Effects of *Acacia mearnsii* added to silages differing in nutrient composition and condensed tannins on ruminal and manure-derived methane emissions of dairy cows

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ABSTRACT

This study investigated the effects of acacia (extract of *Acacia mearnsii*) and sainfoin (*Onobrychis viciifolia*) as condensed tannin (CT)-rich sources on ruminal and manure methane (CH_4) emissions in comparison with non-CT silages characterized by different contents of the cell wall and water-soluble carbohydrates. In a 3 × 6 incomplete Latin square design, 30 Holstein cows (63 ± 23 d in milk; mean ± SD; 33.8 ± 7.6 kg of milk per day, body weight 642 ± 81 kg) were provided with ad libitum access to 1 of 6 total mixed rations comprising 790 g of silage and 210 g of concentrate per kilogram of dry matter (DM). The silages were either rich in sainfoin [neutral detergent fiber (NDF): 349 g/kg of DM], perennial ryegrass (NDF: 420 g/kg of DM), or red clover (NDF: 357 g/kg of DM). Each silage was supplemented with 20 g/kg (of total diet DM) of acacia or straw meal. Feed intake and milk yield were recorded daily. Milk composition and ruminal fluid characteristics and microbiota were analyzed. The individual ruminal CH_4 production was determined using the GreenFeed system, and CH_4 emissions from the manure of cows fed the same diets were measured in a parallel experiment over 30 d at 25°C using a dynamic flux chamber. The CT sources did not reduce CH_4 yield or emission intensity. Acacia reduced milk production (from 26.3 to 23.2 kg/d) and DM intake (from 19.7 to 16.7 kg/d) when supplemented with ryegrass, and both CT sources reduced the milk protein content and yield. Acacia supplementation and ryegrass silage reduced the ruminal acetate:propionate ratio. Furthermore, during acacia treatment, the abundance of *Methanobrevibacter* archaea tended to be lower and that of *Thermoplasmata* was higher. Acacia reduced the CH_4 emissions from manure for the ryegrass group by 17% but not for the sainfoin and clover groups. Feeding sainfoin silage resulted in the lowest manure-derived CH_4 emissions (−47% compared with ryegrass). In conclusion, acacia reduced ruminal CH_4 production by 10%, but not emission intensity, and the mitigation effect of sainfoin depended on the silage to which it was compared. Because mitigation was partially associated with animal productivity losses, careful evaluation is required before the implementation of tanniferous feeds in farm practice.

Key words: environment, feed additive, manure, sainfoin, tannin extract

INTRODUCTION

One of the most outstanding features of ruminant livestock is their ability to convert nonedible plant biomass in the form of herbage-based diets into human foods. However, compared with concentrate-based diets, diets based on herbage typically have a high digestible fiber content, with the result being increased emissions of methane (CH_4), an important greenhouse gas. Therefore, the mitigation of these emissions is an urgent goal. Two sources of CH_4 emissions are relevant in ruminants: enteric (mostly ruminal) fermentation and methanogenic activity in manure, with the first accounting for approximately 71% of direct cattle greenhouse gas emissions (Gerber et al., 2013).

A possible approach to reduce enteric CH_4 emissions is feeding ruminants extracts or plants rich in condensed tannins (**CT**; Bodas et al., 2012), which are polyphenolic compounds found in various plants with an affinity to bind to dietary components, such as pro-

teins and carbohydrates (Waghorn, 2008). Condensed tannins can mitigate ruminal CH_4 emissions by reducing carbohydrate (especially fiber) degradation and by direct action against ruminal methanogens or hydrogen-producing microbes (Bodas et al., 2012). However, the mode of action of CT has not been entirely revealed. Both modes of action might also reduce the energy supply to the animals and cause higher excretion of principally fermentable OM due to decreased diet digestibility, possibly leading to increased manure-derived CH_4 emissions (Huhtanen et al., 2021). In contrast, as CT are mostly excreted undigested (Waghorn, 2008), they may resume their CH_4-mitigating activity in the manure. Whether these compensatory effects result in a net increase or decline in manure-derived CH_4 emissions is unknown.

Among other sources of CT, the powder extract from the bark of *Acacia mearnsii* (hereafter, acacia), which is used as a feed supplement (Carulla et al., 2005; Grainger et al., 2009; Denninger et al., 2020), and the tanniferous legume sainfoin (*Onobrychis viciifolia*; Huyen et al., 2016) have shown potential in mitigating enteric CH_4. However, the effects of acacia on CH_4, although quite consistent in absolute amounts, are less consistent when considering the CH_4 yield (e.g., CH_4/DMI) or emission intensity (e.g., CH_4/milk production; Grainger at al., 2009; Denninger et al., 2020). For sainfoin, the effects on CH_4 have been found to be inconsistent. For instance, Huyen et al. (2016) found a reduction in CH_4 yield in dairy cows consuming sainfoin compared with grass silage, whereas Chung et al. (2013) and Stewart et al. (2019) found that sainfoin had only a slight effect on CH_4 in beef cattle compared with alfalfa. In vitro, no effect on CH_4 production was found when incubating ensiled (Grosse Brinkhaus et al., 2017) or fresh sainfoin (Rufino-Moya et al., 2019) compared with other legumes. This suggests that, apart from possible but still unclear CT-based effects, the effects of sainfoin on CH_4 might rely on other compositional characteristics in cases in which sainfoin is compared as forage with grasses or non-tanniferous legumes, such as clovers.

In previous studies, acacia was found to affect the final composition of the manure but did not significantly affect CH_4 emissions from it (Staerfl et al., 2012; Fagundes et al., 2020). Regarding sainfoin, its potential to mitigate CH_4 emissions from manure has not yet been tested, to our knowledge. Furthermore, there is a lack of knowledge about whether the mitigation of enteric and manure-derived CH_4 emissions can even be enhanced when acacia and sainfoin are combined or whether these 2 CT sources level out or negatively affect animal productivity. In fact, the CT in the acacia extract have a low degree of polymerization and a low procyanidin/prodelphinidin ratio (Venter et al., 2012),

and they are mostly soluble (as the extract is produced by solubilizing the CT bark in water) and likely to be more reactive with feed components than other native CT sources (García et al., 2017). In contrast, the CT of sainfoin have a higher degree of polymerization, higher ratios of procyanidin/prodelphinidin, and are protein- and fiber-bound in greater proportions (Mueller-Harvey et al., 2019), which might make them less reactive with other diet components.

Based on these gaps in knowledge, we hypothesized that (1) Acacia and sainfoin as CT-rich sources mitigate ruminal and manure-derived CH_4 emissions without affecting DMI and milk production, (2) the effects of acacia and sainfoin are additive, and (3) the degree of the acacia effect depends on the type of basal forage used in the diet. The additivity of the effects was expected to result from the increase in dietary tannin proportion when combining the 2 sources and from the specific properties of the 2 tannin sources (concentrated form of tannins without binding to nutrients vs. tannins present in a nutritious matrix), addressing different modes of action in the rumen and manure. Our objectives were to quantify the effects of acacia and sainfoin on ruminal and manure CH_4 emissions compared with non-CT silages characterized by different contents of cell walls and water-soluble carbohydrates (**WSC**). In this context, ruminal fermentation and the microbial community were also studied, as CT may reduce ruminal methanogenesis by affecting the microbial community. As non-CT silages, common forages were chosen, such as perennial ryegrass and red clover. The red clover and sainfoin silages had similar NDF and WSC content, whereas the perennial ryegrass silage had higher NDF and WSC content than the 2 legumes at the expense of CP.

MATERIALS AND METHODS

Design, Animals, and Diets

The experimental protocol complied with the Swiss guidelines for animal welfare and was approved (No. 2018_25_FR) by the Animal Care Authority of the Canton of Fribourg. A total of 30 lactating Swiss Holstein-Friesian cows (63 ± 23 DIM; mean ± SD; 33.8 ± 7.6 kg of milk per day) weighing 642 ± 81 kg were divided into 6 groups of 5 cows, each group receiving 1 of the 6 treatments. The experiments consisted of 3 periods; each period lasted 4 wk, including a 2-wk adaptation and a 2-wk data collection period. At the beginning of each subsequent period, the groups were reassembled so that at the end, each cow received 3 of the 6 treatments, resulting in an incomplete Latin square design (n = 15 per treatment).

