumen microbiota composition can be related to CH₄ emission in three ways. First, because the higher content of lignin, probably related to late mowing (Rinne et al., 1997; Nocera et al., 2005; Muciño-Álvarez et al., 2021), obstructs fiber degradation, the passage rate of feed will decrease which results in a low concentration of H₂ in rumen fluid of cows fed the LMS and HRS diets, as it has been suggested in Kittelmann et al. (2014). Such conditions (low H₂ concentrations) are known to favor the growth of H₂ producers (Janssen, 2010) such as *Christensenellaceae*. This is what we observed in cows fed LMS and HRS diets despite higher compound feed to roughage ratio. Moreover, *Christensenellaceae* harbor a syntrophic relationship with *Methanobrevibacter* (Ruaud et al., 2020). They can form flocs that ease the interspecies transfer of H₂ in *Methanobrevibacter* to produce CH₄. The higher abundance of *Methanobrevibacter* in cows fed the experimental diets tends to confirm this narrative. Second, the decrease of *Succinivibrionaceae* and *Acidaminococcaceae* that ultimately produce propionate, an electron sink (Wang et al., 2023), strengthen the hypothesis of a dietary induced change of microbiota associated with CH₄ emissions. Third, to a lesser extent, the higher Richness and Shannon's index in cows fed the LMS and HRS diets are in accordance with Kruger Ben Shabat et al. (2016) who observed that higher CH₄ emissions were associated with similar indexes. Altogether, these findings suggest higher CH₄ emission associated with the experimental diets but this was not observed on CH₄ production. Such a contradiction could be explained by the fact that the cows fed the experimental diets had a higher intake of compound feed, which would accelerate the passage rate and thus reduce CH₄ emission, but the experimental silages would offset this reduction in CH₄ in the three ways described above. Further investigations should be carried on to decouple the effect of agroecological silages on CH₄ emission from the effect of compound feed.

An upcoming question related to this study is whether the modifications in rumen microbiota induced by agroecological practices might benefit the cows. Two of our results pointed in this direction. First, we measured in cows fed LMS diet, and to a certain extent HRS, a higher abundance of *Bacillaceae*, a family predominantly composed of the genus *Bacillus* which is a rumen probiotic with antibacterial activity (Mingmongkolchai and Panbangred, 2018). Second, we measured higher microbial diversity in rumen of cows fed diets that included medium to high botanical diversity. Community diversity is argued as a stability factor (Costa-Roura et al., 2022) thus we advocate to further explore robustness of the rumen microbiota in the context of agroecology. This could be achieved first through the correlation between plant botanical diversity with the microbial diversity and second by the assessment of microbial functions over the time after a disturbance.

5. Conclusion

Implementing agroecological practices at dietary level increased the diversity and modified the composition of the rumen microbiota in three dairy cattle breeds, although a time effect could not be ruled out. Compositional changes included a decrease in the relative abundance of *Ruminococcaceae* and an increase in the relative abundance of *Christensenellaceae* and *Methanobrevibacter*, which favor conditions for higher CH₄ production. Methane production was not increased by the diets, but neither was it decreased despite the higher intake of compound feed in the agroecological diets. This supports the hypothesis of a balanced effect of

agroecological silages and compound feed on CH₄ production. It is not clear whether the changes in rumen microbiota induced by agroecological practices can benefit cows but our results tend to point toward this and should be assessed further. Finally, this study paths the way to understand the potential of microbiota in alternative farming system such as agroecology.

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Authors’ contributions

L.S. conceptualized the study, L.S. and S.K.K. acquired funding, J.v.R. designed the experiment, L.K. managed the experiment and sampled rumen fluid, S.K.K. managed sequencing of the samples, A.B. managed infrastructure for data analysis, S.R. analyzed and interpreted data, S.R. and L.K. wrote the original draft, S.R., L.K., L.S., S.K.K., D.S., A.B. wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

None.

Data Availability

The data supporting the conclusions of this article are available in the Sequence Read Archive repository under BioProject PRJNA894831, <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA894831>

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.anifeedsci.2023.115716](https://doi.org/10.1016/j.anifeedsci.2023.115716).

