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Methane Emission and Milk Production from Jersey Cows Grazing Perennial Ryegrass–White Clover and Multispecies Forage Mixtures

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Abstract: Methane is a major constituent of greenhouse gas (GHG) emissions from ruminants, and mitigation strategies are needed to alleviate this negative environmental impact while maintaining the environmental and other benefits of grazing systems. Forages containing plant-specialized metabolites (PSM), particularly condensed tannins, may help reduce enteric methane (CH_4) emissions. However, information on in vivo CH_4 emissions from cows grazing mixtures that contain bioactive herbs is scarce. Accordingly, this study compared a binary mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) against a diverse mixture of six additional species, including tannin-rich species like birdsfoot trefoil (*Lotus corniculatus*) and salad burnet (*Sanguisorba minor*), in a full-grazing dairy system. Enteric CH_4 emissions were measured using the SF_6 tracer technique. Cows grazing diverse mixtures increased their energy-corrected milk (ECM) yield by 4% ($p < 0.001$) compared with binary mixtures. However, CH_4 emissions per kg ECM were also 11% greater for the diverse mixtures ($p < 0.05$). The very high feed quality and milk yield from both mixtures explained the low CH_4 emissions recorded relative to the milk output. The addition of forbs did not provide additional benefits at these intensities, as they were maintained in low yield shares throughout.

Keywords: polyphenols; climate smart agriculture; biodiversity; pasture; tannins; forage production

1. Introduction

Enteric methane (CH_4) is a byproduct of ruminal fermentation that allows the ruminal ecosystem to dispose of the metabolic hydrogen produced during microbial metabolism [1,2]. This is problematic in terms of both agricultural utilization and environmental impact. Ruminal CH_4 emissions represent a loss of feed energy through inefficient digestion. This is reported to be equivalent to 144 million tons of oil, globally, and enteric CH_4 is responsible for 44% of the anthropogenic methane emissions [3]. Methane is a potent greenhouse gas, and it has been calculated that 20% of the radiative forcing since 1750 may be attributed to methane emissions [4]. However, despite this environmental impact, ruminants have an important role in supporting human nutrition and, unlike monogastrics, they have the evolutionary advantage of producing high-quality food (meats and milk) from grass.

Thus, otherwise, marginal lands, including sites where other forms of agriculture cannot easily be supported, may be utilized by grazing to produce edible energy and protein for humans. Grasslands are also particularly important in providing ecosystem services, including climate change mitigation. This is linked particularly to their high carbon sequestration potential, with mean rates for the temperate conditions of 0.54 Mg C ha^{-1} yr^{-1} [5]. Other grassland-related ecosystem services include water purification and biodiversity enhancement [6]

The utilization of grasslands by grazing has also shown benefits in terms of the high quality of food products, notably through improved fatty acid profiles of milk and meats by increasing the conjugated linoleic acid (CLA) and polyunsaturated fatty acids (PUFA) concentrations, as compared with silage feeding; these attributes have potential benefits for human health [7–9]. Hence, grass-based dairy systems are considered to be not only highly competitive [10] but also beneficial for environmentally friendly and economically viable production [10,11]. Accordingly, grazing has been shown to be positively linked to consumer preference [12,13].

In the case of high-yielding dairy cows, it is important to determine the extent to which their metabolizable energy requirements can be met by forage. It has been well-established that energy intake is driven by a combination of forage quality (as determined by its fiber and protein contents) and the characteristics of the animals (breed, age, body weight, stage of lactation, and milk yield) that regulate the energy requirements and intake capacity [14–18]. In this way, the maximum energy intakes are achieved by the maximization of the nutrient concentration in the diet combined with a high feed intake. If the nutritional value of the forage is low, then, even with a high feed intake, the total energy intake will be insufficient to achieve the maximum output from high-performance cattle.

A meta-analysis by Peyraud and Delagarde [19] showed that high-quality forages (7.2-MJ Net Energy of Lactation (NEL) kg dry matter (DM^{-1}), on average) can meet the feed energy requirements of 400-kg cows to produce 30 kg of fat-corrected milk (FCM) without supplementation. Similar levels of milk production could be also obtained from pastures with an herbage of lower energy content (6.5–6.8-MJ NEL kg DM^{-1}) and moderate concentrate supplementation (1.0–3.5 kg DM day^{-1}).

However, poorly implemented grazing and pasture management can also result in increased nutrient losses [20] and reductions in digestibility of the herbage in the sward. This can lead to greater GHG emissions [21,22], thereby reducing the efficiency of forage utilization and overall sustainability of the system. A meta-analysis by Lorenz et al. [23] found that the forage efficiency has a significant effect on the carbon footprint of pasture-based systems, with low GHG emissions per product unit being achievable with improved grazing management, whereas the carbon footprint of 1 kg ECM milk can increase two to three-fold if the production system is inefficient. As CH_4 is usually the major source of GHG emissions from dairy production, mitigation strategies are required that alleviate these negative impacts while also maintaining the benefits of grazing-based dairy production. The main factors determining CH_4 emissions are: (a) the quantity and quality of the animal feed consumed, (b) the cattle breed and its yield of product (milk/meat), and (c) the composition of the rumen microbiome, with a focus on the methanogenic archaea. All these factors are interlinked, as the forage intake capacity is affected by the cattle breed and passage rates (which are also affected by the feed), while the feed determines the rumen microbiome and, again, the intake capacity. In those highly productive systems that have the lowest carbon footprints per liter of milk, there is little further potential in reducing emissions by feeding concentrates or using feed additives such as fats and oil seeds [24]. Consequently, the modification of the rumen microbiome has become the focus of research, including the use of halogen compounds and ionophores. However, as these are currently prohibited in the EU, it is the use of phytogenic substances and plants containing plant-specialized metabolites (PSM) such as tannins that may be considered as promising solutions to reduce CH_4 emissions [25–28].