Dietary variation was created by combining 3 types of herbage silage with 2 differently composed energy concentrates, resulting in 6 treatments (3 × 2 factorial arrangement; Table 1). The herbages used for silage making were composed predominantly of perennial ryegrass (*Lolium perenne*), sainfoin (*Onobrychis viciifolia*), or red clover (*Trifolium pratense*). Information about harvest data and the botanical and chemical composition of herbages and silages is given in Supplemental Table S1 (https://doi.org/10.5281/zenodo.7646644). All crops were grown in Posieux, Switzerland (650 m above sea level). The complete diets characterized by silages rich in perennial ryegrass, sainfoin, or red clover are denominated as **RG**, **SF**, and **CL**, respectively. The energy concentrates contained 100 g/kg as fed of either straw meal (control) or a powdery tannin extract obtained from the bark of *Acacia mearnsii* trees (Seta SB, Seta, Estância Velha, Brazil; acacia). The batch of acacia extract used contained 190 g/kg and 476 g/kg DM of total CT (which were entirely soluble) and total polyphenols, respectively, according to the analysis. Otherwise, the 2 energy concentrates had the same composition and contained (per kg as fed): corn meal, 395 g; wheat meal, 268 g; corn gluten meal, 55 g; $CaHPO_4$, 51 g; soybean cake, 46 g; sugar beet molasses, 30 g; $CaCO_3$, 25 g; MgO, 21 g; NaCl, 6 g; Cu, 31 mg; Zn, 260 mg; I, 3.8 mg; Mn, 52 mg; Se, 2 mg; Co, 1.3 mg; vitamin A, 44,200 IU; vitamin D_3, 3,510 IU; vitamin E, 208 mg; β-carotene, 90 mg; and biotin, 6 mg. In the RG diets, 36 g/kg of the energy concentrate was replaced by a protein concentrate to compensate for the low CP content of the ryegrass-rich silage. This concentrate consisted of (g/kg as fed) straw meal or acacia extract, 100; soybean cake, 500; corn gluten meal, 255; potato protein, 85; sugar beet molasses, 36; animal fat, 18; and mineral premix, 10. The calculated proportion of either straw meal or acacia extract in the total diets was always 20 g/kg of DM. This dose of acacia was chosen based on the study by Grainger et al. (2009), in which dairy cows were fed 270 g/d and 540 g/d of acacia extract. We decided to use an intermediate dose, namely 400 g/d, which, for an estimated DMI of 20 kg/d, corresponded to 20 g/kg of DM, considering also that the combination with sainfoin would have increased the CT dose. All diets were calculated to contain 6.2 MJ of NE_L/kg of DM and 97 g of MP/kg of DM (here: absorbable protein at the duodenum; Agroscope, 2021), as required for a milk yield of 30 kg/d. The diets were offered as TMR. The animals were kept in a freestall barn with ad libitum access to feed and drinking water. The TMR was always offered at 0600 h and 1400 h. After each period, the groups were reorganized in such a way that each cow received a different silage type in each of the 3 periods, and each cow had at least 1 change of concentrate type.

Data Recording and Sample Collection from Animals

Individual feed intake was recorded using access-controlled weighing bunks (Insentec RIC system, Hoko-farm Group, Marknesse, the Netherlands). A total of 30 Insentec units were used. For each of the 6 diets, there were 5 Insentec units accessible to all 5 cows assigned to each diet in the respective period. The refilling of the bunks was managed in such a way that roughly 10% of the feed was left. Seventy grams of TMR were collected on Mondays, Tuesdays, Thursdays, and Fridays of each experimental week, stored at −20°C, and finally pooled by treatment and week for later analysis. The BW of the cows was recorded twice daily after milking using an access-controlled walk-through scale (Hoko-farm Group). The milk yields were determined at each milking (0500 and 1600 h) in the milking parlor using a Pulsameter 2 (SAC, A. Bertschy AG, Guschelmuth, Switzerland), and milk samples were collected once per week from 2 consecutive milkings. Depending on the milk yield of the morning and evening milkings, aliquots were prepared and pooled. A subsample was stored in a tube containing 2-bromo-2-nitro-1,3-propanediol (bronopol) for the subsequent determination of the gross composition. The remaining part was stored at −20°C for later determination of MUN.

Ruminal fluid was collected via a stomach tube (Selekt, Virbac, Kolding, Denmark) after morning milking (between 0530 and 0730 h) on d 11 and 14 of each 2-wk collection period. The samples were cooled on ice immediately after sampling. For the VFA and NH_3 analyses, 10 mL of ruminal fluid was mixed with 0.2 mL of 50% (vol/vol) sulfuric acid and 0.2 mL of 50% (vol/vol) trichloroacetic acid, respectively, and stored at −20°C. The untreated samples (50 mL) were immediately frozen at −20°C for later analysis of the ruminal microbiota. For capacity reasons, storage at −80°C was not possible, but all samples were treated in exactly the same way as recommended by Goodrich et al. (2014). The cows had no access to feed and water from 2 h before collection to minimize diet × animal interactions for the analysis of the ruminal microbiota (de Assis Lage et al., 2020).

To measure individual ruminal CH_4 and H_2 emissions, 2 units of the GreenFeed (**GF**) system (C-Lock Technology Inc., Rapid City, SD) permanently installed in the freestall barn were used. Descriptions of the system, the method used to calculate the emissions, and the maintenance calibration operations are given in Denninger et al. (2019). All cows had access to both

Table 1. Ingredients and nutrient composition of the diets, fed as TMR (mean[1] ± SD)

Item	Perennial ryegrass		Sainfoin		Red clover	
	Control[3]	Acacia[4]	Control	Acacia	Control	Acacia
Ingredient of TMR (g/kg of DM)						
Silage	786 ± 6	786 ± 7	788 ± 7	788 ± 8	793 ± 8	793 ± 9
Energy concentrate	174 ± 5	175 ± 6	212 ± 7	212 ± 8	207 ± 8	207 ± 9
Protein concentrate	39 ± 2	39 ± 1	—	—	—	—
Analyzed composition (g/kg of DM, unless stated otherwise)						
DM (g/kg of fresh matter)	450 ± 14	461 ± 30	502 ± 25	514 ± 28	541 ± 49	561 ± 40
OM	905 ± 4	905 ± 4	899 ± 6	897 ± 9	873 ± 1	873 ± 2
CP	129 ± 3	129 ± 2	172 ± 8	170 ± 7	205 ± 6	205 ± 5
NDF	363 ± 13	344 ± 12	307 ± 7	285 ± 9	315 ± 17	301 ± 15
ADF	219 ± 7	209 ± 6	225 ± 8	210 ± 4	191 ± 10	184 ± 10
Water-soluble carbohydrates	105 ± 17	108 ± 18	53 ± 4	60 ± 3	57 ± 7	62 ± 5
Condensed tannins[5]						
Total	0.81 ± 0.26	4.25 ± 0.87	14.10 ± 3.75	17.41 ± 3.93	0.63 ± 0.21	4.14 ± 0.89
Soluble	0.68 ± 0.27	4.02 ± 0.88	8.52 ± 2.33	11.97 ± 5.14	0.39 ± 0.18	3.78 ± 0.90
Protein-bound	0.11 ± 0.10	0.19 ± 0.00	4.14 ± 1.09	3.93 ± 1.69	0.16 ± 0.04	0.26 ± 0.07
Fiber-bound	0.02 ± 0.00	0.04 ± 0.01	1.44 ± 0.34	1.51 ± 0.43	0.08 ± 0.01	0.10 ± 0.03
Calculated energy and protein supply[6] (per kg of DM)						
NE_L	6.25 ± 0.05	6.25 ± 0.05	6.11 ± 0.14	6.11 ± 0.14	6.27 ± 0.00	6.27 ± 0.00
APDE	99.8 ± 1.8	99.8 ± 1.8	96.8 ± 1.8	96.8 ± 1.8	99.0 ± 0.1	99.0 ± 0.1
APDN	88.7 ± 4.33	88.7 ± 4.33	108.8 ± 4.56	108.8 ± 4.56	132.1 ± 0.39	132.1 ± 0.39

[1]Mean over the 3 data collection periods.

[2]Most characteristic forage species in the respective silages.

[3]Control = energy and protein concentrate containing 100 g of wheat straw meal/kg of DM.

[4]Acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract/kg of DM.

[5]Expressed as cyanidine equivalents.

[6]APD = absorbable protein at the duodenum when rumen fermentable energy (APDE) or nitrogen (APDN) is limiting microbial protein synthesis in the rumen, calculated as outlined by Agroscope (2021).

units at any time (except during milking) and were allowed a maximum of 6 visits to the station per day and 1 visit per 4-h time slot. To keep the cows close to the collection manifold, up to 8 portions of 30-g bait feed [pelleted dried whole corn plant (g/kg DM except for DM): DM, 914 ± 5 g/kg (mean ± SD); NDF, 325 ± 14; ADF, 175 ± 12; CP, 79 ± 5; and WSC, 93 ± 3] were released in 20-s intervals per visit. The average number of visits per day was 4.4 ± 1.3 (mean ± SD), and the total bait feed intake per cow ranged from 748 to 1,440 g/d. The DM and nutrient intakes associated with the bait were added to the measured TMR intake (Table 2). The airflow rate was 20 to 40 L/s. The CO_2 recovery used for airflow sensor calibration was determined every 3 wk by repeatedly releasing weighed amounts of CO_2 into the unit's manifold, and amounted to 98 ± 2% (mean ± SD). The CH_4 production data derived from the GF system were averaged by cow over the 14 d of each collection period. If, within the same period, a cow visited the GF fewer than 20 times, the corresponding data were excluded from the analysis. This occurred only in 2 cases (for 1 cow in period 1 and 1 cow in period 2).