References

- Altieri, M.A., 1999. The ecological role of biodiversity in agroecosystems. *Agric. Ecosyst. Environ.* 74, 19–31.
- Beauchemin, K.A., McAllister, T.A., McGinn, S.M., 2009. Dietary mitigation of enteric methane from cattle. *CABI Rev.* 2009, 1–18. <https://doi.org/10.1079/PAVSNR20094035>.
- Bueno de Mesquita, C.P., Wu, D., Tringe, S.G., 2023. Methyl-based methanogenesis: an ecological and genomic review. *Microbiol. Mol. Biol. Rev.* 87, e00024 <https://doi.org/10.1128/mbr.00024-22>.
- Cardinale, B.J., Wright, J.P., Cadotte, M.W., Carroll, I.T., Hector, A., Srivastava, D.S., Loreau, M., Weis, J.J., 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. *Proc. Natl. Acad. Sci. USA* 104, 18123–18128. <https://doi.org/10.1073/pnas.0709069104>.
- Costa-Roura, S., Villalba, D., Balcells, J., De la Fuente, G., 2022. First steps into ruminal microbiota robustness. *Animals* 12, 2366. <https://doi.org/10.3390/ani12182366>.
- CVB Tabellenboek Voeding Herkauwers 2022 voedernormen Rundvee, Schapen, Geiten en voederwaarden voedermiddelen voor Herkauwers CVB-reeks nr. 65 November 2022.
- Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutschord, M., Seifert, J., 2017. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8, 1605. <https://doi.org/10.3389/fmicb.2017.01605>.
- Dumont, B., Fortun-Lamothe, L., Jouven, M., Thomas, M., Tichit, M., 2013. Prospects from agroecology and industrial ecology for animal production in the 21st century. *Animal* 7, 1028–1043. <https://doi.org/10.1017/S1751731112002418>.
- Dumont, B., González-García, E., Thomas, M., Fortun-Lamothe, L., Ducrot, C., Dourmad, J.Y., Tichit, M., 2014. Forty research issues for the redesign of animal production systems in the 21st century. *Animal* 8, 1382–1393. <https://doi.org/10.1017/S1751731114001281>.
- Dumont, B., Puillet, L., Martin, G., Savietto, D., Aubin, J., Ingrand, S., Niderkorn, V., Steinmetz, L., Thomas, M., 2020. Incorporating Diversity Into Animal Production Systems Can Increase Their Performance and Strengthen Their Resilience. *Front. Sustain. Food Syst.* 4, 109. <https://doi.org/10.3389/fsufs.2020.00109>.
- Fernando, S.C., Purvis, H.T., Najjar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G., Roe, B.A., DeSilva, U., 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol.* 76, 7482–7490. <https://doi.org/10.1128/AEM.00388-10>.
- Finn, J.A., Kirwan, L., Connolly, J., Sebastia, M.T., Helgadottir, A., Baadshaug, O.H., Bélanger, G., Black, A., Brophy, C., Collins, R.P., Čop, J., Dalmannsdóttir, S., Delgado, I., Elgersma, A., Fothergill, M., Frankow-Lindberg, B.E., Ghesquiere, A., Golinska, B., Golinski, P., Grieu, P., Gustavsson, A.-M., Höglind, M., Huguenin-Elie, O., Jørgensen, M., Kadziulienė, Z., Kurki, P., Llorba, R., Lunnan, T., Porqueddu, C., Suter, M., Thumm, U., Lüscher, A., 2013. Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J. Appl. Ecol.* 50, 365–375. <https://doi.org/10.1111/1365-2664.12041>.
- Foster, Z., Sharpton, T., Grünwald, N., 2017. Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. *PLoS Comput. Biol.* 13, 1–15. <https://doi.org/10.1371/journal.pcbi.1005404>.
- Friggens, N.C., Blanc, F., Berry, D.P., Puillet, L., 2017. Review: Deciphering animal robustness. A synthesis to facilitate its use in livestock breeding and management. *Animal* 11, 2237–2251. <https://doi.org/10.1017/S175173111700088X>.
- Haas, G., Wetterich, F., Köpke, U., 2001. Comparing intensive, extended and organic grassland farming in southern Germany by process life cycle assessment. *Agric. Ecosyst. Environ.* 83, 43–53. [https://doi.org/10.1016/S0167-8809\(00\)00160-2](https://doi.org/10.1016/S0167-8809(00)00160-2).
- Humbert, J.-Y., Pellet, J., Buri, P., Arlettaz, R., 2012. Does delaying the first mowing date benefit biodiversity in meadowland. *Environ. Evid.* 1, 9. <https://doi.org/10.1186/2047-2382-1-9>.

