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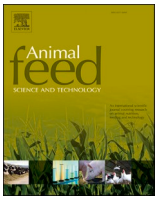

Effects of tanniferous sainfoin and *Acacia mearnsii* extract on urinary N excretion and ammonia volatilization from the slurry of dairy cows

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ABSTRACT

This study examined whether the effects of the tanniferous extract of *Acacia mearnsii* on N partitioning in dairy cows and ammonia (NH_3) volatilization from their excreta are additive to that of the tanniferous legume sainfoin (*Onobrychis viciifolia*) and remain consistent when supplemented to different silage types with varying crude protein (CP) content. In a 6 × 6 Latin Square arrangement, six multiparous Holstein cows (milk yield: 36.6 ± 3.9 kg/d; 70 ± 13 d in milk) were assigned randomly to six treatments. The experiment included a 14-d adaptation and a 7-d data collection period, where intake, milk yield, and composition were recorded daily and excreta were collected. Cows had ad libitum access to one of six total mixed rations containing (dry matter [DM] basis) 750 g/kg DM silage. The silages were rich in either sainfoin (180 g/kg CP of DM), ryegrass (118 g/kg CP) or clover (220 g/kg CP). Each silage was supplemented with 20 g/kg DM of *Acacia mearnsii* extract or straw meal. The effects on N partitioning, ruminal volatile fatty acids (VFA) and microbiota, and NH_3 volatilization from slurry reconstituted from fresh urine and feces were determined. The sainfoin-based diet reduced the apparent digestibility of organic matter, acid detergent fiber, and N compared with the ryegrass- and clover-based diets ($P < 0.001$). Acacia reduced the DM intake ($P < 0.05$) and apparent digestibility of organic matter and N across all silage types ($P < 0.01$). The digestibility of neutral detergent fiber was reduced with sainfoin, and ryegrass with Acacia (Silage × Acacia interaction $P < 0.05$). Acacia reduced the ruminal acetate proportion ($P < 0.05$) but did not affect ruminal propionate and n-butyrate or the microbiota composition. With ryegrass, the rumen fluid had lower acetate ($P < 0.001$) and greater n-butyrate proportions ($P < 0.01$) compared to sainfoin and clover, lower propionate proportions as compared with sainfoin ($P < 0.05$), and the lowest blood urea N concentration ($P < 0.001$).

Abbreviations: AA, amino acids; ADFom, ash-free acid detergent fiber; aNDFom, ash-free neutral detergent fiber assayed with a heat stable amylase; BUN, blood urea nitrogen; CP, crude protein; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; ECM, energy corrected milk; MUN, milk urea nitrogen; NH_3, ammonia; NH_3-N, ammonia-nitrogen; NH_4^+, ammonium; NH_4^+-N, ammonium-nitrogen; NMDS, non-metric multidimensional scaling; OM, organic matter; OTU, operational taxonomic units; TMR, total mixed ratio; VFA, volatile fatty acids; UUN, urinary urea nitrogen.

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Acacia reduced energy-corrected milk yield ($P < 0.05$). Milk protein content was reduced with the sainfoin diet ($P < 0.05$). Sainfoin (compared to clover) and Acacia supplementation shifted the N excretion from urine to feces and decreased urinary urea N excretion and NH_3 volatilization from the slurry ($P < 0.05$). The effects of the two tannin sources were widely additive in mitigating urinary urea N and NH_3 volatilization. The results illustrate that combinatory N-abatement measures based on different CT sources are efficient, but supplementing Acacia resulted in decreasing dry matter intake and milk production, while sainfoin supplementation resulted in lower organic matter digestibility.

1. Introduction

In dairy cows, herbage-based diets may reduce competition in the production of human food and animal feed. However, these diets are often rich in rumen-degradable protein and imbalanced in their ratio of ruminal N to energy substrate, leading to excessive ammonia (NH_3) production in the rumen (Broderick, 2003). Excessive ruminal NH_3 is absorbed and transported via blood circulation to the liver, where it is metabolized into urea. The urea is then mainly excreted as urinary urea N (UUN), which is easily emitted as NH_3 (Dijkstra et al., 2013). Moreover, the high crude protein (CP) content of the diet is a critical driver of low N use efficiency (Huhtanen and Hristov, 2009) and is positively related to N excretion (Yan et al., 2006). However, reducing CP in herbage-based diets is difficult, as high forage yield through N fertilization or the use of forage legumes and low CP content of herbage are often mutually exclusive.

One solution may be dietary supplementation with condensed tannins (CT). The CT bind to dietary CP and reduce its ruminal degradation rate, thus shifting the N excretion pathway from urine to feces (Orlandi et al., 2015) and decreasing the NH_3 volatilization potential of the excreta (Misselbrook et al., 2005). Among sources of CT, the extract of the bark of *Acacia mearnsii* (hereafter "Acacia") and the temperate climate forage legume sainfoin (*Onobrychis viciifolia*) have been investigated for their effects on N partitioning in animals, especially on the excretion of urinary N (Acacia: e.g., Koenig and Beauchemin, 2018a; sainfoin: e.g., Huyen et al., 2016) and, in one study, N emissions from the excreta of CT-fed animals (Acacia: Hao et al., 2011). To the best of our knowledge, the effects of sainfoin and Acacia on animals and their excreta have not been studied within the same experiment. Information is also lacking on whether combinations of different sources of CT in one diet (to maximize beneficial effects) are additive in their effects or whether efficiency declines, rendering this strategy less useful.

Apart from their potential to decrease metabolic and environmental loads, CT may improve N utilization, potentially due to an

Table 1
Ingredients and chemical composition of diets, fed as total mixed ratio (means[1] ± SD).

	Dietary treatment[2]					
	RG		SF		CL	
Item	–	+	–	+	–	+
Ingredients in total diet (g/kg of DM)						
Silage	753 ± 3	753 ± 4	756 ± 15	756 ± 16	750 ± 25	749 ± 26
Whole-corn plant pellets	43 ± 1	43 ± 1	43 ± 2	43 ± 2	45 ± 04	45 ± 4
Energy concentrate	167 ± 2	167 ± 2	201 ± 13	201 ± 14	206 ± 21	206 ± 22
Protein concentrate[3]	37 ± 1	37 ± 1	—	—	—	—
Analyzed composition (g/kg of DM, unless stated otherwise)						
DM (g/kg of wet matter)	453 ± 7	452 ± 13	500 ± 68	496 ± 66	543 ± 52	539 ± 51
Organic matter	906 ± 4	907 ± 4	900 ± 6	901 ± 5	875 ± 2	874 ± 5
Crude protein	128 ± 5	130 ± 7	170 ± 6	168 ± 7	201 ± 9	201 ± 10
Neutreal detergent fiber (aNDFom)	365 ± 10	346 ± 13	307 ± 13	296 ± 13	317 ± 8	299 ± 9
Acid detergent fiber (ADFom)	220 ± 7	206 ± 7	222 ± 13	218 ± 13	194 ± 6	183 ± 9
Water-soluble carbohydrates	98.4 ± 15.4	97.6 ± 18.8	51.1 ± 10.0	53.6 ± 10.6	53.2 ± 7.7	56.6 ± 7.6
Condensed tannins (g/kg)[4]						
Total	0.49 ± 0.22	4.64 ± 0.73	12.22 ± 5.90	17.90 ± 5.80	0.69 ± 0.26	4.23 ± 0.72
Soluble	0.32 ± 0.21	4.41 ± 0.70	7.30 ± 4.35	12.31 ± 4.60	0.43 ± 0.21	3.89 ± 0.69
Protein-bound	0.15 ± 0.10	0.20 ± 0.04	4.16 ± 1.64	4.68 ± 1.33	0.17 ± 0.04	0.26 ± 0.08
Fiber-bound	0.03 ± 0.01	0.03 ± 0.01	0.76 ± 0.40	0.90 ± 0.49	0.09 ± 0.02	0.09 ± 0.03
Calculated energy and protein supply[5]						
NE_L (MJ/kg DM)	6.25 ± 0.04	6.25 ± 0.04	6.15 ± 0.24	6.15 ± 0.24	6.20 ± 0.13	6.20 ± 0.13
APDE (g/kg of DM)	97.5 ± 2.9	97.5 ± 2.9	96.9 ± 2.3	96.9 ± 2.3	96.9 ± 1.4	96.9 ± 1.4
APDN (g/kg of DM)	85.8 ± 5.1	85.8 ± 5.1	107.4 ± 5.4	107.4 ± 5.4	125.9 ± 3.5	125.9 ± 3.5