Manure Incubation Experiment

Samples of feces and urine were collected during a parallel experiment described by Lazzari et al. (2023). Briefly, in that experiment, 6 multiparous Holstein cows (milk yield: 36.6 ± 3.9 kg/d, mean ± SD; 70 ± 13 DIM) were fed the same diets as in the present experiment, and excreta were entirely collected over 7 d after a 14-d adaptation period. Waterproof urinals were used to separate urine from feces. The feces and urine samples used in the present manure incubation experiment were collected on d 5 of each collection period of the parallel experiment and frozen within 30 min after sampling. Later, these samples were thawed and mixed by cow and treatment (n = 36). The feces-to-urine ratio respected the ratios excreted from the cows. The manure was mixed with sterilized water and an inoculum in proportions of 0.626:0.308:0.065. Dilution with water was applied to simulate DM content of slurry collected from commercial cattle farms (Dinuccio et al., 2008). The inoculum had the purpose of stimulating immediate CH_4 production and was prepared according to Sommer et al. (2007). It contained fresh feces and urine

Table 2. Effects of silage type and acacia supplementation on daily intake and enteric methane (CH$_4$) emissions

Item	Perennial ryegrass		Sainfoin		Red clover		SEM	P-value		
	Control[1]	Acacia[2]	Control	Acacia	Control	Acacia		Silage	Acacia	Silage × Acacia
Daily intake per cow[3]										
DM (kg)	19.6^c	16.7^d	19.8^{bc}	20.7^{abc}	21.9^{ab}	22.0^a	0.74	0.004	<0.001	0.004
OM (kg)	17.7^a	15.1^b	17.8^a	18.6^a	19.1^a	19.2^a	0.64	0.072	<0.001	0.004
NDF (kg)	7.11^a	5.72^c	6.09^{bc}	5.93^{bc}	6.89^a	6.62^{ab}	0.232	<0.001	<0.001	0.003
ADF (kg)	4.29^a	3.48^b	4.47^a	4.36^a	4.19^a	4.04^a	0.162	0.230	<0.001	0.012
CP (kg)	2.54^c	2.15^d	3.43^b	3.52^b	4.47^a	4.52^a	0.146	<0.001	0.004	0.034
Condensed tannins (g)										
Total	15.7^y	72.1^y	274.0^x	359.7^x	15.0^y	90.3^y	16.58	<0.001	<0.001	0.300
Soluble	12.7^y	68.6^y	165.5^x	246.7^x	9.96^y	82.2^y	17.9	<0.001	<0.001	0.385
Protein-bound	2.21^y	3.11^y	80.39^x	81.83^x	3.53^y	5.80^y	4.561	<0.001	0.865	0.983
Fiber-bound	0.45^y	0.62^y	28.08^x	31.22^x	1.70^y	2.15^y	5.271	<0.001	0.887	0.261
Ruminal gas emissions										
CH$_4$ production (g/d)	411^y	333^y	377^y	354^y	412^x	393^x	7.2	0.005	<0.001	0.371
CH$_4$ yield										
g/kg DMI	21.2^x	20.4^x	19.3^y	17.1^y	18.8^y	17.8^y	0.29	0.002	0.115	0.434
g/kg NDF intake	58.3	59.2	62.8	60.0	60.0	59.1	0.76	0.104	0.467	0.270
g/kg ADF intake	96.4^x	97.6^x	86.0^y	81.4^y	98.4^x	96.9^x	1.40	<0.001	0.835	0.378
CH$_4$ emission intensity (g/kg ECM)	15.4	14.2	13.7	14.1	14.7	13.2	0.23	0.086	0.102	0.084
H$_2$ production (g/d)	1.71	1.34	1.57	1.44	1.56	1.61	0.138	0.474	0.011	0.130

[a-d]Means within a row with different superscripts are different due to the silage × acacia interaction at $P < 0.05$.

[x,y]Means within a row with different superscripts are different due to the silage effect at $P < 0.05$.

[1]Control = energy and protein concentrate containing 100 g of wheat straw meal/kg of DM.

[2]Acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract/kg of DM.

[3]Including bait feed consumption when visiting the GreenFeed.

(separately collected from the dairy cows fed TMR at the Agroscope experimental farm in Posieux, Switzerland) and water in proportions of 0.46:0.30:0.25. After preparation, the inoculum was stored in sealed cylindric 3-L containers (25 cm height and 14 cm diameter) for 8 wk at 25°C before use. During the 8 wk, the containers were shaken daily. The excreta–water–inoculum mix was blended using a commercial single-blade mixer (Bosch, Gerlingen, Germany). Each sample was then split into 2,500-g subsamples and transferred into 1-L jars (100-1000-01, Savillex, Eden Prairie, MN) in such a way that each sample was incubated in duplicate. The remaining manure was immediately frozen for chemical analysis. The pH of the samples was measured at the start and end of the incubation experiment using a portable pH meter (Hanna Instruments, Woonsocket, RI).

The CH_4 emissions from the manure were measured in a self-constructed dynamic chamber setup adapted from Dinuccio et al. (2008). The chamber consisted of 13 jars of 1 L each (Supplemental Figure S1 https://doi.org/10.5281/zenodo.7646644). The jars were sealed with screw caps (600-110-28, Savillex), which had 2 quarter-inch tube ports on the top for air inlet and outlet. Twelve jars contained manure samples, and one was kept empty for the background measurement. The inlet ports of the jar caps were connected to the air supply system. The outlet ports were connected to a multichannel sampler (model A0311-s1, Picarro, Santa Clara, CA) with 16 inlet ports (of which only 13 were used in this experiment) and 2 outlet ports, with the latter being called the active and exhaust ports. The active port connected the active sampling line to the measurement device, whereas the exhaust port collected all the exhaust gas coming from the non-active lines. Airflow through the active sampling line was monitored using a mass flow meter (GSM, Vögtlin Instruments Inc., Muttenz, Switzerland). The concentration of CH_4 was monitored using a cavity ring-down spectrometer (model G2301, Picarro). A total of 72 manure-containing jars and 6 empty jars were used, and they were divided into batches of 13 jars (12 containing samples and 1 empty). Each batch included 6 cows in duplicate (with 1 cow in each treatment) and 1 empty jar. During the manure incubation experiment, a constant flow of synthetic air (PanGas, Dagmersellen, Switzerland) adjusted using critical orifices (hole diameter 100 μm, Lenox Laser, Glen Arm, MD) was injected into the jars. Each batch of 13 jars was subjected to measurements over 30 d: continuously for the first 48 h of the experiment and then, in turn, for 12 h every 36 h for the remaining 28 d. The entire manure storage experiment lasted 2 mo, with 3 batches of jars being measured each month. During the measurements, the multichannel sampler successively connected the jars to the cavity ring-down spectrometer for 10 min each, and then the cycle started again. When connected to the multichannel sampler, the samples were kept in a water bath at 25°C; when not connected, they were kept at 25°C in a thermal incubator (Liebherr-Hausgeräte, Ochsenhausen, Germany). Before and after being connected to the multichannel sampler, the samples were manually stirred with a small spoon to homogenize the manure matter. The room temperature was kept constant and monitored using data loggers (model 175-h1, Testo, Mönchaltorf, Switzerland). The mean of the CH_4 concentrations recorded in the final 30 s of each 10-min period was used for data evaluation. For each measuring session (initially 48 h and subsequently 12 h every 36 h), the individual area under the curve was calculated and used in the statistical evaluation. Daily emissions fluxes were calculated as the concentration of CH_4 (in mg/m^3) in the air exiting the jars multiplied by the airflow through the jars (in m^3/d). The cumulative emissions were calculated from the daily fluxes. The jars containing the manure were weighed at the beginning and end of the incubation experiment to calculate the losses of wet and dry weight, DM, C, and N.

Laboratory Analyses

The diet components, TMR, and manure samples were lyophilized (Delta 1-24 LSC, Christ, Osterode, Germany) and ground to pass through a 1.0-mm screen (Brabender mill with titanium blades, Brabender, Duisburg, Germany). The DM and ash contents were determined by drying for 3 h at 105°C (prepASH 229, Precisa, Dietikon, Switzerland), with subsequent incineration at 550°C until reaching a constant weight. The OM was defined as the difference between DM and ash. The NDF and ADF contents (method ISO 16472:2006 for NDF and ISO 13906:2008 for ADF) were determined using Fibertherm (Gerhardt, Königswinter, Germany) and corrected for ash content. The NDF content was analyzed with the addition of heat-stable amylase and sodium sulfite. The WSC content was determined based on the method of Hall et al. (1999). The total N content of the diet components and TMR was analyzed using the Kjeldahl method (AOAC International, 1995; method 988.05) and multiplied by 6.25 to calculate the CP. The total C and N contents of the manure were analyzed using the Dumas method (AOAC International, 1995; method 988.06) on a C/N analyzer (Trumac CNS, Leco Instruments, St. Joseph, MI). The soluble, protein-bound, fiber-bound, and total CT contents were determined in triplicate in diet items and feces using the HCl–butanol method, as described by Terrill et al. (1992). The CT contents of the diluted manure was calculated considering the dilution of the feces with

urine, water, and inoculum, and its DM content. The total extractable phenols in diet items were analyzed using the Folin–Ciocalteau assay, following Salminen and Karonen (2011). Both CT and total extractable phenols were expressed as cyanidine equivalents. The ruminal VFA were analyzed using HPLC (Shodex RI, Denko KK, Minato, Japan) equipped with a refractive index detector and a Nucleogel ION column (300 OA 300 × 7.8 mm, Macherey–Nagel, Düren, Germany). The concentrations of NH_3-N in ruminal fluid were analyzed colorimetrically using a commercial test kit (Urea Liquicolor 10505, Human, Wiesbaden, Germany). The fat, protein, and lactose contents of the milk were analyzed using Fourier-transformed infrared spectrometry (Milkoscan FT 6000, Foss, Hillerød, Denmark). The MUN content was determined using the differential pH method with a Microlab EFA kit (Hamilton, Bonaduz, Switzerland) according to ISO 14637 (ISO, 2004).