- Huws, S.A., Creevey, C.J., Oyama, L.B., Mizrahi, I., Denman, S.E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S.M., Hess, M., Tapio, I., Smidt, H., Krizsan, S.J., Yáñez-Ruiz, D.R., Belanche, A., Guan, L., Gruninger, R.J., McAllister, T.A., Newbold, C.J., Roehe, R., Dewhurst, R.J., Snelling, T.J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A.H., Scollan, N.D., do Prado, R.M., Pilau, E.J., Mantovani, H.C., Attwood, G.T., Edwards, J.E., McEwan, N.R., Morrisson, S., Mayorga, O.L., Elliott, C., Morgavi, D.P., 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9, 2161. <https://doi.org/10.3389/fmicb.2018.02161>.
- Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160, 1–22. <https://doi.org/10.1016/j.anifeeds.2010.07.002>.
- Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P.H., Shi, W., Froula, J., Rubin, E.M., Attwood, G.T., 2016. Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a *Sharpea*-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4, 56. <https://doi.org/10.1186/s40168-016-0201-2>.
- Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P.H., 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS One* 9, e103171. <https://doi.org/10.1371/journal.pone.0103171>.
- Kurth, J.M., Op den Camp, H.J.M., Welte, C.U., 2020. Several ways one goal—methanogenesis from unconventional substrates. *Appl. Microbiol. Biotechnol.* 104, 6839–6854. <https://doi.org/10.1007/s00253-020-10724-7>.
- Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest package: tests in linear mixed effects models. *J. Stat. Softw.* 82, 1–26. <https://doi.org/10.18637/jss.v082.i13>.
- Lenth, R.V., 2021. emmeans: Estimated Marginal Means, aka Least-Squares Means.
- Lourenço, M., Van Ranst, G., Vlaeminck, B., De Smet, S., Fievez, V., 2008. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. *Anim. Feed Sci. Technol.* 145, 418–437. <https://doi.org/10.1016/j.anifeeds.2007.05.043>.
- Magne, M.A., Thénard, V., Mihout, S., 2016. Initial insights on the performances and management of dairy cattle herds combining two breeds with contrasting features. *Animal* 10, 892–901. <https://doi.org/10.1017/S1751731115002840>.
- McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8, e61217.
- Mingmongkolchai, S., Panbangred, W., 2018. *Bacillus* probiotics: an alternative to antibiotics for livestock production. *J. Appl. Microbiol.* 124, 1334–1346. <https://doi.org/10.1111/jam.13690>.
- Mizrahi, I., Jami, E., 2018. Review: The compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal* 12, s220–s232. <https://doi.org/10.1017/S1751731118001957>.
- Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024–1036. <https://doi.org/10.1017/S1751731110000546>.
- Mucio-Álvarez, M., Albarrán-Portillo, B., López-González, F., Arriaga-Jordán, C.M., 2021. Multi-species pastures for grazing dairy cows in small-scale dairy systems in the highlands of Mexico. *Trop. Anim. Health Prod.* 53, 113. <https://doi.org/10.1007/s11250-021-02564-y>.
- Muizelaar, W., Bani, P., Kuhla, B., Larsen, M., Tapio, I., Yáñez-Ruiz, D., van Gastelen, S., 2020. Rumen fluid sampling via oral stomach tubing method. *Methods Cattle Physiol. Behav. Res. - Recomm. SmartCow Consort.* <https://doi.org/10.5680/mcpb008>.
- Newbold, C.J., Ramos-Morales, E., 2020. Review: Ruminant microbiome and microbial metabolome: effects of diet and ruminant host. *Animal* 14, s78–s86. <https://doi.org/10.1017/S1751731119003252>.
- Nocera, J.J., Parsons, G.J., Milton, G.R., Fredeen, A.H., 2005. Compatibility of delayed cutting regime with bird breeding and hay nutritional quality. *Agric. Ecosyst. Environ.* 107, 245–253. <https://doi.org/10.1016/j.agee.2004.11.001>.
- Paz, H.A., Anderson, C.L., Muller, M.J., Kononoff, P.J., Fernando, S.C., 2016. Rumen bacterial community composition in holstein and jersey cows is different under same dietary condition and is not affected by sampling method. *Front. Microbiol.* 7. <https://doi.org/10.3389/fmicb.2016.01206>.
- Peratoner, G., Pötsch, E.M., 2019. Methods to describe the botanical composition of vegetation in grassland research. *Die Bodenkult.: J. Land Manag., Food Environ.* 70, 1–18. <https://doi.org/10.2478/boku-2019-0001>.
- Prescott, M.J., Lidster, K., 2017. Improving quality of science through better animal welfare: the NC3Rs strategy. *Lab Anim.* 46, 152–156. <https://doi.org/10.1038/labana.1217>.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>.