[1] Mean over the 6 periods of the Latin square.
[2] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets; "–" = energy concentrate without Acacia extract; "+ " = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.
[3] Added to the diets from the second period onwards.
[4] As cyanidin equivalent.
[5] Calculated according to Agroscope (2020) analogous to MP. APDE/APDN = absorbable protein at the duodenum consisting of rumen-bypass protein and microbial protein from fermentable energy/rumen-degradable protein.

increased amino acid (AA) supply in the small intestine (Min et al., 2003). This has been shown for Acacia and sainfoin tannins, although the effects have not been consistent across studies. An increase in body N retention when supplementing diets with Acacia was found by Orlandi et al. (2015) and Carulla et al. (2005) in steers and sheep, respectively, but not by Kozloski et al. (2012) in sheep. When feeding sainfoin, Egan and Ulyatt (1980) found a greater body N retention in sheep, whereas Grosse Brinkhaus et al. (2016) and Huyen et al. (2016) did not find effects on N retention or the efficiency of N utilization for milk protein synthesis in dairy cows. This inconsistency, among other factors, might be related to variations in forage type (Grosse Brinkhaus et al., 2016) or dietary CP content, with which CT strongly interact.

Accordingly, the goal of the present study was to quantify the effects of Acacia supplementation to a sainfoin-rich silage (medium-high CP) and to compare the effects of Acacia to those occurring using silages rich in ryegrass (low CP) or clover (high CP) in terms of N partitioning and ruminal microbiota and fermentation in dairy cows as well as NH_3 volatilization from their excreta. We tested the following hypotheses: i) Acacia and sainfoin reduce urinary N losses and NH_3 volatilization from cows' excreta; ii) both CT sources have only minor adverse effects on N utilization and efficiency; and iii) the effects of these two sources are fully additive.

2. Materials and methods

2.1. Experimental design, diets, and animals

All procedures were carried out in accordance with Swiss guidelines for animal welfare and were approved (No. 2018_25_FR) by the Animal Care Authority of Canton Fribourg, Switzerland. The experiments were conducted at the experimental farm of Agroscope, Posieux, Switzerland.

Six multiparous Swiss Holstein-Friesian cows were assigned randomly to the six treatments in a 6 × 6 Latin square arrangement. At the start of the experiment, the cows were 70 ± 13 days in milk (mean ± SD), weighed 649 ± 47 kg, and produced 36.6 ± 3.9 kg/d of milk. Each of the six consecutive experimental periods consisted of 21 d, including a 14-d adaptation period, where the cows were tethered in individual stalls equipped with rubber mat flooring, and a 7-d data collection period, where each cow was kept in a metabolism stand fitted with a partially slatted floor. Cows were offered a total mixed ratio (TMR) with ad libitum access and had unrestricted access to tap water throughout the experiment. During adaptation, they were milked in the milking parlor and in their metabolism stand during collection periods. Prior to the experiment, the animals were accustomed to the metabolism stand for 1 d.

The experimental variation was created by combining three types of herbage silages with two differently composed energy concentrates, resulting in six different treatments (Table 1). The herbage silages were either rich in ryegrass (representing a negative control in terms of CP) or red clover (positive control) or were composed mainly of sainfoin. The chemical compositions of the silages are presented in Supplementary file S1. The ryegrass-rich material was harvested as first cut at the stage of beginning ear emergence and consisted (DM basis) of 610 g/kg perennial ryegrass (*Lolium perenne*), 250 g/kg other grasses, 120 g/kg red clover (*Trifolium pratense*), and 20 g/kg other dicotyledons. The sainfoin-rich material was harvested at the late bud stage as first, second, or third cut on two adjacent fields and had an average composition (DM basis) of 800 g/kg sainfoin (*Onobrychis viciifolia*), 30 g/kg grasses, 70 g/kg other legumes, and 100 g/kg other dicotyledons. The first cut was fed in collection periods 2, 4, 5, and 6, and the second and third cuts were fed in periods 3 and 1, respectively. The clover-rich material was a fifth cut harvested at the late bud stage and composed (DM basis) of 450 g/kg red clover, 140 g/kg white clover (*Trifolium repens*), 390 g/kg grasses, and 30 g/kg other dicotyledons. All herbages were grown in Posieux, Switzerland (650 m a.s.l.) and ensiled in vertical silos (ryegrass-rich and clover-rich material) or bales (sainfoin-rich material). The complete diets were denominated "RG," "CL," and "SF." The energy concentrates contained the following (per kg as fed): corn, 395 g; wheat, 268 g; wheat straw meal (—) or Acacia extract (+), 100 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, 44200 IU; vitamin D_3, 3510 IU; vitamin E, 208 mg; β-carotene, 90 mg; and biotin, 6 mg. The Acacia extract was a commercial product (Seta SB, Seta, Estância Velha, Brazil), originating from the bark of *A. mearnsii* trees. The batch was analyzed and contained 190 and 476 g/kg DM of soluble CT and total polyphenols, respectively.

In the RG treatment, 36 (± 1) g/kg of the energy concentrate was replaced with a protein concentrate, which consisted (per kg as fed) of soybean cake (500 g), corn gluten meal (260 g), wheat straw meal or tannin-rich extract (100 g), potato protein (90 g), sugar beet molasses (36 g), and animal fat (18 g). Adding Acacia extract to both types of concentrates ensured that the proportion always amounted to 100 g/kg in the concentrate and, consequently, to 20 g/kg of the entire diets. The diets were calculated to meet the nutritional requirements for a cow producing 30 kg/d of milk (Agroscope, 2021).