In particular, the potential of plants containing PSM is an interesting solution, as these species (a) may be incorporated into diets within both grazing and confinement systems, (b) may provide additional benefits for ecosystem services, such as potential increments in carbon immobilization [29,30], and (c) provide animal health benefits, such as anthelmintic effects and frothy bloat prevention [31]. In the rumen, PSM and, particularly, proanthocyanidins (PAs) (syn. condensed tannins) bind with the dietary protein, protecting it from enzymatic hydrolysis, hence reducing the enteric methane production [26]. These complexes dissociate in the intestines at low pH, thus enabling the protein to be absorbed. As well as suppressing CH_4, this increases the concentration of utilizable protein at the duodenum (uCP) and, hence, enhances the forage nutritive value without depressing the rumen fiber digestion or daily intake [32,33]. These beneficial properties are dependent on both the concentration and structural composition of the PAs and, hence, are plant species-dependent [31,34,35]. Additionally, red clover contains polyphenol oxidase (PPO), which also has the potential to increase uCP and to increase the nitrogen use efficiency along the digestive tract [36].

Introducing herbs and legumes into mixtures for grasslands provides numerous benefits for productivity [37], yield stability [38], carbon inputs [39], and resource use efficiency as a result of the maximized complementarity in niche exploitation. However, these benefits do not necessarily continue indefinitely, and the forage quality of multispecies mixtures can be reduced due to difficulties in determining the optimal utilization timepoint as a result of differences in the maturation speed. There are also potential problems from introducing species if other aspects of their forage quality compare unfavorably to perennial ryegrass. Yet, if it were possible to combine the agronomic benefits of mixed-species swards with the bioactive benefits due to additionally increased PSM contents in the pasture, this could substantially reduce the environmental impact of pasture-based dairy production. For this to be feasible, the plant species in a sward mixture need to be agronomically competitive and have a growth strategy adapted to grazing, in addition to properties for rumen bioactivity. If these attributes can be met, the target species could be included in sward mixtures with high yields and energy densities, while also alleviating negative externalities, such as methane emissions. Due to the limited persistence of many broad-leaved species, such as red clover and herbs, this potential is likely to be limited to ley systems of a maximum of two to three years [37]. Information on methane emissions from cow grazing mixtures, including bioactive herbs, is scarce, and almost all information has been obtained from in vitro studies using the herbs only or herb extracts [40,41].

Therefore, we designed an in vivo experiment based on the SF_6 tracer technique [42] on an intensive pasture-based dairy farm to identify the potential for increased pasture diversity, using herbs containing PSM with the potential to lower the CH_4 emissions per kg of milk.

We hypothesized that:

I. Diverse mixtures, including species rich in PSM and PPO-rich red clover (RC), will improve the protein use efficiency, thus increasing milk yields.
II. The inclusion of PSM-rich legumes and herbs will reduce the methane intensity (g CH_4 kg FCM^{-1}) as a result of both the increased milk yield and the anti-methanogenic effect from polyphenols.
III. Despite the increased species number, diverse mixtures would not decrease the energy yields compared to binary mixtures if the grazing intervals are short enough to provide only material with high digestibility.

2. Materials and Methods

The experiment was conducted at the Lindhof experimental farm in Northern Germany (54°27′56.0″ N 9°57′56.0″ E) from March to September 2019. The climate at the Lindhof is temperate maritime (8.7 °C and 785-mm annual means, 1981–2010), and soil types vary between Cambisols, Luvisols, Stagnosols, and Coluvic Regosols. The soil texture at the experimental site is sandy loam and loamy sand.

2.1. Weather Conditions

According to Germany's National Meteorological Service (Deutscher Wetterdienst (DWD)), mean temperatures for the "Kiel-Holtenau" weather station were 1.3 °C below the 30-year average (1981–2010) in both of the experimental periods (P1 and P2) during the year (Table 1). Accumulated rainfall was greater (+16 mm) than the long-term average for P1 and lower than the 30-year average for P2 (−9 mm).

Table 1. Mean temperature and cumulative precipitation during the experimental months (May and August) in the experimental year 2019 and the 30-year average.

	Mean Temp (°C)		**Rain (mm)**	
	2019	**1981–2010**	**2019**	**1981–2010**
May (P1)	10.6	11.9	70.4	54.0
August (P2)	17.0	18.3	74.0	82.5

2.2. Pasture and Grazing Management

The Lindhof grazing system is arranged as a two-year grass–clover ley system in an organic 4-year crop rotation (2 years of grass–clover, followed by 2 years of crops: oats/winter triticale and faba beans/winter spelt, consecutively). As the site is under organic management, no mineral N fertilization is added, but limestone, rock phosphate, and potassium sulphate are applied in biannual intervals. The average soil nutrient contents at 0–30-cm-depths were 19.4-g P_2O_5, 17.6-g K_2O, and 12.0 g Mg per 100-g dry soil; the pH was 6.2, and the average C/N ratio was 10.9.

The experimental grazing area comprised two paddocks of approx. 2.2 ha per treatment subdivided into daily strips, grazed successively. A new strip was offered to the cows after each morning of milking. Back grazing was prevented by electric fencing. The area of each strip was calculated based on the available pregrazing herbage mass (HM) and the established daily allowance. The target forage allowances for the experimental swards were 18 and 14-kg DM per cow and day for P1 and P2, respectively, and the target residue was generally set at 4-kg DM per cow and per day. The composition and sowing rate of the experimental sward is given in Table 2.

Table 2. Composition and sowing rate (kg ha^{-1}) of the binary and diverse seed mixtures used in the experiment.