Ruminal Microbiota Analyses

To characterize the ruminal bacteria and archaea community, the samples were thawed and DNA was extracted from 250 mg of ruminal fluid using the FastDNA SPIN Kit for Soil (MP Biomedical, Solon; Deusch et al., 2017). The quality and purity of the DNA extracts were analyzed using a NanoDrop ONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The V1–2 region of the 16S rRNA gene was amplified by PCR, and Illumina amplicon sequencing library preparation was performed as described by Deusch et al. (2017). The PCR product integrity was assessed by gel electrophoresis. Amplicons were purified and normalized using a SequalPrep Normalization Kit (Invitrogen, Carlsbad, CA) and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform. The sequences were submitted to the European Nucleotide Archive under accession number PRJEB51040. The raw sequencing data were processed using the Mothur pipeline (Deusch et al., 2017). We used UCHIME to detect possible chimeras, and the SILVA reference database (release 132) was used for taxonomic assignation. Reads were clustered at 97% identity into 3,162 bacterial operational taxonomic units (**OTU**) and 419 archaeal OTU. Those OTU with a sequence length >250 bp and an average abundance higher than 0.0001% were used for further analysis.

Calculations and Statistical Analyses

All statistical analyses were performed using R v. 4.1.2 (R Core Team, 2021). The data collected in the animal experiment over several days of the same collection period were averaged per cow per period before the statistical analysis. For all the variables (except for rumen bacterial and archaeal sequencing data), a linear mixed model was used for ANOVA with silage type, acacia supplementation, the interaction of both, and the sequence of silage type and acacia supplementation treatments as fixed effects. Silage type and acacia supplementation sequence effects were found to be insignificant and were removed from the model. Experimental period and cow were considered random effects, and cow was considered the experimental unit. Effects were considered to be statistically significant at $P < 0.05$. The results in the tables are presented as the least squares means of the 6 treatment groups and the overall standard error of the mean. Tukey's procedure was used as a post hoc test.

The rumen bacterial and archaeal sequencing data were analyzed using PRIMER 6 (v. 6.1.16) software with the Permanova+ (v. 1.0.6) add-on package (PRIMER-E, Plymouth, UK) and R. A Bray–Curtis similarity matrix was created, and the distances among the centroids were calculated. The centroids represent the average plotting positions of each dietary treatment, and they were visualized using principal coordinate analysis. A permutational multivariate ANOVA was used for this statistical evaluation. Using R, a Kruskal–Wallis test was performed to determine differences among taxonomic groups across silage types, and a pairwise Wilcoxon test was employed for post hoc comparisons. Linear discriminant analysis effect size was used to determine the bacteria and archaea most likely to explain the differences between treatments (Segata et al., 2011). A linear discriminant analysis score threshold of >2 was used. The Pearson correlation coefficients between microbiota and feed composition, ruminal emissions, and production variables were calculated using the Hmisc R package.

RESULTS

The diets differed largely in CP content from low in RG to medium-high in SF and high in CL (Table 1). The WSC content of RG was almost twice that of SF and CL, and the NDF content of RG was also higher than that of the 2 legume-based diets. Among the control treatments, SF had a notable CT concentration, whereas those of RG and CL were negligible. Acacia increased the CT content in all silage types but only from 14 g/kg to 17 g/kg of DM in SF and did not change the nutrient composition of the diets compared with the control group.

The daily intakes of DM and CP were greatest for CL, followed by SF and RG ($P < 0.01$), showing particularly low values for RG + acacia (silage × acacia, $P = 0.004$; Table 2). Acacia affected the intakes of DM, OM, and CP (−15% each), as well as NDF and ADF

(-19%), but only with RG (silage × acacia, $P < 0.05$) and not with SF and CL. The intake of CT was greater for SF compared with CL and RG (316 g/d vs. 53 g/d and 44 g/d, respectively, $P < 0.001$) and greater for acacia compared with the control group across all silages ($P < 0.001$).

Ruminal CH_4 production was greater for CL (403 g/d) compared with RG (372 g/d) and SF (366 g/d, $P = 0.005$; Table 2). The CH_4 yield per kilogram of DMI was higher in RG-fed cows (20.8 g) compared with CL-fed (18.3 g) and SF-fed cows (18.2 g; $P < 0.01$). No diet effect was found for CH_4 expressed as grams per kilogram of NDF intake or for CH_4 emission intensity per kilogram of ECM, whereas CH_4 in grams per kilogram of ADF intake was lower for SF (83.7 g) compared with RG (97.0 g) and CL (97.6 g; $P < 0.001$). Acacia reduced the CH_4 production by, on average, 10% (360 vs. 400 g/d; $P < 0.001$) but had no effect on CH_4 yield and intensity. Additionally, H_2 emission was reduced by acacia compared with the control (1.46 vs. 1.61 g/d; $P = 0.011$) but was not affected by silage type.

The total milk yield (kg/d) was greatest for CL (29.4), lower for SF (26.5), and the lowest for RG (24.8; $P < 0.001$), and ECM yield (kg/d) showed the same pattern (CL: 29.4, SF: 26.6, RG: 25.5; $P < 0.001$). The same order was also found for milk protein and lactose yield, whereas milk fat yield was not affected by silage type ($P = 0.737$). The effects of acacia on total milk, ECM, and milk component yield were not consistent across silage types (silage × acacia, $P < 0.05$; Table 3). Acacia reduced the yield of total milk and milk components in RG and CL (except fat yield in CL), whereas no such effect was found for SF. Milk fat content was greater ($P < 0.05$) for RG (4.34%) compared with SF (4.16%) and CL (4.08%). In addition, milk protein content was greater for CL (3.28%) than for SF (3.20%; $P = 0.006$) and ranged in between for RG (3.25%). The MUN content was greatest for CL, lower for SF, and lowest for RG ($P < 0.001$). Acacia reduced the milk fat content with RG and SF and increased the milk fat content with CL (silage × acacia, $P = 0.004$). Moreover, it reduced milk protein and MUN contents across all silage types, on average, by 3.0% and 17.0%, respectively ($P < 0.01$). The treatments had no effect on the lactose content.

The ruminal NH_3 concentration was greatest for CL, lower for SF, and lowest for RG ($P < 0.001$; Table 4). The total VFA concentration was greater for RG compared with CL (72.1 vs. 95.2 mM/L; $P = 0.013$), whereas SF took an intermediate position (68.2 mM/L). The RG-fed cows had a lower proportion of acetate and a greater proportion of propionate, n-butyrate, and n-valerate of total VFA compared with SF-fed and CL-fed cows ($P < 0.01$). Consequently, the acetate:propionate

Table 3. Effects of silage type and acacia supplementation on milk yield and composition

Item	Perennial ryegrass Control[1]	Perennial ryegrass Acacia[2]	Sainfoin Control	Sainfoin Acacia	Red clover Control	Red clover Acacia	SEM	P-value Silage	P-value Acacia	P-value Silage × Acacia
Daily yield per cow (kg)										
Total milk	26.3^c	23.2^d	27.0^c	26.0^c	30.2^a	28.5^b	1.01	<0.001	<0.001	0.038
ECM[3]	27.5^b	23.5^d	27.4^bc	25.7^c	29.8^ab	29.0^ab	1.01	<0.001	<0.001	0.002
Milk fat	1.16^a	0.98^c	1.15^ab	1.05^bc	1.18^a	1.21^a	0.050	0.737	<0.001	<0.001
Milk protein	0.87^bc	0.73^d	0.86^c	0.82^c	1.00^a	0.92^b	0.028	<0.001	<0.001	0.014
Milk lactose	1.25^c	1.10^d	1.28^c	1.24^c	1.45^a	1.36^b	0.049	<0.001	<0.001	0.034
Milk composition										
Fat (%)	4.44^a	4.24^ab	4.26^ab	4.07^b	3.93^b	4.23^ab	0.103	<0.001	0.091	0.004
Protein (%)	3.33^xy	3.16^xy	3.22^y	3.17^y	3.33^x	3.23^x	0.062	0.016	<0.001	0.256
Lactose (%)	4.76	4.75	4.75	4.78	4.82	4.76	0.040	0.338	0.885	0.138
MUN (mg/kg)	40.6^z	28.9^z	68.6^y	57.8^y	130.2^x	111.5^x	4.57	<0.001	0.006	0.421

a–d Means within a row with different superscripts are different due to the silage × acacia interaction at $P < 0.05$.

x–z Means within a row with different superscripts are different due to the silage effect at $P < 0.05$.

[1] Control = energy and protein concentrate containing 100 g of wheat straw meal/kg of DM.

[2] Acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract/kg of DM.