2.2. Data recording and sample collection

Individual feed intake was recorded daily throughout the experiment by weighing the TMR distributed and leftovers removed immediately before new supply (at 1500 h). At the same time, samples of TMR and leftovers were collected, and they were pooled by cow across the entire collection periods and stored at − 20 °C for later analysis. The body weight of the cows was recorded twice a day after milking during the adaptation periods using a walk-over scale (Hokofarm Group, Marknesse, The Netherlands). Milk yields were determined at each milking (0500 and 1600 h), and milk samples were collected daily during each collection period. Samples from the evening and morning milkings were pooled proportional to the milk yield. Sub-samples were preserved with 2-bromo-2-nitro-1,3-propanediol (Bronopol®) for analysis of milk gross constituents, and further sub-samples were conserved at − 20 °C for analysis of N and urea content.

Feces and urine were collected quantitatively every day of the collection period. To separate urine from the feces, waterproof

urinals were used, as described by Grosse Brinkhaus et al. (2016). At the end of the urinal, urine was split into two sub-samples, one remaining non-acidified and the other being immediately acidified with 50 g of 3 *M* sulfuric acid to avoid N loss. Acidified and non-acidified urine and feces samples were collected by cow proportionally to the quantities excreted daily, pooled by cow per collection period, and stored at − 20 °C for later analysis. On day five of each collection period, additional feces and non-acidified urine were collected from each cow for immediate slurry incubation.

Ruminal fluid was collected via a stomach tube (Selekt, Virbac, Kolding, Denmark) after morning milking (between 0530 and 0630 h) at the beginning and end of each collection period. The first 0.5 L of ruminal fluid collected was discarded to avoid saliva contamination. The samples were cooled on ice directly after sampling. For analysis of VFA and NH_3 nitrogen (NH_3-N) concentrations, 10 mL of ruminal fluid were mixed with 0.2 mL of 500 mL/L sulfuric acid or with 0.2 mL of 500 mL/L trichloroacetic acid, respectively, and stored at − 20 °C. Untreated samples (50 mL) were immediately frozen at − 20 °C for later analysis of ruminal microbiota. Access to feed and water was terminated 2 h before ruminal fluid collection for minimizing diet by animal interactions for the analysis of the ruminal microbiota (de Assis Lage et al., 2020).

On the same days, immediately before ruminal fluid collection, blood was sampled from the jugular vein using serum tubes (art. n. 455092, Greiner Bio-One, St-Gallen, Switzerland; Grosse Brinkhaus et al., 2016). After 1 h, the tubes were centrifuged for 15 and 2 min at 3000 and 4000g, respectively, at room temperature (Grosse Brinkhaus et al., 2016). The retrieved serum was stored at − 20 °C for later analysis of blood urea nitrogen (BUN).

2.3. Dynamic chamber measurements

Approximately 100 g of fresh feces were mixed with urine in the ratio excreted within the preceding 24 h. These mixed samples were diluted with distilled water in a 1:1 ratio to achieve an approximate DM content of 5% to avoid crusting. This level of dilution is typical in Swiss dairy housing with cubicles. The diluted samples were blended using a commercial single-blade mixer (Bosch, Gerlingen, Germany). Two 50 g sub-samples were taken and transferred into 500 mL jars (100–0500–01, Savillex, Eden Prairie, MN, USA), so each sample was incubated in duplicate. The remaining slurry was immediately frozen at − 20 °C for later chemical analysis.

A dynamic chamber system, adapted from Misselbrook et al. (2005), was used to measure the NH_3 volatilized from the slurry. Briefly, a series of 12 jars was kept in a water bath at 25 °C. The jars had been sealed with screw caps (600–089–01, Savillex) equipped with two ¼" tube ports (Swagelok, Solon, OH, USA) on the top for air inlet and outlet. All jars, pipelines, and fittings were manufactured in perfluoroalkoxy alkanes to avoid adsorption of NH_3 to the surfaces. The outlet ports of the jar caps were connected to a multi-channel sampler (model A0311-S1, Picarro, CA, USA), and an attached pump (KNF, Sursee, Switzerland) created constant airflow through the jars across the slurry surface. The NH_3 concentration of the outgoing airflow was analyzed using a cavity ring-down spectrometer (model G2103, Picarro, CA, USA). An additional port of the multi-channel sampler was kept open to ambient air to measure the background NH_3 concentration. Each jar was measured for 10 min sequentially, and the measuring cycle was repeated over 48 h.

2.4. Laboratory analyses

Feed samples (ingredients, TMR, and leftovers) and fecal samples were lyophilized (Delta 1–24 LSC; Christ, Osterode, Germany) and ground to pass 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 340, Precisa, Dietikon, Switzerland), with subsequent incineration at 550 °C until reaching a constant weight. The difference between DM and ash was defined as organic matter (OM). The neutral detergent fiber (aNDFom, method ISO 16472:2006) and acid detergent fiber (aADF, method ISO 13906:2008) contents were determined using a Fibertherm (Gerhardt, Königswinter, Germany) and corrected for ash content. The aNDFom was analyzed with the addition of heat-stable amylase and sodium sulfite. Water-soluble carbohydrates were determined following Hall et al. (1999). The total N content of the TMR, leftovers, feces, urine, slurry, and milk was analyzed using the Kjeldahl method (AOAC International, 1995; method 988.05). The CP content of feed items was calculated by multiplying the N content with 6.25. The ammonium (NH_4^+) content of the slurry was analyzed by Kjeldahl colorimetric titration after alkalinization by MgO (VDLUFA MB2.1–3.2.2), and the pH was measured using a portable pH meter (Hanna Instruments, Woonsocket, RI, USA). The content of soluble, protein-bound, fiber-bound, and total CT was determined in triplicate using the HCl-butanol method, as described by Terrill et al. (1992). The CT content was expressed as cyanidin equivalents. Total phenols were analyzed using the Folin–Ciocalteau assay, according to Salminen and Karonen (2011), and were expressed as cyanidin equivalents.

Ruminal VFA were analyzed using HPLC equipped with a refractive index detector (Shodex RI, Denko KK, Minato, Japan) and a Nucleogel ION column (300 OA 300 × 7.8 mm, Macherey-Nagel, Düren, Germany). The samples were thawed to characterize ruminal bacteria and archaea, and 250 mg of ruminal fluid was used to extract DNA with the FastDNA™ SPIN Kit for Soil (MP Biomedical, Solon, OH, USA), as previously described by Deusch et al. (2017). The quality and purity of the DNA extracts were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V1–2 region of the 16 S rRNA gene was amplified by PCR, and Illumina library preparation was performed as described by Kaewtapee et al. (2017) for bacteria and by Deusch et al. (2017) for archaea. The PCR product integrity was analyzed by gel electrophoresis. Amplicons were purified and normalized using a SequalPrep Normalization Kit (Invitrogen, Carlsbad, CA, USA) and paired-end sequenced (2 ×250 bp) on an Illumina Miseq platform. Sequences were submitted to the European Nucleotide Archive under accession number PRJEB51039. Raw sequencing data were processed with the Mothur pipeline (Kozich et al., 2013). UCHIME was used to identify possible chimeras, and taxonomic assignation was performed using the SILVA reference database (release 132). Reads were clustered at 97% identity into 3048 bacterial

operational taxonomic units (OTU) and 374 archaeal OTUs. OTUs with a sequence length > 250 bp and an average abundance higher than 0.0001% were used for further analysis. Concentrations of NH_3-N in ruminal fluid and urea N in blood and urine were analyzed colorimetrically with commercial test kits (for ruminal NH_3-N: Urea liquicolor 10505, Human, Wiesbaden, Germany; for blood and urinary urea N: Urea Greiner Diagnostic 147 116, Greiner Diagnostic, Langenthal, Switzerland).