Species	**Variety**	**Ploidy**		**Seed Mixture**	
			Abbr [1]	**Binary**	**Diverse**
Perennial ryegrass (*Lolium perenne*)	Discus	2n [2]	PRG	6.0	4.0
	Calvano1	2n	PRG	6.0	4.0
	Astonenergy	4n [3]	PRG	6.0	4.0
	Astonhockey	4n	PRG	6.0	4.0
White clover (*Trifolium repens*)	Vysocan		WC	2.0	0.65
	Liflex		WC	2.0	0.65
Red clover (*Trifolium pratense*)	Harmonie	2n	RC	—	1.5
	Larus	4n	RC	—	1.5
Ribwort plantain (*Plantago lanceolata*)	"native"		LP	—	1.0
Chicory (*Cichorium intybus*)	Spadona		CI	—	2.0
Salad burnet (*Sanguisorba minor*)	Burnet		SB	—	2.0
Caraway (*Carum carvi*)	Volhouden		CC	—	2.0
Birdsfoot trefoil (*Lotus corniculatus*)	Lotanava		BFT	—	3.0

[1] Abbreviation of each species used in this experiment. [2] 2n: diploid cultivars. [3] 4n: tetraploid cultivars.

Five weeks prior to the first measurement period, all animals started grazing on the same paddocks, which were a selection of both the binary and diverse mixture (for details, see Table 2). This procedure was applied to ensure the adaptation of the ruminal microbiota to a pasture-based diet and to all species that were subsequently included in the treatments.

2.3. Animals, Experimental Design, and Treatment

Animal procedures were conducted in accordance with the German Animal Welfare Act (TierSchG) and were authorized by the Animal Welfare Commission of the Ministry of Energy, Agriculture, Environment, and Rural Areas of the Federal State of Schleswig-Holstein, Germany (V 242-32938/2018). Twenty-four multiparous lactating Jersey cows were selected for the experiment. On average (±SD), they had pre-experimental milk yields of 17.4 ± 2.7 kg day^{-1}, days in milk (DIM) of 45 d ± 28, body weights of 448 kg ± 51, and parities of 3 ± 1. For the measurement periods, the animals were grouped into homogeneous pairs, based on all the above-mentioned factors, one of which was assigned randomly to each of the two groups to create comparable subgroups. The experimental periods were conducted using a balanced crossover design (two treatments × two subperiods, with each subperiod consisting of 4 days of adaptation followed by 4 days of measurements). As both mixtures were grazed by all cows for at least the previous 5 weeks, the rumen biome was considered to be stable and adjusted to both mixtures. Based on the observed metabolic plasticity of the rumen microbes, the four days were then considered to be sufficient for the stable and preadapted microbes to adapt to the specific substrate and shift the formation of the fermentation products accordingly [43]. All the cows were given 2 kg (±0.5) of concentrate (mixture of faba bean, triticale, lupins, and maize) on a fresh matter basis per day, in two feedings, during milking. Details of the compositions of the seed mixtures representing the two treatments in this experiment are detailed in Table 2.

2.4. Herbage Measurements

Due to the research farm being certified as organic in accordance with EU standards (Regulation (EC) No 834/2007), as well as in accordance with the stricter regulations of the "Bioland" certification (the largest organic food association in Germany), the use of tracers of any kind to measure grass intake is prohibited. As this prevents the measuring of individual feed intake in the grazing animals, the individual DM intake (DMI) for each cow and subperiod was estimated according to the model developed by Gruber et al. [44] based on the days in milk (DIM), cow breed, parity, body weight (BW), and milk yield (MY). Gruber et al. [44] was selected after the assessment of the performance of four different models, having provided the most accurate results [45]. To validate the model for the experimental conditions, the biomass on offer and residues were measured using both an electronic rising platemeter (Grasshopper, True North Technologies, Shannon, Ireland), taking 100 measurements per treatment made at random intervals while traversing the plot in a zigzag across each grazing area and by cutting ten randomly chosen quadrats of 0.25 m^2 per treatment to a height of 4 cm by means of hand-operated grass clippers. These methods allowed the calculation of a group intake and, thus, a calculation of the average consumption per cow. The cut samples were subsequently sorted to determine the botanical composition. For this procedure, each sample was collected in plastic bags and weighed immediately after cutting. From the hand-cut samples, a 100-g subsample was taken for hand separation to determine the botanical composition. Unsown species were collected separately. All fractions were weighed and dried at 58 °C for 48 h. After drying, samples were weighed for DM determination and milled to a particle size of 1 mm (Ultra-centrifugal mill, ZM200, Retsch GmbH, Haan, Germany) for chemical analysis. Forage use efficiency (FUE) was estimated as the difference between the offered and the remaining herbage, as determined by the cut quadrats, according to the following equation:

Forage use efficiency (%) = (offered herbage − remaining herbage)/offered herbage, where offered herbage is the herbage offered to the cows before grazing, and remaining herbage is the herbage remaining after grazing, both expressed in kg DM/ha.

2.5. Animal Measurements

Throughout the study, cows were milked twice a day (6 a.m. and 4 p.m.) (DeLaval MPC 680, DeLaval AB Tumba, Sweden) and individual milk yield was recorded automatically (DeLaval MM15, DeLaval AB Tumba, Sweden) every time, during 4 consecutive days (d5 to d8 of each subperiod, for both periods). Milk composition (fat, protein, and lactose) was determined daily from morning and afternoon milk samples (d5 to d8 of each subperiod), and energy-corrected milk (ECM) was estimated according to Sjaunja [46] as: ECM = MY $\times$ (0.2534 + 0.1226 $\times$ fat + 0.0776 $\times$ protein), where MY is the measured milk yield (kg cow day^{-1}), and fat and protein are the daily concentrations (%) of fat and protein, respectively. Body weight was recorded on each day of the experimental period after each milking. Variation in BW was calculated as the difference of the individual mean weight at the beginning and the end of each period.