- R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ramayo-Caldas, Y., Zingaretti, L., Popova, M., Estellé, J., Bernard, A., Pons, N., Bellot, P., Mach, N., Rau, A., Roume, H., Perez-Enciso, M., Faverdin, P., Edouard, N., Ehrlich, D., Morgavi, D.P., Renand, G., 2020. Identification of rumen microbial biomarkers linked to methane emission in Holstein dairy cows. *J. Anim. Breed. Genet.* 137, 49–59. <https://doi.org/10.1111/jbg.12427>.
- Rinne, M., Jaakkola, S., Huhtanen, P., 1997. Grass maturity effects on cattle fed silage-based diets. 1. Organic matter digestion, rumen fermentation and nitrogen utilization. *Anim. Feed Sci. Technol.* 67, 1–17. [https://doi.org/10.1016/S0377-8401\(96\)01141-8](https://doi.org/10.1016/S0377-8401(96)01141-8).
- Roca-Fernández, A.I., Peyraud, J.L., Delaby, L., Delagarde, R., 2016. Pasture intake and milk production of dairy cows rotationally grazing on multi-species swards. *Animal* 10, 1448–1456. <https://doi.org/10.1017/S1751731116000331>.
- Ruad, A., Esquivel-Elizondo, S., de la Cuesta-Zuluaga, J., Waters, J.L., Angenot, L.T., Youngblut, N.D., Ley, R.E., 2020. Syntrophy via interspecies H₂ transfer between *Christensenella* and *Methanobrevibacter* underlies their global cooccurrence in the human gut. *mBio* 11, e03235. <https://doi.org/10.1128/mBio.03235-19>.
- Shabat, S.K.B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M.E., White, B.A., Shterzer, N., Mizrahi, I., 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. <https://doi.org/10.1038/ismej.2016.62>.
- Smith, P.G.R., Wells, M., Cant, J.P., Wright, T., Kyle, J., Roberts, P., Giraldo, M.R., 2022. Hay nutritional quality and grassland bird nesting: impact of delaying first hay cut on dairy and beef production in Ontario. *SAR* 11, 14. <https://doi.org/10.5539/sar.v11n2p14>.
- Tamminga, S., Van Straalen, W.M., Subnel, A.P.J., Meijer, R.G.M., Steg, A., Wever, C.J.G., Blok, M.C., 1994. The Dutch protein evaluation system: the DVE/OEB-system. *Livest. Prod. Sci.* 40, 139–155. [https://doi.org/10.1016/0301-6226\(94\)90043-4](https://doi.org/10.1016/0301-6226(94)90043-4).
- Tapio, I., Snelling, T.J., Strozzi, F., Wallace, R.J., 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8, 7. <https://doi.org/10.1186/s40104-017-0141-0>.
- Totty, V.K., Greenwood, S.L., Bryant, R.H., Edwards, G.R., 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *J. Dairy Sci.* 96, 141–149. <https://doi.org/10.3168/jds.2012-5504>.
- Ungerfeld, E.M., 2020. Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front. Microbiol.* 11, 589. <https://doi.org/10.3389/fmicb.2020.00589>.
- Vaidya, J.D., van Gastelen, S., Smidt, H., Plugge, C.M., Edwards, J.E., 2020. Characterization of dairy cow rumen bacterial and archaeal communities associated with grass silage and maize silage based diets. *PLoS ONE* 15, e0229887. <https://doi.org/10.1371/journal.pone.0229887>.
- van Groenigen, J.W., Lubbers, I.M., Vos, H.M.J., Brown, G.G., De Deyn, G.B., van Groenigen, K.J., 2014. Earthworms increase plant production: a meta-analysis. *Sci. Rep.* 4, 6365. <https://doi.org/10.1038/srep06365>.
- Villalba, J.J., Ates, S., MacAdam, J.W., 2021. Non-fiber Carbohydrates in Forages and Their Influence on Beef Production Systems. *Front. Sustain. Food Syst.* 5, 566338. <https://doi.org/10.3389/fsufs.2021.566338>.
- Volaire, F., Barkaoui, K., Norton, M., 2014. Designing resilient and sustainable grasslands for a drier future: Adaptive strategies, functional traits and biotic interactions. *Eur. J. Agron.* 52, 81–89. <https://doi.org/10.1016/j.eja.2013.10.002>.
- Wang, K., Xiong, B., Zhao, X., 2023. Could propionate formation be used to reduce enteric methane emission in ruminants. *Sci. Total Environ.* 855, 158867. <https://doi.org/10.1016/j.scitotenv.2022.158867>.

- Weimer, P.J., 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.00296>.
- Willem Erisman, J., van Eekeren, N., de Wit, J., Koopmans, C., Cuijpers, W., Oerlemans, N., Koks, J., 2016. Agriculture and biodiversity: a better balance benefits both. B., 1 Louis Bolk Institute, Hoofdstraat24, 3972 LA Driebergen, The Netherlands *AIMS Agric. Food* 1, 157–174. <https://doi.org/10.3934/agrfood.2016.2.157>.
- Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., He, Y., Zhang, H., 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in holstein heifers. *Front. Microbiol.* 8, 2206. <https://doi.org/10.3389/fmicb.2017.02206>.