Milk samples were analyzed for fat, protein, and lactose content using Fourier-transformed infrared spectrometry (Milkoscan FT 6000, Foss, Hillerød, Denmark). The milk urea nitrogen (MUN) was determined using the differential pH method with MICROLAB EFA (Hamilton, Bonaduz, Switzerland; method ISO 14637).

2.5. Calculations and statistical analysis

All data collected over several days were averaged per cow per period before statistical analysis. The NH_3 concentration of the air entering the slurry jars was subtracted from the NH_3 concentration of the exiting air. The mean values recorded over the final 30 s of each 10-min period were interpolated on an hourly basis. The area under every curve was calculated and used in the statistical analysis.

All statistical analyses were performed using the statistical software platform R (R Core Team, 2020). A linear mixed model (package "lme4″, function "lmer") was used for the ANOVA (package "car", function "Anova"), with silage type, Acacia supplementation, and their interaction as fixed effects. Experimental period and cow were considered random effects. Effects were considered statistically significant at $P < 0.05$. To verify the normal distribution of the residuals a Shapiro–Wilk normality test was performed, and residual plots were created. In the tables, data are presented as Least Square Means for the effect of silage type across Acacia treatments, the effect of Acacia supplementation across silage treatments, and the overall standard error of the mean (SEM). A Tukey test was used for multiple comparisons among silage types when the silage effect but not the interaction was significant and among treatments in the few cases when a silage × Acacia interaction occurred. Where interactions between silage type and Acacia supplementation occurred, they are mentioned in the text. Means by treatment and multiple comparisons of variables that showed significant interactions are available as supplementary material (Supplementary file S1).

Rumen bacterial and archaeal sequencing data were analyzed using Primer6 (v. 6.1.16) software, the Permanova+ (v. 1.0.6) statistical software package (PRIMER-E, Plymouth, UK), and R. A Bray–Curtis similarity matrix was created, and data were graphically represented by applying non-metric multidimensional scaling (NMDS). A permutational multivariate ANOVA was performed for statistical evaluation. Using R, the Kruskal–Wallis (package "stats," function "kruskal.test") test was conducted to determine differences among taxonomic groups across silage types, and a pairwise Wilcoxon test (package "stats," function "pairwise.wilcox.test") was applied for post hoc comparisons.

3. Results

There were large differences among diets with different silage types in CP content (low with RG, medium-high with SF, and high with CL) as well as in CT content (high in SF) (Table 1). Acacia supplementation increased CT in all silage types, but the associated exchange of straw meal did not change the nutritional value of the diets.

No difference in DM, OM, aNDFom, and ADFom intake caused by silage type was detected (Table 2). On average, the apparent

Table 2
Effects of silage type and Acacia supplementation on intake and apparent digestibility.

	Dietary treatment								
	Silage type[1]			Acacia[2]			P-value		
Item	RG	SF	CL	–	+	SEM	Silage (S)	Acacia (A)	S × A
Daily intake per cow									
Total DM (kg)	22.6	24.9	26.2	25.2	23.9	1.03	0.191	0.010	0.112
Organic matter (kg)	20.6	22.5	23.0	22.6	21.4	0.92	0.435	0.009	0.117
Neutreal detergent fiber (kg; aNDFom)	8.09	7.62	8.31	8.40	7.61	0.372	0.088	0.001	0.145
Acid detergent fiber (kg; ADFom)	4.83	5.50	5.08	5.38	4.90	0.255	0.313	0.005	0.269
Condensed tannins[3] (g)									
Total	54.2[b]	372.8[a]	64.9[b]	115.3	212.7	33.28	< 0.001	0.036	0.927
Soluble	50.2[b]	245.9[a]	57.2[b]	70.7	164.8	25.81	< 0.001	0.006	0.934
Protein-bound	3.8[b]	110.8[a]	5.6[b]	38.8	41.3	8.31	< 0.001	0.958	0.953
Fiber-bound	0.6[b]	16.7[a]	2.1[b]	6.0	6.9	2.31	< 0.001	0.989	0.837
Apparent digestibility (g/kg intake)									
Organic matter	744[a]	660[b]	726[a]	720	700	12.5	< 0.001	0.002	0.099
Neutreal detergent fiber (aNDFom)	652	485	704	618	608	21.4	< 0.001	0.014	0.028
Acid detergent fiber (ADFom)	712[a]	481[b]	746[a]	645	648	26.0	< 0.001	0.100	0.064
N	565[b]	509[c]	628[a]	597	538	17.6	< 0.001	< 0.001	0.643

[a,b]Means within a row with different superscripts are significantly different for silage type ($P < 0.05$).

[1] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.

[2] "–" = energy concentrate containing 100 g/kg DM of wheat straw meal; "+" = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.

[3] As cyanidin equivalent.

digestibility of OM and ADFom was reduced by 10% and 34%, respectively, with SF compared to RG and CL ($P < 0.001$). We found a silage × Acacia interaction in aNDFom apparent digestibility that was lower with RG + Acacia and both SF variants compared with RG withoutAcacia and both CL variants ($P = 0.028$, Supplementary file S1). The apparent N digestibility was greater for CL than for SF, whereas RG took an intermediate position ($P < 0.001$). The total CT intake and that of all fractions were greater for SF as compared with RG and CL ($P < 0.001$). Acacia supplementation reduced DMI and consequently OM, aNDFom, and ADFom intake by, on average, 5.0%, 5.1%, 9.4%, and 9.0%, respectively, ($P < 0.01$). The apparent digestibility of OM and N was reduced by 2.7% and 9.8%, respectively, on average ($P < 0.01$). Acacia supplementation increased the total and soluble CT intake ($P < 0.05$) but not the intake of protein- and fiber-bound CT.