Enteric CH_4 emissions were measured using the SF_6 tracer technique following the manual of Global Research Alliance for Greenhouse Gases on Agriculture [47] and using the adaptation of Gere and Gratton [42] for a 4-day collection period. Seven days prior to the beginning of the first measurements, a precalibrated SF_6 permeation tube was introduced per os into the rumen of each cow using a plastic dosing applicator. The mean permeation rate of the SF_6 capsules used in this experiment was 4.5 $\pm$ 0.97 mg d^{-1}. Permeation rates were validated gravimetrically by serial weighing of the SF_6 permeation tubes (kept at 39 °C (the nominal rumen temperature in cattle) in a water bath) with an analytical balance over a period of ~6 weeks. Only capsules with R^2 values exceeding 0.9995 were selected to be used in the experiment. The sampling system for the collection of the exhaled air consisted of 0.5-L canisters (made of stainless steel or polyvinyl chloride (PVC)) and a ball baring inflow restrictor adjusted to accumulate 0.5 bar of air sample during a 4-day period (d5 to d8 of each subperiod) and a 6-mm Ø polyethylene (PE) tube, used to connect both. The gas sampling tube was protected by a flexible PVC tube (16-mm inner Ø). The inflow restrictor was protected against water and dust by a double filter [42] and was located above the animal's nostrils, using a canvas halter adapted for this purpose. To ensure the comfort of the cows, as well as the reliability of the sampling, two canisters were fitted on the back of each cow using a foam-filled canvas belt, also adapted for this purpose. Each belt was mounted between the shoulders and hipbones and secured using an adjustable leather strap extending from the top of the belt to a padded strap around the base of the cows' tail. Simultaneously (d5 to d8), background air samples were collected at two points in each experimental plot, using the same system as for the breath samples. Immediately prior to the sampling period, each collecting canister was evacuated (<0.5 mb) after being cleaned with high-purity nitrogen gas (N_2). The exhaled and background air samples collected were analyzed for concentrations of CH_4 (ppm, parts per million by volume) and SF_6 (ppt, parts per trillion by volume) by gas chromatography (SCION 456-GC, Bruker, Leiderdorp, The Netherlands) immediately after the end of each measuring period. The method was calibrated using four CH_4 standards (Linde GmbH, Pullach, Germany) with defined concentrations, as well as a 10-point dilution curve of an SF_6 standard (Linde GmbH, Pullach, Germany), covering the entire expected range of SF_6 concentrations in the samples. After correction for background gas concentrations, the daily CH_4 emissions were calculated according to the following equation from Gere and Gratton [42]: Q_{CH4} = $Q_{SF6} \times ([CH_4]/([SF_6])$, where Q_{CH4} is the daily methane emission (g d^{-1}), Q_{SF6} is the SF_6 permeation rate of the inserted capsules, and $[CH_4]$ and $[SF_6]$ are the concentrations of these gases above the atmospheric concentration.

2.6. Chemical Analysis

Forage quality parameters of all samples were determined by near-infrared reflectance spectroscopy (NIRS) with a NIRSystems 5000 monochromator (FOSS, Laurel, MD, USA). Mathematical evaluation of the spectra was performed using the Modified Partial Least Squares method (WinISI software version 3, Infrasoft International, Mumbai, MH, India).

Calibration and validation were based on sample subsets of perennial ryegrass, legumes, and forage herb species, which represented the whole spectral and chemical variability.

The following analyses of the subset samples were performed as follows: The N concentration was directly determined with an elemental analyzer (Vario Max CN, Elementar Analysensysteme, Hanau, Germany); the crude protein content (CP) was calculated from the N content (CP = N · 6.25). The concentrations of NDF (assayed with heat-stable amylase (aNDF)) and ADF were analyzed using the Fiber Analyzer Ankom A2000 (Ankom Technology, Macedon, NY, USA). The ADF values were expressed exclusive from the residual ash (ADFom). Ash (A) was determined by combustion in a muffle furnace (24 h at 550 °C). Digestibility and metabolizable energy (ME) content of herbage samples was determined using the in vitro cellulase technique developed by De Boever, Cottyn [48]. The percentage of digestible organic matter (DOM) was calculated using the enzymatic soluble organic matter (ELOS) and the enzymatic insoluble organic matter (EULOS).

The following equations were used:

$$\text{DOM (\%)} = 100 \times (940 - \text{A-}0.62 \times \text{EULOS} - 0.000221 \times [\text{EULOS}]\hat{}2) / (1000 - \text{A}) \quad (1)$$

$$\text{EULOS (g kg}^{-1}\text{ DM)} = 1000 - \text{A} - \text{ELOS} \quad (2)$$

$$\text{ME (MJ kg}^{-1}\text{ DM)} = 5.51 + 0.00828 \times \text{ELOS} - 0.00511 \times \text{A} + 0.02507 \times \text{CL} - 0.00392 \times \text{ADFom} \quad (3)$$

The net energy lactation content was derived from the metabolizable energy content (ME) and was estimated using the following equation: NEL (MJ kg^{-1} DM) = ME (0.46 + 12.38 ME/(1000 − A).

The statistical key figures of the NIRS calibration and validation are given in Table A1 of the Appendix A.

The concentrations of CH_4 and SF_6 were determined by gas chromatography (SCION 456-GC, Bruker, Leiderdorp, The Netherlands). The samples were injected in two different setups: a 3-mL loop, a HP-PLOT Q column, and a flame ionization detector (FID) for CH_4 and a 10-mL loop, a HP-MOLSIV column, and an electron capture detector (ECD) for SF_6. Each sample was analyzed at least twice, and the average values were used to obtain the CH_4 concentration and CH_4 emissions. The maximum delay between the collection and the determination of the CH_4 and SF_6 concentrations was 15 days.

2.7. Statistical Analysis

All experimental data were analyzed with statistical software R [49].

Sward characteristics and chemical composition for pre- and post-grazing HM were analyzed with ANOVA followed by multiple contrast tests, using a linear mixed model, including the treatment (i.e., the mixture) and period (spring vs. autumn) and their interaction as fixed factors and the date of sampling within each subperiod as the random factor.

Milk data were also analyzed with ANOVA followed by multiple contrast tests, using a linear mixed model, including the treatment (i.e., the mixture) and period (May vs. August) and their interaction as fixed factors. However, the random factor was the experimental period nested in the individual cow. Additionally, the correlations of the measurement values due to the repeated measurements of each individual cow were considered.