[3] Here defined as milk yield (kg) × [0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%)]/3.14.

(**A:P**) ratio was lower for RG (3.54) compared with CL (4.09) and SF (4.27; $P < 0.001$). Acacia had no effect on ruminal NH_3-N and total VFA concentration; however, it caused a decrease in the acetate (65.7% vs. 67.9%) and an increase in the propionate (17.5% vs. 16.6%) proportions and consequently decreased the A:P ratio ($P < 0.001$). Although the proportion of n-valerate decreased with acacia (1.15% vs. 1.41%; $P < 0.001$), the differences between the 2 RG treatments were more evident than between those of SF and CL (silage × acacia, $P = 0.003$). No treatment effect was found on the proportions of isobutyrate and isovalerate.

Concerning the ruminal microbiota, 3,162 bacterial and 419 archaeal OTU were assigned to the samples. The principal coordinate analysis of the distances among the centroids of the single treatments showed a clear separation according to silage type for both community types (PERMANOVA, $P < 0.001$; Figure 1; Supplemental Figure S2, https://doi.org/10.5281/zenodo.7646644). In contrast, the effect of acacia ($P = 0.012$) and a silage × acacia interaction ($P = 0.002$) were identified only for the archaeal community. At the phylum level, the bacterial community structure composition was dominated by *Bacteroidetes* (42.3%), followed by *Firmicutes* (38.3%; Figure 2A; Supplemental Tables S2 and S3, https://doi.org/10.5281/zenodo.7646644). Less-abundant phyla were *Actinobacteria*, *Spirochaetes*, *Candidatus Saccharibacteria*, *Tenericutes*, and *Fibrobacteres*. We found 85 genera, 17 of which contributed more than 1% to the total abundance. Unclassified (**unc.**) *Prevotellaceae*, unc. *Clostridiales*, unc. *Bacteroidetes*, unc. *Lachnospiraceae*, unc. *Bacteroidales*, unc. *Bacteria*, *Prevotella*, unc. *Firmicutes*, unc. *Ruminococcaceae*, *Olsenella*, *Treponema*, *Bifidobacterium*, *Butyrivibrio*, *Succiniclasticum*, unc. *Coriobacteriaceae*, and unc. *Bacteroidia* contributed more than 90% to the total relative abundance. For cows fed RG-based diets, lower abundances of *Butyrivibrio* and *Prevotella* were detected than for cows fed SF and CL ($P < 0.01$; Figure 2A). Unclassified *Firmicutes* and unc. *Lachnospiraceae* showed the opposite response, being more abundant in RG compared with the 2 legumes ($P < 0.001$; Figure 2A). For bacteria belonging to the phylum *Actinobacteria*, SF-fed cows had a lower abundance of *Olsenella* ($P < 0.001$) than those fed CL and RG, whereas unc. *Coriobacteriaceae* was more abundant for SF, followed by RG and CL ($P < 0.001$; Figure 2A). The contribution of the bacterial taxa toward the changes in the bacterial communities with respect to silage type was shown by the linear discriminant analysis effect size (Figure 3A) and confirmed the silage-specific abundance of a few bacterial taxa. The archaeal community was dominated by the genera *Methanobrevibacter*, *Methanosphaera*, and *Methanomassiliicoccus* (72.2%, 17.2%, and 5.7%, on

Table 4. Effects of silage type and acacia supplementation on ruminal fluid variables

Item	Perennial ryegrass Control[1]	Perennial ryegrass Acacia[2]	Sainfoin Control	Sainfoin Acacia	Red clover Control	Red clover Acacia	SEM	Silage	Acacia	Silage × Acacia
Ammonia N (mM)	1.10[z]	0.94[z]	2.59[y]	2.36[y]	3.45[x]	3.86[x]	0.291	<0.001	0.668	0.420
VFA										
Total (mM)	72.7[x]	71.4[x]	66.1[xy]	70.3[xy]	62.3[y]	68.0[y]	3.38	<0.001	0.703	0.369
Acetate (mol %)	65.1[y]	62.5[y]	69.5[x]	67.4[x]	69.0[x]	67.2[x]	0.57	0.011	<0.001	0.605
Propionate (mol %)	17.5[x]	19.0[x]	16.0[y]	16.4[y]	16.3[y]	17.2[y]	0.34	<0.001	<0.001	0.244
n-Butyrate (mol %)	14.3[x]	15.1[x]	11.6[y]	13.4[y]	11.8[y]	12.6[y]	0.53	<0.001	0.190	0.436
Isobutyrate (mol %)	0.76	0.78	0.85	0.69	0.81	0.77	0.038	0.192	0.771	0.061
n-Valerate (mol %)	1.31[b]	1.74[a]	1.05[d]	1.23[bc]	1.08[cd]	1.27[b]	0.064	<0.001	<0.001	0.003
Isovalerate (mol %)	0.98	0.89	1.03	0.85	1.04	0.94	0.071	0.802	0.359	0.800
Acetate:Propionate	3.75[y]	3.33[y]	4.39[x]	4.14[x]	4.26[x]	3.93[x]	0.102	<0.001	<0.001	0.638

a–dMeans within a row with different superscripts are different due to the silage × acacia interaction at $P < 0.05$.

x–zMeans within a row with different superscripts are different due to the silage effect at $P < 0.05$.

[1]Control = energy and protein concentrate containing 100 g of wheat straw meal/kg of DM.

[2]Acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract/kg of DM.
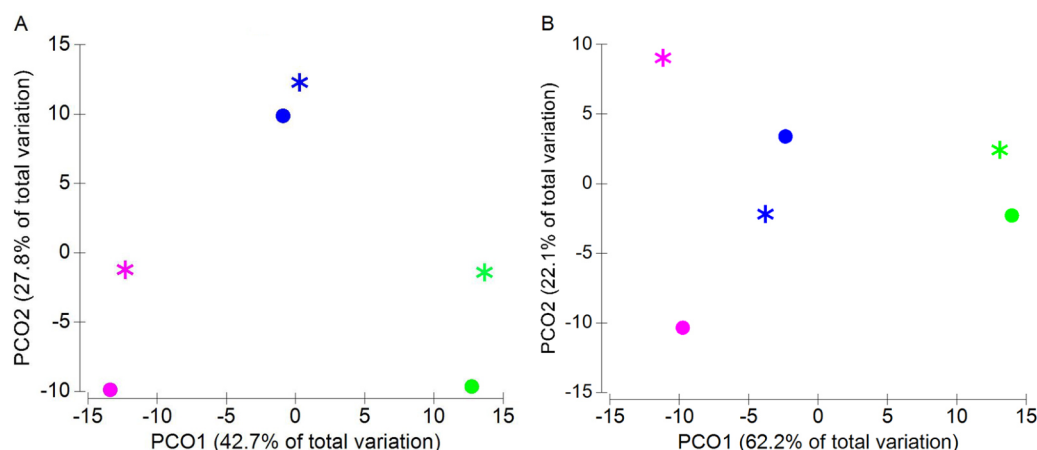
Figure 1. Principal coordinate analysis (PCO) plots depicting the centroids of each dietary treatment on the bacterial (A) and archaeal (B) community structures. Green = diet with perennial ryegrass-rich silage; purple = diet with sainfoin-rich silage; blue = diet with red clover-rich silage; asterisks = diet with energy and protein concentrate containing 100 g of wheat straw meal per kilogram of DM (control); circles = diet with energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract per kilogram of DM (acacia).

average, respectively; Figure 2B; Supplemental Tables S4 and S5, https://doi.org/10.5281/zenodo.7646644). *Methanobrevibacter* were more abundant in RG, followed by SF and CL ($P < 0.001$) and numerically less abundant with acacia ($P = 0.091$; Figure 2B). In addition, *Methanomassiliicoccus* were more abundant in RG and SF compared with CL ($P < 0.05$). *Methanosphaera* were most abundant in SF, intermediate in CL, and lowest in RG ($P < 0.001$). Unclassified *Thermoplasmata* were more abundant in RG than in CL ($P = 0.002$), whereas they were intermediate in SF and more abundant in acacia compared with the control across all silages by 31% ($P = 0.017$). The differences within the archaeal communities with respect to treatment effects are represented by 3 genera: *Methanobrevibacter, Methanomassiliicoccus,* and *Methanosphaera* (Figure 3B). The abundance of archaeal community members was significantly correlated with feed and fermentation variables on 10 occasions (Supplemental Table S6, https://doi.org/10.5281/zenodo.7646644). Accordingly, the abundance of *Methanobrevibacter* was correlated with the intake of WSC ($r = 0.61$; $P < 0.001$) and NDF ($r = 0.66$; $P < 0.001$; Supplemental Table S6). Furthermore, the abundance of *Methanosphaera* was positively correlated with CP intake ($r = 0.62$), ruminal NH_3 concentration ($r = 0.51$), ruminal acetate proportion ($r = 0.58$), and the A:P ratio ($r = 0.58$) and negatively correlated with WSC ($r = -0.80$) and NDF intake ($r = -0.83$) and the proportions of ruminal propionate ($r = -0.51$) and valerate ($r = 0.52$; all $P < 0.001$).