The concentration of BUN was affected by Acacia only with SF (reduction from 10.2 to 7.1 mg/dL, Silage × Acacia interaction $P = 0.045$, Supplementary file S1). Cows fed RG had the lowest ruminal NH_3-N and BUN concentrations, followed by cows fed SF and CL ($P < 0.001$) (Table 3). Total VFA concentration was not affected by silage type. Cows fed CL and SF had higher ($P < 0.001$) molar proportion of acetate and propionate (significant only for SF) and a lower ($P < 0.01$) proportion of n-butyrate compared with cows fed RG. In the case of n-valerate, a silage × Acacia interaction occurred ($P = 0.007$) due to the stronger effect of the Acacia supplementation with RG compared to those of the other two treatments (Supplementary file S1). The acetate proportion decreased ($P = 0.023$) while the valerate proportion increased ($P < 0.001$) with Acacia. From the ruminal fluid, a total of 3048 bacterial and 374 archaeal OTUs were taxonomically assigned (Supplementary file S2). Permutational multivariate ANOVA results revealed differences between silage types ($P < 0.01$) but no effect of Acacia supplementation or Silage × Acacia interaction on bacterial and archaeal communities. Therefore, the following analysis is focused on silage type. The NMDS plots based on Bray–Curtis similarity show the sample distributions by silage type for the bacterial (Fig. 1A) and archaeal (Fig. 1B) community structures. Unclassified *Bacteroidales* were more abundant with RG when compared with CL and SF ($P = 0.001$, Fig. 2A). *Butyrivibrio* were more abundant with CL and SF compared with RG ($P < 0.05$). Unclassified *Coriobacteriaceae* were less abundant with CL than with SF ($P = 0.004$), while in RG this genus took an intermediate position. Unclassified *Firmicutes* were more abundant with RG than with CL ($P = 0.032$); the abundance of the genus in SF took an intermediate position. *Methanobrevibacter* were more abundant in RG, followed by CL and SF ($P < 0.001$), and the opposite trend was observed for *Methanosphera* ($P < 0.001$). Unclassified *Thermoplasmata* were more abundant in RG and SF compared with CL ($P = 0.002$); *Methanomassiliicoccus* showed the same trend, but the differences were only numeric ($P = 0.068$).

Daily yields of milk, energy corrected milk (ECM), and milk fat, protein, and lactose were highest for CL, followed by SF and RG ($P < 0.001$) (Table 4). Milk protein content was lower with SF than with CL ($P = 0.047$), with RG being intermediate. The yield and concentration of MUN were highest ($P < 0.001$) with CL, followed by SF and RG, but they were not affected by Acacia supplementation. In addition, Acacia supplementation had no effect ($P = 0.117$) on milk yield. However, the milk fat and protein contents were reduced by Acacia ($P = 0.013$). Accordingly, yields of ECM, fat, and protein were also reduced ($P < 0.05$).

N intake was highest when cows were fed CL, followed by SF and RG ($P < 0.001$, Table 5). Fecal N excretion was similar with CL and SF but higher than that with RG ($P < 0.001$). Cows fed CL had the highest urinary and total N excretion ($P < 0.001$), followed by SF and RG. The proportions of fecal and urinary N excretion in relation to N intake were lower with RG than with SF and CL ($P < 0.001$). The proportion of fecal N excretion was highest for SF, followed by CL and SF ($P < 0.001$). The proportion of urinary N was highest ($P < 0.001$) with CL (29.4%), followed by SF (21.9%) and RG (15.6%). Regarding UUN excretion, a silage × Acacia interaction occurred ($P = 0.028$), as the effect of Acacia supplementation was pronounced with CL (reduction from 209 to 162 g/d) and SF (96–61 g/d), whereas it was not significant with RG (31–15 g/d) and exhibited only a trend of a general effect ($P = 0.077$) (Supplementary file S1). The proportion of UUN in total urinary N was highest ($P < 0.001$) when cows were fed CL, followed by SF and RG. The amount of N excreted in milk was highest ($P < 0.001$) when cows were fed CL, followed by SF and RG. The proportion of milk N excretion related to N intake was higher when cows were fed RG, followed by SF and CL. Body N retention was not affected by silage

Table 3
Effects of silage type and Acacia supplementation on ruminal fluid variables and blood urea nitrogen concentration.

	Dietary treatment								
	Silage type[1]			Acacia[2]			P-value		
Item	RG	SF	CL	–	+	SEM	Silage (S)	Acacia (A)	S × A
Ruminal fluid variables									
NH_3-N (mM/L)	0.72[c]	1.65[b]	2.51[a]	1.70	1.55	0.161	< 0.001	0.983	0.392
VFA									
Total (mM/L)	78.5	79.0	73.4	78.9	74.9	6.09	0.065	0.086	0.152
Acetate (mol/ 100 mol)	64.2[b]	68.3[a]	68.0[a]	67.5	66.2	0.73	< 0.001	0.023	0.498
Propionate (mol/ 100 mol)	17.4[a]	16.2[b]	17.1[ab]	16.4	17.4	0.43	0.014	0.233	0.671
n-Butyrate (mol/ 100 mol)	15.3[a]	12.6[b]	11.9[b]	13.2	13.3	0.73	0.006	0.098	0.172
iso-Butyrate (mol/ 100 mol)	0.79	0.80	0.80	0.81	0.78	0.058	0.909	0.594	0.958
n-Valerate (mol/ 100 mol)	1.51[a]	1.19[c]	1.29[b]	1.19	1.46	0.052	< 0.001	< 0.001	0.007
iso-Valerate (mol/ 100 mol)	0.92	1.00	0.92	0.99	0.90	0.116	0.781	0.076	0.418
Blood urea nitrogen (mg/dL)	4.93[c]	10.24[b]	14.27[a]	10.12	9.13	0.700	< 0.001	0.372	0.045

[a,b]Means within a row with different superscripts are significantly different for silage type ($P < 0.05$).
[1] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.
[2] "–" = energy concentrate containing 100 g/kg DM of wheat straw meal; "+" = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.

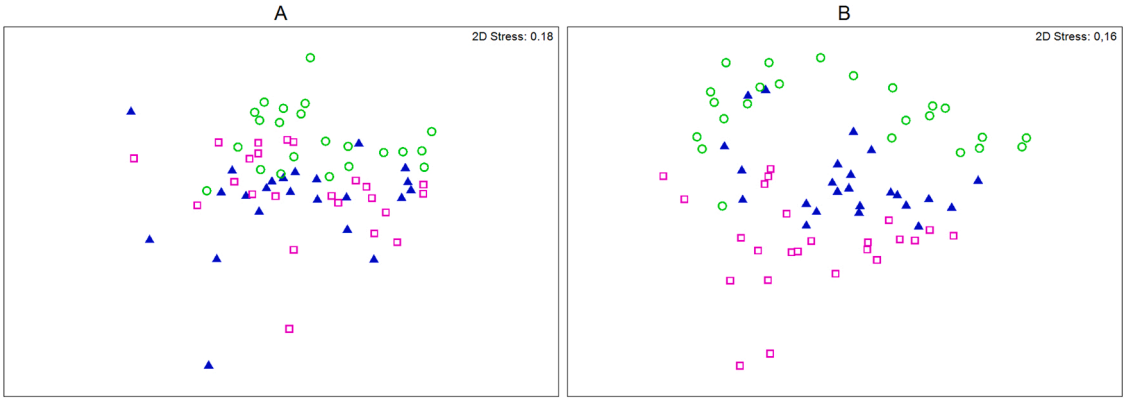

Fig. 1. Non-metric multidimensional scaling (NMDS) plots of the bacterial (A) and archaeal (B) community structures affected by the dietary treatments. Each point represents a single ruminal fluid sample. Green circles: ryegrass-rich silage-based diets; purple squares: sainfoin-rich silage-based diets; blue triangles: clover-rich silage-based diets.