The same model was also used for the methane data, without the correlation factor, however, as the methane values were sampled cumulatively over the sampling period.

All models were analyzed as split-plot designs. Based on graphical residual analyses, data were assumed to be normally distributed and heteroscedastic.

3. Results

3.1. Methane Emissions and Sward Characteristics

Information regarding the sward yield and quality are shown in Table 3. The mean values of the herbage mass per ha did not differ between treatments for P1 and was 2308-kg DM. However, it was significantly greater ($p < 0.001$) for the diverse mixture compared to the binary mixture in P2, with 1218- and 677-kg DM ha^{-1}, respectively. The paddock sizes were adjusted accordingly to provide an equal herbage mass on offer per cow. The FUE was similar between treatments over both periods, yet with a nonsignificant tendency of a greater FUE in the diverse pasture.

Table 3. Forage characteristics, milk production, target daily herbage availability, DM intake, herbage utilization, methane emissions, and body weight (BW) variation of dairy cows grazing on binary and diverse mixtures. Abbreviations are as follows (sorted alphabetically): ADF: acid detergent fiber, CP: crude protein, DHA: daily herbage allowance, DMI: dry matter intake, ECM: energy-corrected milk, FUE: forage use efficiency, HM: herbage mass, ME: metabolizable energy, NDF: neutral detergent fiber, and NEL: net energy for lactation.

	P1 (2–8 May 2019)		**P2 (15–30 August 2019)**	
	Binary	**Diverse**	**Binary**	**Diverse**
	Mean (SEM)	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**
Forage characteristics				
HM (kg DM ha^{-1})	2460 (177) ^{Aa}	2157 (68) ^{Aa}	677 (95) ^{Bb}	1218 (151) ^{Aa}
OM digestibility	87.6 (0.25) ^{Aa}	84.4 (0.17) ^{Ba}	80.2 (0.36) ^{Ab}	77.9 (0.44) ^{Bb}
ME (MJ kg DM^{-1})	12.5 (0.03) ^{Aa}	12.1 (0.01) ^{Ba}	11.3 (0.09) ^{Ab}	11.1 (0.05) ^{Bb}
NEL (MJ kg DM^{-1})	7.7 (0.01) ^{Aa}	7.5 (0.01) ^{Ba}	6.9 (0.06) ^{Ab}	6.7 (0.03) ^{Bb}
Chemical composition (g kg^{-1})				
CP	11.5 (0.52) ^{Ba}	15.6 (0.14) ^{Aa}	18.5 (0.79) ^{Bb}	20.3 (0.51) ^{Ab}
NDF	35.5 (0.29) ^{Ba}	38 (0.30) ^{Aa}	49.9 (0.66) ^{Bb}	45.3 (0.46) ^{Ab}
ADF	16.6 (0.20) ^{Ba}	19.5 (0.17) ^{Aa}	22.8 (0.40) ^{Bb}	26.7 (0.33) ^{Ab}
Fat	2.7 (0.1) ^{Bb}	3.1 (0.05) ^{Aa}	4.1(0.08) ^{Aa}	3.6 (0.06) ^{Ba}
Milk production				
Days in milk	49 (28)	49 (28)	154 (26)	154 (26)
Milk yield (kg cow^{-1} day^{-1})	23.4 (0.77) ^{Ba}	24.9 (0.86) ^{Aa}	18.6 (0.71) ^{Bb}	19.8 (0.66) ^{Ab}
ECM yield (kg cow^{-1} day^{-1})	29.4 (0.91) ^{Ba}	30.3 (0.98) ^{Aa}	22.1 (0.61) ^{Bb}	23.5 (0.64) ^{Ab}
Milk composition				
Fat content (g kg DM^{-1})	59.2 (1.09) ^{Aa}	56.6 (1.08) ^{Ba}	53.6 (1.19) ^{Bb}	53.7 (1.08) ^{Ab}
Protein content (g $kgDM^{-1}$)	35.7 (0.60) ^{Ab}	35.1 (0.51) ^{Bb}	38.0 (0.71) ^{Aa}	36.8 (0.66) ^{Ba}
Lactose content (g $kgDM^{-1}$)	47.9 (0.19) ^{Aa}	46.0 (0.21) ^{Ba}	45.8 (0.17) ^{Ab}	44.7 (0.17) ^{Bb}
Fat yield (kg day^{-1})	1.38 (0.05) ^{Ba}	1.41(0.05) ^{Aa}	0.98 (0.03) ^{Ba}	1.05 (0.03) ^{Ba}
Protein yield (kg day^{-1})	0.83 (0.02) ^{Ba}	0.87 (0.03) ^{Aa}	0.69 (0.02) ^{Ba}	0.71 (0.02) ^{Ba}
Lactose yield (kg day^{-1})	1.12 (0.04) ^{Ba}	1.15 (0.04) ^{Aa}	0.85 (0.03) ^{Ba}	0.89 (0.03) ^{Ba}
Target DHA (kg DM cow day^{-1})	18	18	14	14
DMI (kg DM cow day^{-1}) [1]	16.7	16.8	11.5	11.5
DMI (kg DM cow day^{-1}) [2]	13.0	15.0	10.6	13.0
FUE (%)	58	66	69	77
Methane emissions				
g CH_4 cow^{-1} day^{-1}	239 (8) ^{Bb}	277 (11) ^{Aa}	203 (8) ^{Bb}	242 (15) ^{Aa}
g CH_4 kg $milk^{-1}$	10.4 (0.4) ^{Ba}	11.2 (0.4) ^{Aa}	11.2 (0.4) ^{Ba}	12.6 (0.8) ^{Aa}
g CH_4 kg ECM^{-1}	8.3 (0.4) ^{Bb}	9.2 (0.3) ^{Aa}	9.3 (0.6) ^{Bb}	10.4 (0.3) ^{Aa}
g CH_4 kg DMI^{-1}	14.3	16.5	17.7	21
BW variation (kg day^{-1})	−0.2 ^{Aa}	−0.3 ^{Aa}	0.1 ^{Aa}	0.3 ^{Aa}

[1] Estimated according to Gruber et al. (2004) and includes 2-kg concentrate. [2] Estimated with pre- and post-grazing measurements of the herbage mass in addition to 2 kg of concentrate. ^{A,B} Differences between treatment, according to the adjusted *p*-value method. ^{a,b} Differences between the periods, according to the adjusted *p*-value method.