Feeding the cows SF promoted the excretion of wet feces (61.6 kg/d) compared with RG (51.4 kg/d; $P < 0.001$), whereas CL feed took an intermediate position (56.8 kg/d; Table 5). Urine excretion was greatest for CL (44.2 kg/d), followed by SF (33.8 kg/d) and RG (29.7 kg/d; $P < 0.001$). It was lower with acacia than with the control (34.2 vs. 37.7 kg/d; $P = 0.034$), which resulted in a 5% higher ($P = 0.043$) initial DM content of the manure. Manure samples from the SF feed had the greatest initial DM content [76.4 g/kg of fresh matter (**FM**)], followed by CL (67.4 g/kg of FM) and RG (63.2 g/kg of FM; $P < 0.001$). Manure from SF-fed cows had the greatest initial C content (438 g/kg) followed by those from RG-fed (413 g/kg) and CL-fed cows (408 g/kg; $P < 0.001$). Manure from SF-fed and RG-fed cows had a greater initial NDF content than that from CL-fed cows ($P < 0.001$). Manure from SF-fed cows had the greatest initial ADF content (299 g/kg) followed by those from RG-fed (192 g/kg) and CL-fed cows (143 g/kg; $P < 0.001$). Acacia increased the initial manure content of DM (70.7 vs. 67.4 g/kg of FM; $P = 0.043$) and C (425 vs. 415 g/kg of DM; $P = 0.009$) compared with the control-fed cows. The initial N content of the manure was greatest for CL (41.3 g/kg), lower for SF (36.9 g/kg), and lowest for RG (33.7 g/kg; $P < 0.001$); however, it was not affected by acacia. No significant differences among treatments were detected in the contents of total, soluble, protein-bound, and fiber-bound CT of the initial manure, although CT contents were numerically elevated when feeding of CT-containing feeds (acacia and sainfoin). Treatments had no effect on C, N, and wet and dry matter losses during storage, whereas acacia caused a reduction in N losses by 6% on average ($P = 0.042$). The initial C:N ratio of the manure was lower with CL (10.0) compared with SF (11.9) and RG (12.3; $P < 0.001$). These differences
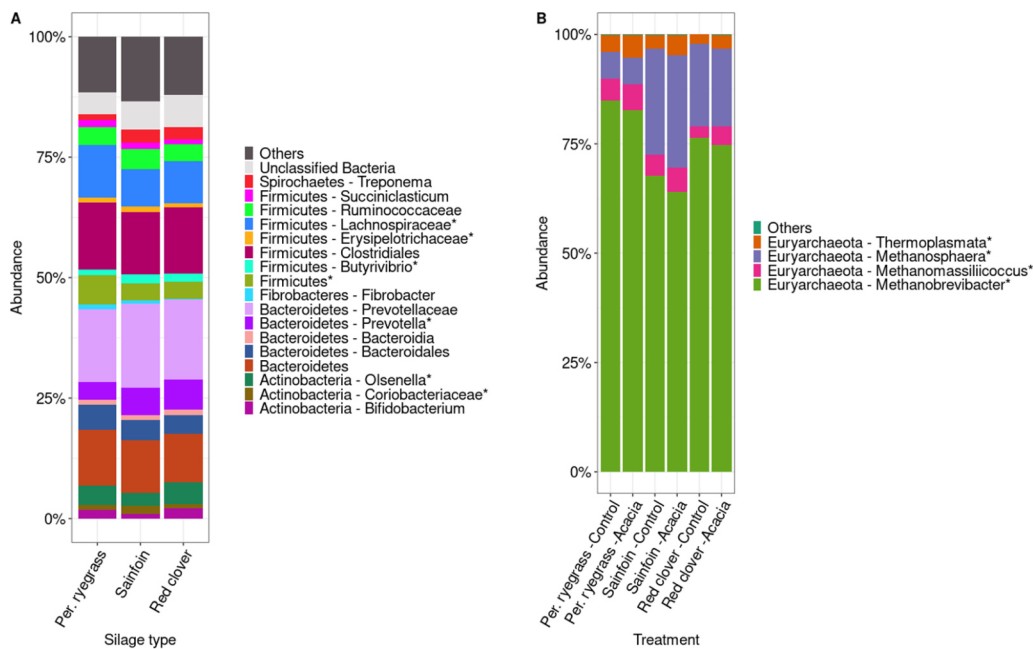
Figure 2. Bacterial (A) and archaeal (B) phylogenetic distributions obtained from 16S rRNA gene sequencing. The taxa are presented at their lowest level of taxonomic identification. The asterisks indicate the taxa showing significant differences ($P < 0.05$) between silage types (for bacteria) and between treatments (for archaea). Taxa with a contribution of <1% are summed up in "others." Control = energy and protein concentrate containing 100 g of wheat straw meal per kilogram of DM; acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract per kilogram of DM. Per. = perennial.

remained largely unchanged until the end of the storage experiment (CL: 10.0; SF: 11.6; RG: 11.2; $P < 0.001$). In contrast, acacia decreased the final C:N ratio (10.5 vs. 11.4; $P = 0.001$). Silage type had an effect on the initial and final manure pH ($P = 0.001$), and acacia decreased the final pH ($P = 0.006$). The cumulative CH_4 emissions from the manure were lowest for SF, followed by CL and RG ($P < 0.001$; Figure 4). The effect of acacia ($P = 0.015$) was not consistent across silage types, as it decreased manure-derived CH_4 only with RG (silage × acacia, $P = 0.009$).

DISCUSSION

Depending on the dose and source, CT may mitigate ruminal CH_4 emissions but also reduce diet digestibility. Emissions of CH_4 from manure depend, among other factors, on the amount of OM excreted in the feces, which is affected by diet composition, digestibility, and feeding level (Huhtanen et al., 2021). However, the extra amount of OM excreted has to be fermentable in the manure; otherwise CH_4 production does not increase. Therefore, quantifying CH_4 production from the excreta is important in order to comprehensively assess dietary CH_4 mitigation measures, such as CT supplementation. We followed this comprehensive ap-

proach in our study. The levels of production, yield, and emission intensity of the CH_4 measured in our cows were in the range of values reported in other studies on dairy cows fed herbage-based diets (van Dorland et al., 2007; Enriquez-Hidalgo et al., 2014). The CT amounts provided were 4 times higher with SF than with acacia, but the latter was at least similarly effective in causing changes similar to SF. The CT of acacia were found to be entirely soluble, whereas almost half of the CT in the sainfoin-rich silage were protein- or fiber-bound. Therefore, the acacia CT might be more reactive than the CT in SF (García et al., 2017), also due to the different CT structure (Venter et al., 2012; Mueller-Harvey et al., 2019).

Effects of Acacia Supplementation and Additivity with Sainfoin

Acacia extract, 1 of the 2 forms of CT used in this experiment, probably reduced ruminal CP degradation, as suggested by the lower MUN and the lower urine amount excreted by acacia-fed cows. Likewise, in a parallel experiment, Lazzari et al. (2023) found a shift from urine N to feces N when feeding cows the same diet. This was expected from the use of CT (Carulla et al., 2005; Grainger et al., 2009) and indicates its benefi-
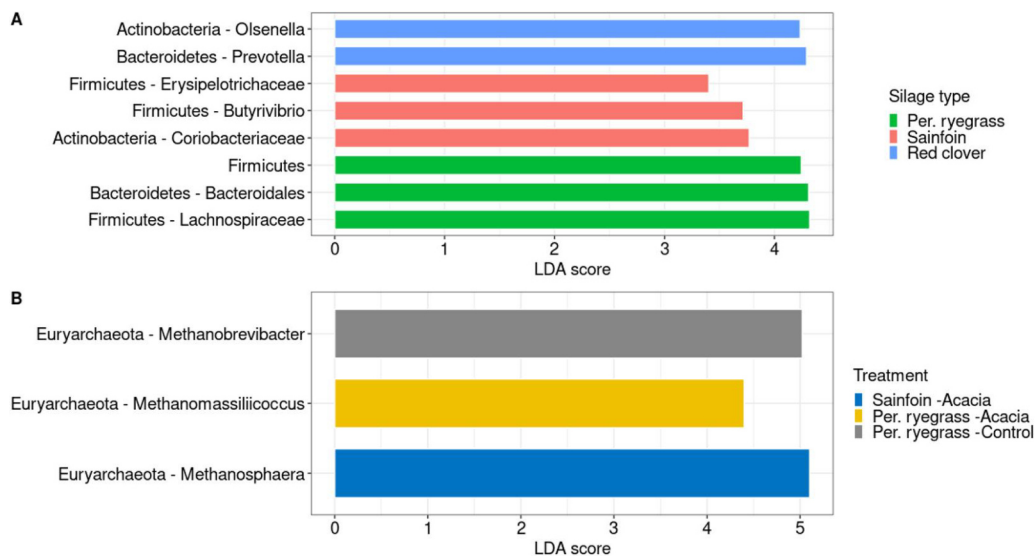
Figure 3. Bacterial (A) and archaeal (B) taxa that showed statistical significance ($P < 0.05$) based on linear discriminant analysis (LDA) effect size. The LDA score is shown. An LDA score threshold of >2 was used. The taxa are presented at their lowest level of taxonomic identification. Per. = perennial.

cial effect on the N emission potential of manure from acacia-fed cows. In this context, acacia must have acted additively to sainfoin, as MUN, milk protein content, and lower urine excretion were affected for all 3 silages (no silage × acacia interaction).