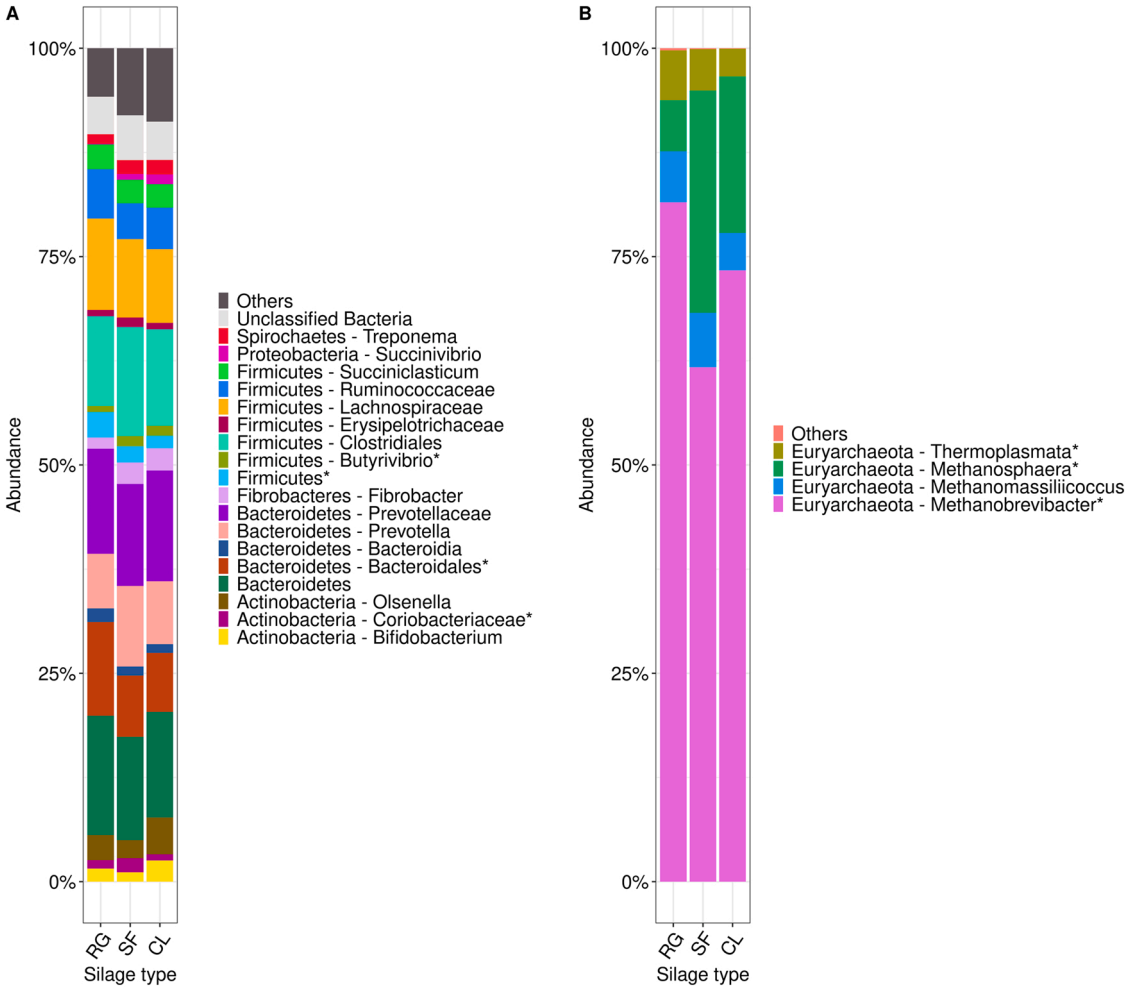

Fig. 2. Bacterial (A) and archaeal (B) phylogenetic distributions obtained from 16 S rRNA gene sequencing. Taxa are presented at their lowest level of taxonomic identification. Asterisks indicate taxa showing significant differences ($P < 0.05$) between silage types. Taxa with a contribution of $< 1\%$ are summed up in "Others." RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.

Table 4
Effects of silage type and Acacia supplementation on milk yield and composition.

	Dietary treatment								
	Silage type[1]			Acacia[2]			P-value		
Item	RG	SF	CL	–	+	SEM	Silage (S)	Acacia (A)	S × A
Daily yield per cow									
Total milk (kg)	24.8[c]	27.7[b]	30.5[a]	28.3	26.9	1.15	< 0.001	0.117	0.531
Energy corrected milk (kg)[3]	28.2[c]	31.1[b]	34.8[a]	32.8	29.9	1.48	< 0.001	0.022	0.657
Fat (kg)	1.27[c]	1.39[b]	1.55[a]	1.48	1.32	0.089	< 0.001	0.035	0.876
Protein (kg)	0.83[c]	0.90[b]	1.03[a]	0.96	0.88	0.039	< 0.001	0.017	0.708
Lactose (kg)	1.19[c]	1.31[b]	1.46[a]	1.35	1.28	0.063	< 0.001	0.268	0.664
Milk urea nitrogen (g)	0.65[c]	1.93[b]	3.97[a]	2.55	1.81	0.227	< 0.001	0.216	0.101
Milk composition									
Fat (g/kg)	51.5	50.6	51.1	52.4	49.7	2.38	0.521	0.013	0.664
Protein (g/kg)	33.4[ab]	32.9[b]	34.0[a]	33.8	33.0	1.02	0.047	0.013	0.864
Lactose (g/kg)	48.1	47.7	47.8	47.8	47.8	0.62	0.766	0.501	0.673
Milk urea nitrogen (mg/kg)	26.4[c]	68.3[b]	129.2[a]	85.4	63.9	5.51	< 0.001	0.095	0.252

[a,b]Means within a row with different superscripts are significantly different for silage type ($P < 0.05$).
[1] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.
[2] "–" = energy concentrate containing 100 g/kg DM of wheat straw meal; "+" = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.
[3] ECM is defined here as milk yield (kg) × [0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%)] / 3.14

Table 5
Effects of silage type and Acacia supplementation on N turnover.

	Dietary treatment								
	Silage type[1]			Acacia[2]			P-value		
Item	RG	SF	CL	–	+	SEM	Silage (S)	Acacia (A)	S × A
N balance (g/d)									
N intake	458[c]	660[b]	829[a]	663	635	28.6	< 0.001	0.200	0.331
Fecal N	197[b]	321[a]	307[a]	264	287	11.0	< 0.001	0.144	0.143
Urinary N	72[c]	141[b]	244[a]	170	134	10.0	< 0.001	0.028	0.808
Urinary urea N	23[c]	79[b]	186[a]	112	80	6.7	< 0.001	0.077	0.028
Fecal N & urinary N	269[c]	462[b]	551[a]	434	421	17.1	< 0.001	0.594	0.378
Milk N	130[c]	141[b]	165[a]	151	139	6.3	< 0.001	0.041	0.459
Body N retention[3]	59	58	113	78	75	19.7	0.272	0.409	0.489
N balance (g/kg of N intake)									
Fecal N	435[b]	491[a]	372[c]	403	462	17.6	< 0.001	< 0.001	0.643
Urinary N	157[c]	212[b]	294[a]	24.5	196	10.9	< 0.001	< 0.001	0.984
Fecal N & urinary N	592[b]	703[a]	666[a]	648	658	21.3	< 0.001	0.358	0.770
Milk N	286[a]	214[b]	199[b]	235	231	9.34	< 0.001	0.625	0.278
Urinary urea N (g/kg of urinary N)	308[c]	545[b]	761[a]	582	494	21.2	< 0.001	< 0.001	0.715
Urinary N (g/kg of fecal N & urinary N)	266[c]	302[b]	442[a]	376	297	13.2	< 0.001	< 0.001	0.533