The chemical composition of both herbage mixtures on offer generally identified a high quality for both mixtures across P1 and P2, despite decrements over time. On average, across P1 and P2, the organic matter digestibility (OMD) and energy content (ME and NEL) mean values of the binary mixture were 3.5% greater ($p < 0.001$) than that of the diverse mixtures. In contrast, the crude protein and ADF contents were, on average, ~ 15% lower ($p < 0.001$) for the binary mixtures across both periods.

The botanical composition of the offered forage is shown in Table 4. Perennial ryegrass was the predominant species in both swards. When comparing the botanical composition of the residual swards after grazing with that of the offered forage, a positive selection by grazing animals for the herb species in diverse mixtures could be identified, with herb shares decreasing by 66% in P1 and 55% in P2 when compared to the offered herbage. The preference for white clover was intermediate, and perennial ryegrass was the least-preferred species by the cows in this mixture. In the binary mixtures, the data indicate a preference towards white clover over perennial ryegrass.

Table 4. Botanical composition (%) of the offered (Offer) and remaining (rest) herbage for the binary and diverse mixtures during both experimental periods. Abbreviations are as follows: PRG: perennial ryegrass, WC: white clover, RC: red clover, and BFT: birdsfoot trefoil.

	P1 (2–18 May 2019)				**P2 (15–30 August 2019)**			
	Binary		**Diverse**		**Binary**		**Diverse**	
	Offer	**Rest**	**Offer**	**Rest**	**Offer**	**Rest**	**Offer**	**Rest**
	Mean (SEM)	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**	**Mean (SEM)**
PRG	87.1 (2.0) ^{aBx}	96.4 (0.6) ^{aBy}	43.1 (2.2) ^{aAx}	74.0 (1.2) ^{aAy}	51.6 (3.3) ^{aBx}	61.1 (3.4) ^{aBy}	25.4 (2.2) ^{aAx}	36.8 (2.6) ^{aAx}
WC	12.5 (2.0) ^{aAx}	3.1 (0.5) ^{aBy}	15.0 (1.5) ^{aAx}	7.5 (0.7) ^{aAy}	47.6 (3.2) ^{aBx}	36.9 (3.3) ^{aBy}	20.4 (2.0) ^{aAx}	17.9 (1.7) ^{aAx}
RC	—	—	18.0 (1.8) ^{ax}	9.9 (1.0) ^{ay}	—	—	40.4 (2.5) ^{ax}	36.5 (2.9) ^{ax}
BFT	—	—	7.0 (0.9) ^{ax}	2.2 (0.4) ^{ay}	—	—	3.6 (0.8) ^{ax}	2.6 (0.5) ^{ax}
LP	—	—	8.6 (1.0) ^{ax}	3.8 (0.6) ^{ay}	—	—	4.0 (0.7) ^{ax}	2.6 (0.7) ^{ax}
CI	—	—	7.5 (1.3) ^{ax}	1.9 (0.3) ^{ay}	—	—	5.6 (1.2) ^{ax}	3.4 (1.3) ^{ax}
Other forbs [1]	—	—	0.1 (0.1) ^{ax}	0.1 (0.1) ^{ax}	—	—	0.2 (0.1) ^{ax}	0.0 (0.0) ^{ax}
others	0.3 (0.1) ^{aAx}	0.6 (0.4) ^{aAx}	0.7 (0.2) ^{aAx}	0.6 (0.2) ^{aAx}	0.8 (0.3) ^{aAx}	2.0 (0.7) ^{aBx}	0.3 (0.2) ^{aAx}	0.2 (0.1) ^{aAx}

[1] Salad burnet and caraway. ^{a,b} Differences between the periods, according to the adjusted *p*-value method. ^{A,B} Differences between treatments, according to the adjusted *p*-value method. ^{x,y} Differences between offered and rest, according to the adjusted *p*-value method.

The daily methane emissions increased with the pasture diversity, and the mean values were 18% greater ($p < 0.01$) in diverse pastures, with mean values of 221- and 260-g CH_4 day^{-1} for the binary and diverse mixtures, respectively. The emissions generally decreased ($p < 0.01$) over time, with mean values of 258- and 223-g CH_4 day^{-1} for P1 and P2, respectively. The estimated DMI was unaffected by the pasture diversity for both periods, but they were 45% greater in P1 compared to P2. There was no significant treatment $\times$ period interaction for any of the fixed variables.

3.2. *Milk Yield, Milk Composition, and Methane Intensity*

The mean values for the methane emissions per kg ECM were 11% greater ($p < 0.01$) in the diverse, compared to binary, mixtures, with 9.8- and 8.8-g CH_4 kg ECM^{-1} on average, respectively (Table 3). Over time, the methane intensity generally increased and was 11% lower ($p < 0.05$) in P1 compared to P2, with mean values of 8.8- and 9.9-g CH_4 kg ECM^{-1}, respectively. As shown in Table 3, the milk yield and ECM yields increased when the cows grazed the diverse mixtures in both P1 and P2 (+4%, $p < 0.001$). The milk composition was also affected by the pasture diversity; while the fat and lactose contents decreased by 4.2% on average ($p < 0.05$), the protein decreased by 1.7% ($p < 0.001$) when the diversity increased. Nevertheless, the yields of the milk solids were greater in cows grazing the diverse mixtures (2.4% on average for fat and lactose and 4.8% for protein; $p < 0.05$) as a result of the greater milk yields. Across the lactation stage, the milk solid contents generally decreased from P1 to P2 by 3% ($p < 0.001$) as the days in milk (DIM) advanced.