Acacia also seemed to reduce carbohydrate degradation (especially fiber) in the rumen for all silages, as indicated by the lower acetate proportion of total VFA and the lower A:P ratio when compared with control diets. Indeed, Carulla et al. (2005) and Koenig and Beauchemin (2018) found that cows had lower feed digestibility when fed acacia extracts, and the effects on acetate, propionate, and n-butyrate proportions resembled those found by Carulla et al. (2005). The lower fiber degradation caused by acacia probably decreased the H_2 production—that is, the main substrate for the methanogens—and thus explains a large part of the reduction in ruminal CH_4 production. In this case, acacia acted additively, showing an effect with all 3 types of silages on ruminal H_2 and CH_4 production. The lower ruminal H_2 availability might have led to the lower abundance of *Methanobrevibacter*, which are CO_2-reducing hydrogenotrophic strains with higher thresholds for H_2 compared with methylotrophic archaea, such as *Methanosphaera, Methanomassiliicoccus*, and unc. *Thermoplasmata* (Pitta et al., 2022). Accordingly, when H_2 concentrations are low, *Methanobrevibacter* are outcompeted (Pitta et al., 2022). Across experiments, the effects of acacia were much less consistent with regard to the mitigation of CH_4 yield and emission intensity. In dairy cows, CH_4/DMI was unaffected in

our study, whereas it was reduced in the studies by Grainger et al. (2009; DMI only estimated) and Denninger et al. (2020) and showed a tendency to decrease in the study by Alves et al. (2017). Furthermore, acacia did not reduce the emission intensity per unit of ECM, thereby confirming the findings of Grainger et al. (2009) and Denninger et al. (2020), who used similar doses to the ones fed in the present experiment, whereas emission intensity tended to decrease in the study by Alves et al. (2017), in which a lower dose of acacia was fed (0.7% extract in diet DM). The majority of studies that have tested acacia found that it did not decrease DMI (Griffiths et al., 2013; Orlandi et al., 2015; Gerlach et al., 2018), as was also the case in 2 (SF and CL) of the 3 silages tested in this experiment. In all these cases, acacia was supplemented to diets with CP content above 15%, whereas with the present RG diet (13% CP) DMI was decreased when feeding acacia. Our interpretation is that, due to the high affinity of CT for CP, supplementing acacia to low-protein diets critically decreases ruminal N availability for microbial growth, consequently hindering feed degradation and the feed passage rate through the gut, thus lowering DMI. Concerning variables related to N losses, the detrimental effect of acacia on low CP diets is less clear, as also shown by Lazzari et al. (2023), where most of N loss-related variables showed no clear silage × acacia interaction. The effects of acacia supplementation on milk yield are not consistent across studies. Gerlach et al. (2018) found no effects, whereas Grainger et al. (2009) measured a lower milk yield. Griffiths et al.

Table 5. Effects of silage type and acacia supplementation on the composition and pH of manure

| | Treatment | | | | | | | P-values | | |
| | Perennial ryegrass | | Sainfoin | | Red clover | | | | | |
Item	Control[1]	Acacia[2]	Control	Acacia	Control	Acacia	SEM	Silage	Acacia	Silage × Acacia
Feces (kg of wet matter/cow per day)	50.9^y	51.8^y	62.5^x	60.7^x	55.3^{xy}	58.2^{xy}	2.43	0.001	0.757	0.550
Urine (kg/cow per day)	31.9^z	27.6^z	35.4^y	32.2^y	45.8^x	42.6^x	1.76	<0.001	0.034	0.906
Initial manure composition										
DM (g/kg of original matter)	61.3^z	65.1^z	75.2^x	77.7^x	65.7^y	69.2^y	1.89	<0.001	0.043	0.884
OM (g/kg of DM)	762^y	786^y	788^x	798^x	718^z	735^z	5.9	<0.001	<0.001	0.313
NDF (g/kg of DM)	391^x	395^x	404^x	405^x	266^y	262^y	8.1	<0.001	0.723	0.882
ADF (g/kg of DM)	194^y	190^y	291^x	306^x	143^z	143^z	9.1	<0.001	0.641	0.345
C (g/kg of DM)	408^y	419^y	435^x	442^x	403^y	413^y	3.1	<0.001	0.009	0.778
N (g/kg of DM)	32.9^z	34.4^z	36.3^y	37.5^y	40.7^x	41.8^x	1.11	<0.001	0.223	0.974
Condensed tannins[3] (g/kg of DM)										
Total	0.39	1.50	4.04	5.49	0.42	1.41	2.390	0.363	0.470	0.928
Soluble	0.16	0.83	0.47	1.21	0.09	0.57	0.783	0.936	0.511	0.992
Protein-bound	0.18	0.54	2.16	2.80	0.22	0.64	1.201	0.347	0.525	0.929
Fiber-bound	0.06	0.13	1.42	1.48	0.12	0.20	0.809	0.291	0.558	0.922
Cumulative CH$_4$ (mg/500 g of manure)	355^a	296^b	188^d	225^{cd}	238^{bc}	249^{bc}	31.6	<0.001	0.015	0.009
Losses during 30 d of manure storage (% of initial)										
C	78.4	77.9	81.8	80.7	79.7	80.3	1.88	0.395	0.864	0.890
N	83.1	88.5	82.3	83.4	75.6	84.2	2.72	0.086	0.042	0.392
Wet weight	81.9	81.7	82.0	82.0	82.2	82.2	0.35	0.294	0.194	0.686
Dry weight	82.7	80.8	85.4	84.0	83.7	83.9	1.67	0.474	0.420	0.789
C:N ratio										
Initial	12.4^x	12.2^x	12.0^x	11.8^x	10.0^y	9.9^y	0.30	<0.001	0.558	0.952
Final	11.7^x	10.7^x	11.9^x	11.3^x	10.5^y	9.5^y	0.27	<0.001	0.001	0.502
pH										
Initial	7.78^z	7.79^z	8.00^x	7.98^x	7.86^y	7.88^y	0.026	<0.001	0.791	0.640
Final	7.64^z	7.50^z	7.66^y	7.66^y	7.97^x	7.87^x	0.072	<0.001	0.006	0.138

a–dMeans within a row with different superscripts are different due to the silage × acacia interaction at $P < 0.05$.

x–zMeans within a row with different superscripts are different due to the silage effect at $P < 0.05$.

[1]Control = energy and protein concentrate containing 100 g of wheat straw meal/kg of DM.

[2]Acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* condensed tannin–rich extract/kg of DM.
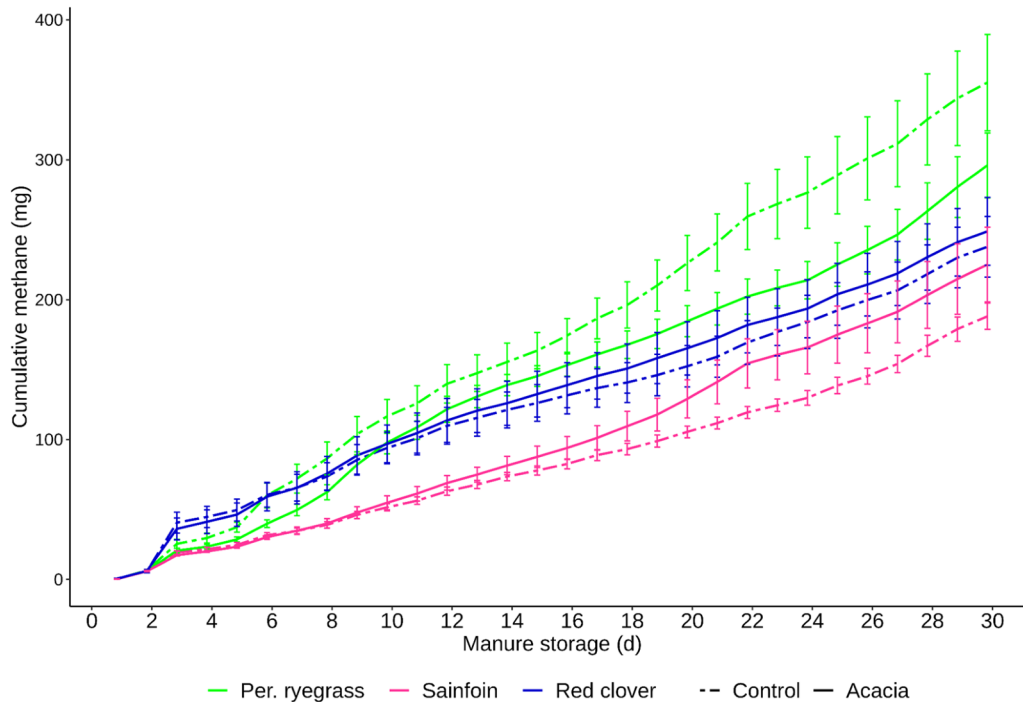
[3]Expressed as cyanidine equivalents.