[a,b]Means within a row with different superscripts are significantly different for silage type ($P < 0.05$).
[1] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.
[2] "–" = energy concentrate containing 100 g/kg DM of wheat straw meal; "+" = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.
[3] Body N retention is defined here as N intake – fecal N – urinary N – milk N.

type or Acacia supplementation. Acacia supplementation reduced ($P < 0.05$) N excretion via urine and milk. Related to N intake, Acacia supplementation caused a shift ($P < 0.05$) from urinary N excretion to fecal N excretion. Urinary N, as a percentage of fecal and urinary N, and UUN, as a percentage of urinary N, were reduced ($P < 0.05$) with Acacia supplementation.

Silage type influenced the DM and N contents of the slurry ($P < 0.001$, Table 6), whereas Acacia did not affect them. Acacia supplementation reduced the NH_4^+-N concentration significantly only with CL and SF (−33% and −37% respectively, Silage × Acacia interaction $P = 0.004$) (Supplementary file S1). The NH_4^+-N as a proportion of total N was influenced by both silage and Acacia ($P < 0.05$). The reducing effect of Acacia on NH_3 volatilization was not entirely the same across silage types (Silage × Acacia interaction $P = 0.007$, Fig. 3), accounting for −31, −38, and −61% in CL, SF, and RG, respectively (Supplementary file S1). In contrast, the absolute concentration values had the opposite ranking. Overall, Acacia reduced NH_3 volatilization across all silage types by an average of 37.1% ($P < 0.001$, Fig. 3).

Table 6
Effects of silage type and Acacia supplementation on composition and pH of slurry as well as ammonia (NH_3) concentration in the air sample from the headspace of slurry jars[1].

	Dietary treatment								
	Silage type[2]			Acacia[3]			P-value		
Item	RG	SF	CL	–	+	SEM	Silage (S)	Acacia (A)	S × A
Initial slurry composition									
DM (g/kg of wet matter)	48.8^{c}	56.7^{a}	52.6^{b}	52.1	53.3	14.57	< 0.001	0.165	0.434
N (g/kg of DM)	3.37^{c}	3.85^{b}	4.81^{a}	3.97	4.04	0.159	< 0.001	0.375	0.113
NH_4^+-N (g/kg of DM)	0.307^{c}	0.674^{b}	1.595^{a}	1.042	0.675	0.073	< 0.001	0.104	0.004
NH_4^+-N (g/kg of total N)	91.0^{c}	180^{b}	330^{a}	245	156	17.2	< 0.001	0.028	0.177
pH at start of incubation	7.68^{b}	7.95^{a}	7.77^{b}	7.80	7.80	0.067	< 0.001	0.478	0.671
pH at end of incubation	8.56^{c}	8.69^{b}	8.86^{a}	8.78	8.62	0.050	< 0.001	< 0.001	0.003
NH_3 concentration (mg/kg)									
1 h of incubation	0.80^{b}	1.27^{b}	2.50^{a}	1.88	1.17	0.248	< 0.001	0.005	0.930
12 h of incubation	1.28^{c}	3.31^{b}	10.05^{a}	6.12	3.64	0.562	< 0.001	0.140	0.010
24 h of incubation	2.22^{c}	4.40^{b}	10.63^{a}	7.27	4.23	0.615	< 0.001	0.015	0.025
48 h of incubation	3.22^{c}	6.04^{b}	12.95^{a}	9.06	5.75	0.721	< 0.001	0.003	0.010

^{a,b}Means within a row with different superscripts are significantly different for silage type ($P < 0.05$).
[1] Slurry reconstituted by mixing fresh feces and urine according to respective excretion ratio and diluted at a rate of 1:1 with distilled water.
[2] RG = ryegrass-rich silage-based diets; SF = sainfoin-rich silage-based diets; CL = clover-rich silage-based diets.
[3] "–" = energy concentrate containing 100 g/kg DM of wheat straw meal; "+" = energy concentrate containing 100 g/kg DM of Acacia CT-rich extract.

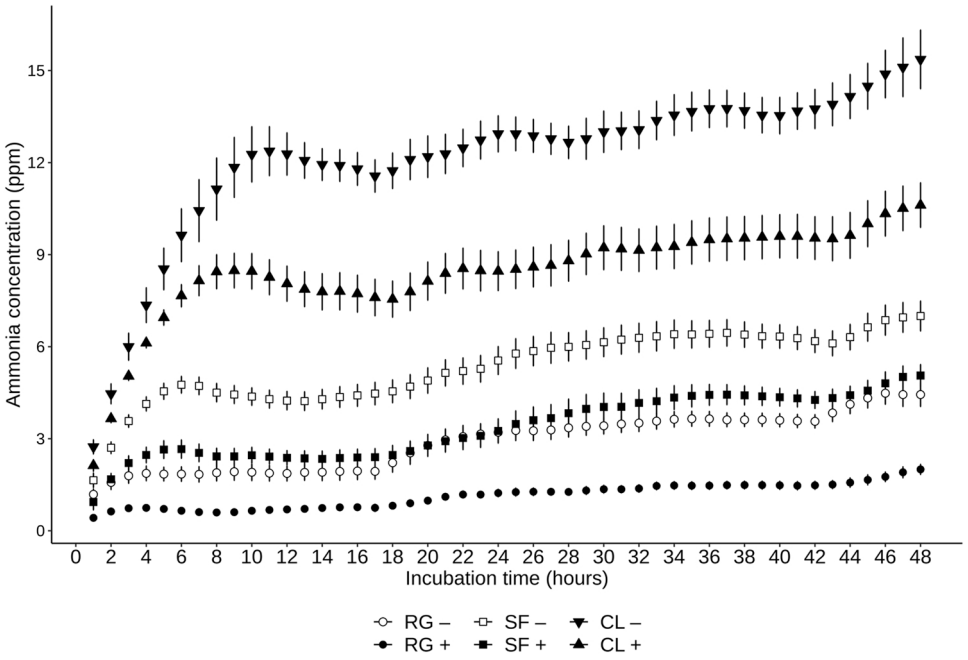

Fig. 3. Hourly (NH_3) concentration from fresh slurry (mean ± SE) incubated over a 48-h period (P Silage <0.001, P Acacia <0.001, P Silage × Acacia =0.007). RG = ryegrass-rich silage-based diets without Acacia extract; CL = clover-rich silage-based diets; SF = sainfoin-rich silage-based diets. "–" = energy concentrate containing 100 g/kg DM wheat straw meal; "+" = energy concentrate containing 100 g/kg DM Acacia CT-rich extract.