The body weights were not affected by the treatments. However, cows lost weight during P1 (-0.2 kg day^{-1}), coinciding with the peak of lactation, and they gained weight (+0.2 kg day^{-1}) during P2.

4. Discussion

4.1. Excellent Forage Quality Might Explain Generally High Milk Yields and Low Methane Intensities

As stated, our results show that cows grazing the diverse pasture sward increased their ECM yield compared to the binary mixtures. However, generally, the milk yields were very high in both treatments. Accordingly, the mean values during P2 (DIM = 154) of 22.1- and 23.5-kg ECM cow^{-1} day^{-1} for the binary and diverse pastures, respectively, were greater than those reported for grazing Jersey cows in mid-lactation by Münger and Kreuzer [50] of 17.8-kg ECM cow^{-1} day^{-1} at 161 DIM and by van Wyngaard et al. [51] of 13.8- or 19.0-kg ECM cow^{-1} day^{-1} (without concentrate supplementation and with 4 kg of concentrate per day, respectively). They were, nevertheless, comparable to the milk yields of Jerseys fed total mixed ration (TMR) diets with 32% concentrate ad libitum of 22.9–23.4-kg ECM cow^{-1} day^{-1} at 187 DIM [52]. This may be explained, at least in part, by the body weights of the Jerseys, which, in the latter study, were, on average, 469 kg and, therefore, similar to the 442 kg in the present study and much higher than the 354 kg of Münger and Kreuzer [50] or the 398 kg of van Wyngaard et al. [51]. The cows' mean BW, as an indicator of size, is one of the main drivers of the DMI (when comparing feeds with the same quality) and, consequently, of the methane emissions and milk yield. Nevertheless, the fact that the Jerseys in the present study, with being fed 2 kg of concentrate (approximately 13–15% of the DMI), produced equal milk yields to Jerseys being fed a TMR with approximatively one-third of the concentrate, and having a greater BW indicates a forage of excellent quality was available in the pastures in general, as well as the cows having a high genetic potential for milk production.

The high forage quality and genetic potential of the Jersey cows is illustrated in the milk yield, and this can also explain, at least partially, the very low CH_4 emissions and, particularly, the methane intensities. Generally, the CH_4 emissions in the present study of 203–277-g CH_4 cow^{-1} day^{-1} were similar to the values reported previously for Jersey cows, ranging from 258- to 321-g CH_4 day^{-1} [50,52]. However, when compared to cattle with similar BW, these emissions are substantially lower than the ~400-g CH_4 day^{-1} reported by Jonker et al., 2019. Additionally, due to the very high milk yields of 29.8-kg ECM cow^{-1} day^{-1} and 22.8-kg ECM cow^{-1} day^{-1}, on average, during periods P1 and P2, respectively, the emissions per unit of milk were low, with 8.8-g CH_4 kg ECM and 9.8-g CH_4 cow^{-1} day^{-1}, on average, for P1 and P2, respectively. These values were much lower than the previously reported 17.9- and 17.4-g CH_4 kg ECM^{-1} for Jersey cows being fed 0 and 4 kg of concentrate, as reported by van Wyngaard et al. [51], and even lower than the 13.4-g CH_4 kg ECM^{-1} reported for Jersey cows fed 61% of the concentrate [52]. Like the very high milk yields, these low emissions are a combined result of the genetic potential of the cows and the very high energy concentrations and organic matter digestibility of up to 12.5-MJ ME and 87.6%, respectively. These high qualities were a result of the very high use intensity, where each sward was grazed eight to nine times per year, with regrowth periods of 7–15 days dependent on the regrowth rates and, thus, always providing young, leafy materials. Under this pasture management regime, the herbage generally has very low contents of fiber and has very high concentrations of water-soluble carbohydrates (WSC). This is also consistent with the findings for sheep, where the grazing of tetraploid ryegrasses exhibited 12% lower methane emissions compared to conventional ryegrass cultivars [53]. This was likely to be due, in part, to higher WSC concentrations, although WSC alone was not sufficient to explain the differences in methane emissions. Additionally, when the remaining statistical uncertainty is considered, the generally low methane emissions of the cows are in accordance with the observed very high milk yields and the excellent forage quality.

4.2. Increasing Pasture Diversity Did Not Reduce Methane Emissions Further

The aim of this study was to investigate the potential for botanically diverse mixtures when presented as sown swards in dairy systems to reduce the enteric CH_4 intensity and, hence, to decrease the entire GHG emissions from the system relative to a binary perennial ryegrass–white clover sward. Our results showed that the CH_4 emissions were greater for the cows grazing diverse swards. This was despite their content of species containing plant-specialized metabolites, especially tannins, which have repeatedly been shown in previous studies to result in lower CH_4 emissions per kg DMI (e.g., Piluzza et al. [54]). The species examined in the present study were birdsfoot trefoil, which contains intermediate concentrations of condensed tannins and which has previously shown potential for ruminant production [35], and salad burnet, which has high concentrations of hydrolysable tannins [55]. Additionally, ribwort plantain and chicory were included, as these species have previously been shown to produce low in vitro methane emissions, despite their relatively low or no tannin concentrations [56]. In chicory, however, this effect was cultivar-dependent. The reason that the methane emissions were not reduced despite the inclusion of these herbs and legumes is likely due to the low proportions at which they were present in the grazed pastures. As shown in the study of Jonker et al. [57], which fed ribwort plantain and chicory in mixtures in vivo, even moderately low proportions of 13% and 15%, respectively, were not sufficient to reduce the CH_4 emissions compared to the freshly cut perennial ryegrass–white clover material. With the even lower proportions of species with anti-methanogenic potential in the present study (maxima of 7%, 8%, and 9% for birdsfoot trefoil, chicory, and ribwort plantain, respectively), these clearly were insufficient to affect the CH_4 emission from cows that grazed these swards. A drawback of the management intensity of the rotational grazing system with 9–10 grazing cycles per year in total is that the selected herbs were unable to persist. Thus, species such as birdsfoot trefoil and salad burnet are outcompeted by species that have a growth strategy that is able to adapt to greater grazing pressures, particularly perennial ryegrass but, also, red and white clover. Thus, herbs containing anti-methanogenic compounds will require either enhanced concentrations of tannins or will be an appropriate solution for grassland systems with lower grazing pressures, where (a) the forage quality is lower due to more fibrous material and, hence, the CH_4 emissions would be greater to begin with, and (b) the proportions of the bioactive species can be greater, due to a more suitable use intensity.