Figure 4. Cumulative methane emissions from 535 g of reconstituted manure (mean ± SE; silage, $P < 0.001$; acacia, $P = 0.015$; silage × acacia, $P = 0.009$). Control = energy and protein concentrate containing 100 g of wheat straw meal per kilogram of DM; acacia = energy and protein concentrate containing 100 g of *Acacia mearnsii* CT-rich extract per kilogram of DM. Per. = perennial.

(2013) noted that milk yield declined only at high doses of acacia (740 g of CT-rich extract/cow per day). In the present study, at much lower levels (on average, 402 g of CT-rich extract/cow per d), acacia reduced ECM yield only with RG, possibly because the rumen-available N with RG was close to a critical threshold.

From the few studies investigating the effects of tannins on CH_4 emissions from manure, it seems that tannins are more efficient in emission mitigation when they are directly added to the excreta than when they are supplemented in the diet. Accordingly, the CT-rich quebracho extract added at several dosages strongly reduced the CH_4 emission from the manure in vitro in the studies by Whitehead et al. (2013; 0.25% and 0.5% wt/vol) and Min et al. (2022; 4% and 8% wt/wt). Conversely, Staerfl et al. (2012) and Fagundes et al. (2020) found a reduction of enteric CH_4 emissions from beef cattle supplemented with acacia but no effects on manure-derived CH_4. With our diets, acacia reduced OM digestibility (Lazzari et al., 2023), slightly increasing the OM content in the manure. However, manure CH_4 emissions did not increase, and acacia even caused a decline in the case of RG. Here the additional, widely indigestible CT excreted with the manure (also indicated by the numerically higher CT contents of the

manure of the acacia-fed cows) seemed to have counteracted additional CH_4 emission from the extra OM.

A possible explanation for this is that when CP is low, CT might bind more intensively to other dietary compounds (such as NDF) or remain free in the rumen, interacting with dietary compounds in the lower gut or after excretion. With RG, methanogenesis might have been reduced due to less substrate available and a lack of soluble N sources (mostly of NH_3) deriving from urinary urea (Lazzari et al., 2023), which is necessary for microbial growth and methanogenesis (Procházka et al., 2012). The total N, soluble or inorganic N, was limiting in RG + acacia, whereas the manure C:N ratio was not affected by acacia at the beginning of the incubation experiment.

Effects of Silage Type

The SF and CL diets were accepted by the cows, as can be concluded from the same level of DMI in the 2 groups. This agrees with Huyen et al. (2016) but is in contrast to Scharenberg et al. (2009) and Chung et al. (2013), who noted lower intakes with sainfoin. The inconsistency of the results between the studies might be explained by the characteristics of the batches fed or by

the forage to which sainfoin was compared. In fact, accession and climate conditions during growing and forage conservation can affect CT content, structure, and solubility, potentially affecting CT effects on the animal (Mueller-Harvey et al., 2019). Huyen et al. (2016) used sainfoin silage as in our experiment. However, Scharenberg et al. (2009) found intake declines with sainfoin hay compared with grass hay, and Chung et al. (2013) found intake declines with fresh sainfoin compared with fresh alfalfa. The SF diets had some mitigation effects on ruminal CH_4 production compared with CL; however, they did not exhibit a mitigating effect on CH_4 emission intensity. Other studies that compared sainfoin to non-tanniferous legumes on enteric CH_4 in vivo have found no or very weak effects of sainfoin. Chung et al. (2013) observed that sainfoin had a slight mitigating effect on CH_4 emissions compared with alfalfa in beef heifers. Stewart et al. (2019) compared sainfoin hay with non-tanniferous legumes and grass hays and found a lower CH_4 yield only in comparison with grass hay with a high content of highly digestible NDF (meadow bromegrass) in beef heifers. The effects of sainfoin on methanogenesis may be modulated by the direct action of the CT but also by a possibly lower nutritive quality of the forage, particularly in terms of the digestibility of the fiber. In support of this, Scharenberg et al. (2009) and Huyen et al. (2016) reported lower digestibility of sainfoin compared with grasses (hay and silage, respectively). As cows fed SF showed the lowest CH_4 yield per fiber intake at NDF and ADF intakes quite similar to those in the RG and CL diets, this also points toward a lower fiber digestibility.

Compared with the 2 legume-based diets, the DMI was clearly lower with RG. In contrast, van Dorland et al. (2007) found a similar DMI of ryegrass and red clover silages. These differences in DMI might have been due to differences in CP content (Hristov et al., 2004) and to the stage and quality (fiber content and lignification) of the forages at harvest. The lower DMI of RG might have caused a slower passage rate in these cows (Van Soest, 1994). Moreover, grasses (such as ryegrass) are known to have a slower passage rate and particle breakdown than legumes due to their higher cell wall content (Van Soest, 1994). This slows down rumen emptying and explains the greater VFA concentration in rumen fluid from RG-fed cows, especially when compared with CL-fed cows. The coarser structure and higher fiber content of the RG diets compared with the legume-rich diets might also have resulted in lower digestibility (Enriquez-Hidalgo et al., 2014). Meanwhile, the CT present in SF must have had a negative effect on protein digestibility (as also shown by Lazzari et al.,

2023), as cows fed SF showed a milk protein content similar to those fed RG despite the greater dietary CP content of SF compared with RG (17% vs. 13%). The concomitantly higher dietary content of fiber, the major substrate of the methanogens (Janssen, 2010), and of the RG diet probably also explains the mitigation effects of SF on ruminal CH_4 yield per kilogram of DMI compared with RG, which was not found with CL. In addition, the probable slower passage rate of RG compared with SF might explain the higher CH_4 per gram of DMI with RG compared with SF (Janssen, 2010). These results confirm those of Huyen et al. (2016) when comparing sainfoin with ryegrass in dairy cows. In contrast, Stewart et al. (2019) found no effects on CH_4 yield when comparing sainfoin, alfalfa, and bromegrass hays. When comparing RG with non-tanniferous CL, the differences in ruminal CH_4 were rather small in the present study and were absent in the study by van Dorland et al. (2007) when comparing red clover and ryegrass silages with similar NDF contents (possibly because of similar CP contents). The RG diet also contained higher WSC content than legume diets, probably leading to a higher ruminal A:P (Janssen, 2010). Notably, Belanche et al. (2013) compared ryegrass and red clover (with chemical compositions similar to those of our silages) in vitro and found similar results for the total VFA and A:P ratio. The higher CP content of SF and CL might have increased the abundance of *Butyrivibrio* and *Prevotella*, which are generally considered proteolytic (Wallace, 1996). Moreover, these bacteria are also reported to form cohorts with specific methanogens, such as *Methanosphaera*, in low CH_4-yield phenotype cows (Pitta et al., 2022) and to be pectinolytic species that yield high amounts of acetate (Matthews et al., 2019). Acetate (higher in SF and CL than in RG) is positively correlated with *Methanosphaera*, which are obligate hydrogenotrophic methanogens, and methylotrophic archaea (Fricke et al., 2006), which strictly require acetate as a carbon source for growth (Saengkerdsub and Ricke, 2014). *Methanosphaera* reduces H_2 to CH_4, thus consuming methanol as a substrate, which is a hydrolysis product of methyl esters produced during the catabolism of pectin (Saengkerdsub and Ricke, 2014). Pectin is known to be more abundant in legumes than in grasses. An RG diet showed greater CH_4 yield per kilogram of DMI than an SF diet in the current study and also showed a higher abundance of *Lachnospiraceae* (described by Tapio et al., 2017, and Pitta et al., 2022, as a member of a high-CH_4 ruminotype) and *Methanobrevibacter* (described as hydrogenotrophic high CH_4-producing archaea; Pitta et al., 2022). We also found *Methanobrevibacter* to be

positively correlated with dietary WSC and NDF, the degradation of which is known to produce substrates for methanogenesis.

The ADF content of the manure from the SF-fed cows used for the incubation experiment, despite being greater than that in RG and CL, did not lead to greater CH_4 emissions from the manure. This indicates that the ADF of SF was more resistant to fermentation than that of RG and CL; it was more resistant not only to ruminal degradation (as measured by Lazzari et al., 2023) but also to fermentation in the manure.

When considering the effects of the 3 diets characterized by the different forages, it should be noted that they did not consist of monocultures of ryegrass, sainfoin, and clovers. Therefore, the small contribution of other forage species might also have had a certain influence.

CONCLUSIONS

Acacia supplementation showed no mitigation effects on ruminal CH_4 emission intensity. The same was true for SF, which at least had some mitigation potential concerning CH_4 yield per unit of DMI. Combining the results of the present study with those of a parallel study in which the digestibility of the same diet was measured, we were able to show that acacia and sainfoin decrease OM digestibility and consequently lead to higher OM amounts in the manure. However, this did not increase the CH_4 emissions from manure. Supplementation of acacia to low-protein silages must be carefully evaluated, as, in the present study, it drastically reduced feed intake and milk production. Both ruminal and manure-derived CH_4 emissions were dependent on the fiber content of the animals' diets. The level of effect of acacia depends on the characteristics of the forage to which it is added. The same was observed for sainfoin, whose effect depends on the silage type with which it is compared. Further studies should prolong the manure experiment and investigate the utility of supplementing acacia directly to the manure as a measure to mitigate CH_4 and N emissions.

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