The reasons for increased CH_4 emissions from cows that grazed the diverse swards are likely to be a combination of two factors. Firstly, the diverse mixture swards had a lower OM digestibility, which was likely a result of increased structural carbohydrates. This is to be expected, due to the share of herbs rich in structural carbohydrates, such as ribwort plantain, and the temporal asynchrony between the species and the corresponding difficulty in utilizing all the species under optimal conditions, thus resulting in increased proportions of stem fractions in many species [58]. Greater contents of structural carbohydrates result in increased CH_4 formation during fermentation in the rumen [59]. Secondly, cows grazing the botanically diverse pasture had greater DMI. This was apparent from the group intakes as calculated from the measurements of the offered and remaining biomass (Table 3). Despite the OM digestibility being less for the diverse swards, it is worth mentioning that it was still highly digestible, especially during P1. Although the most appropriate methodology for a precise determination of the individual DMI is generally the use of external markers, the regulations for organic systems prevented their use here. Thus, due to the large variability and uncertainties connected to the intake calculations from the measurements of the herbage on offer and the residues after grazing, and due to the inability of the method to enable accurate calculations of the individual intakes per cow, a dual approach was selected as the most suitable option for the present study. In this approach, the model developed by Gruber et al. [44] was used to estimate the individual intake and to be compared against the group intake estimates as derived by the cut samples of herbage on offer and the residues after grazing. This model has proven to be the most accurate estimation of DMI for systems with similar conditions to those used in this study. Despite the apparent inability of the model to detect differences in the forage intake of mixtures with only small variations in quality, as apparent from the very small differences in the estimated intake among the treatments, the estimated intake in general appears to be reasonable, given the amount of milk produced. However, the greater DMI of herbage from the diverse mixture sward may better explain the greater methane emissions and greater milk yields from the diverse mixtures. Thus, the measured intakes from the measurements in the pasture are likely to be a slight underestimation, while their semiquantitative differences offer a realistic explanation for the observed differences in the animal performances and emissions. Similarly, the DMI likely resulted in higher milk yields from the diverse pastures. Nevertheless, despite the potential differences in the DMI, these findings are also in accordance with Totty et al. [60], who found that milk yields increase when cows graze diverse pasture mixtures that include high-sugar ryegrass and white clover, chicory, plantain, and birdsfoot trefoil (thus being comparable to the diverse mixture in the present study) compared to either high-sugar ryegrass monocultures or binary mixtures of ryegrass and white clover. These greater milk yields of up to 15% were obtained despite the equal energy concentrations and were due to a higher nutrient use efficiency, with the nitrogen excretions being reduced by almost 20% [60].

5. Conclusions

The present study showed that dairy cows grazing sown grass–white clover and grass–clover–herb leys based on highly improved perennial ryegrass cultivars achieve high milk production levels with low methane emissions per kg of ECM. These beneficial outcomes were achieved under high grazing pressure with short-interval rotational stocking and low external inputs, which ensured that a very high forage quality from young and leafy materials was maintained during the grazing periods. The management system provided a feed of high metabolizable energy value, and low methane emissions were likely a result of this very high forage quality. Contrary to our hypotheses, the addition to the grass–white clover mixture of low proportions of forbs that contain plant-specialized metabolites did not, under the conditions of this experiment, provide any additional benefits. Hence, further research is needed to identify whether different mixture strategies or lower grazing intensities are other options for an increased role of forbs in grazing systems for dairy cows.

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Appendix A

Table A1. Statistical data of the NIRS calibration and validation (SEC, standard error of calibration; SEV, standard error of prediction) for the relevant quality parameters by plant group.

Parameter	Plant Group	N	Mean	Range	SEC	R^2	SEP
ME (MJ/kg DM)	whole sward	251	10.810	8.38–12.62	0.179	0.959	0.195
ME (MJ/kg DM)	Grasses	248	10.806	8.38–12.62	0.173	0.956	0.194
	Legumes	168	10.737	8.55–12.41	0.15	0.961	0.196
	Herbs	117	10.691	8.38–12.54	0.154	0.963	0.211
NEL (MJ/kg DM)	whole sward	249	6.567	4.79–7.81	0.138	0.955	0.173
NEL (MJ/kg DM)	Grasses	250	6.569	4.79–7.87	0.136	0.949	0.193
	Legumes	167	6.512	4.92–7.77	0.106	0.965	0.152
	Herbs	115	6.514	4.80–7.784	0.112	0.964	0.163
DOM (g/kg DM)	whole sward	249	809.45	642.6–919.0	8.85	0.967	9.14
DOM (g/kg DM)	Grasses	248	821.04	632.1–918.8	10.83	0.956	10.97
	Legumes	173	803.63	657.6–902.6	10.36	0.944	12.96
	Herbs	118	817.42	642.6–904.3	13.51	0.943	12.45
N (g/kg DM)	whole sward	268	26.69	8.7–54.2	0.951	0.991	1.071
N (g/kg DM)	Grasses	277	22.59	9–54.9	0.802	0.991	0.914
	legumes	178	35.07	14.7–57.1	1.116	0.981	1.131
	herbs	86	26.39	10.3–39.9	0.749	0.995	1.317

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