4. Discussion

In order to investigate the effect of sainfoin and Acacia on N losses, it seemed important to us to include measurements of the fresh slurry of cows, which, to our knowledge, has not been done previously. The measurements were conducted immediately after excretion to prevent losses caused by manipulation during freezing and thawing or a late onset of emissions until microbial activity is fully re-established (Hristov et al., 2019). Further, the use of a cavity ring-down spectrometer, compared to the acid impingers used in other studies (Misselbrook et al., 2005; Powell et al., 2011), allowed for high temporal resolution monitoring of NH_3 concentrations.

The concentration of CT found in the Acacia extract was similar to that reported in other studies (Denninger et al., 2020; Kozloski

et al., 2012; Minho et al., 2010), namely, between 150 and 190 g/kg DM. However, Carulla et al. (2005), Grainger et al. (2009), and Griffiths et al. (2013) found much higher CT concentrations, between 601 and 615 g/kg DM. This discrepancy is probably due to the standard used for the HCl-BuOH essay (i.e., cyanidin, leucocyanidin, or purified extract from *Acacia mearnsii*). The CT in Acacia (entirely soluble) were likely more reactive compared with the ones in sainfoin (which were, for almost the half of the total CT, protein or fiber bound) (García et al., 2017).

4.1. Effects of sainfoin and Acacia on urine N losses and NH_3 volatilization from slurry

To separate the effects of CP and CT, SF was compared to RG (low CP, low CT) and CL (high CP, low CT). The findings illustrate the known promotion of urinary N losses by a high-CP diet (Külling et al., 2001; Lee et al., 2012); the CT of SF were not sufficient to compensate (compared to CL) for the effect of its high CP content, as shown in other studies on dairy cows (Grosse Brinkhaus et al., 2016; Huyen et al., 2016) and beef heifers (Chung et al., 2013). Instead, all effects of the Acacia treatment in any of the silage types should exclusively reflect CT effects, as dietary CP content remained unchanged. Supplementation of 20 g/kg DM of Acacia extract decreased the urinary N excretion in the present study, as demonstrated previously in sheep (Carulla et al., 2005), beef cattle (Orlandi et al., 2015; Koenig and Beauchemin, 2018a), and dairy cows (Grainger et al., 2009).

Both SF and Acacia additively reduced the proportion of UUN in total urinary N. The reduction was also observed with a low dietary CP content (RG vs. CL [and SF]) (Dijkstra et al., 2013). Reductions of dietary CP may even have a more than proportionate mitigation effect on N emissions (Külling et al., 2001), as UUN and its metabolite NH_4^+-N in slurry are the most important drivers of NH_3 emissions from slurry. Still, a lower UUN might not be the only factor affecting NH_3 volatilization from manure. This is obvious from the observation that the level of effect of the two CT sources, SF (vs. CL) and Acacia, on mitigating NH_3 volatilization was clearly different from that on UUN. This could explain the interaction we found in NH_3 volatilization, which reflected an increasing reduction effect for greater N and NH_4^+-N concentrations in the slurry of CL. As CT are mostly excreted un-degraded (Makkar, 2003), they could inhibit urease production pre-excretion (anti-microbial activity), inhibit urease activity after excretion (anti-enzymatic activity) (Powell et al., 2011), and trap free NH_3 after urea hydrolysis by adsorption (Sepperer et al., 2020). Acacia supplemented to SF showed an additive effect of the two CT sources, enhancing the mitigation of NH_3 volatilization when compared to SF alone. The only other studies investigating the effects of sainfoin or Acacia in manure provided controversial results (Koenig et al., 2018b; Hao et al., 2011; Clemensen et al., 2020).

The results of the present study also provide information about the value of indicator traits for the N emission potential of CT-fed dairy cows. Previously, it was shown that MUN and BUN were related to UUN (Dijkstra et al., 2013), reflecting a decline in dietary CP or UUN. This was also noted in the present study when comparing CL and RG (MUN −80%, BUN −65%). Tanniferous SF also reduced MUN and BUN, but, unexpectedly, this phenomenon was not observed with Acacia. Therefore, the effect of CT is unclear, as the use of SF also resulted in a lower CP content compared to CL.

4.2. Effects of sainfoin and Acacia on N utilization and milk production

The persuasiveness of suggested dietary measures for mitigating N emissions from manure also depends on undesired side effects, such as reduced palatability and apparent digestibility, both of which impair nutrient uptake and, consequently, performance. In the present study, the higher milk yield of cows fed CL, compared to cows fed RG, confirms positive relationships between dietary CP, DMI, and milk production (Hristov et al., 2004).

Acacia supplemented at 20 g/kg DM consistently reduced DM and nutrient intake across all silages. Other studies that fed similar doses of CT extract found controversial effects. Grainger et al. (2009) and Kozloski et al. (2012) found a lower DM intake, but Orlandi et al. (2015) and Gerlach et al. (2018) did not. Carulla et al. (2005) even reported elevated DM intake with Acacia. Different from the decline in apparent digestibility found with Acacia, which is likely due to the effects of CT (Waghorn, 2008), the low apparent digestibility of the SF diet, as described by Scharenberg et al. (2009) and Huyen et al. (2016), could also be a result of the lower nutritional quality of sainfoin compared to high-quality forages, such as ryegrass and clover. The apparent digestibility of aNDFom was reduced by Acacia with RG but remained unchanged with SF and CL, indicating that there were no additive effects of the two CT sources. We interpret this as an antinutritional effect of Acacia, which might occur when it is supplemented to low-CP diets because cellulolytic microbes are particularly susceptible to rumen-degradable protein deficiency. Accordingly, this suggests that the CP content of the ryegrass treatment was borderline, even without extra tannins, and it became deficient with Acacia supplementation. However, the lack of a Silage × Acacia interaction in milk yield remains puzzling and indicates that metabolizable energy rather than metabolizable protein was limiting milk yield in the ryegrass treatment, although, as discussed below, this may not be true for milk protein production.

As N utilization for milk protein synthesis commonly declines with increasing levels of dietary CP (CL vs. RG) (Huhtanen and Hristov, 2009), SF should also be superior to CL in terms of N utilization, but due to high fecal N losses, these cows were less efficient in using dietary N for milk protein synthesis. No additive effects of the two CT sources were found on N utilization, as Acacia did not affect this variable. This is consistent with Kozloski et al. (2012), who found no improvements in N utilization, but it is in contrast with Carulla et al. (2005) and Orlandi et al. (2015), who found an increased duodenal flux of amino acids. The reduction of milk protein content caused by Acacia and SF suggests a lower supply of essential AA in the small intestine with CT diets, even at a high level of dietary CP. It could be that, different from abomasal pH, the high intestinal pH caused CT to bind to dietary or microbial protein. Otherwise, CP released from the bond with CT may not have been digested and absorbed in the small intestine (Waghorn, 2008). Finally, the CT might have reduced rumen microbial growth and protein synthesis (Min et al., 2003).

4.3. Effect of sainfoin and Acacia on ruminal microbiota

Although Acacia led to significant changes in apparent aNDFom and N digestibility, the bacterial community only responded to SF. Acacia may have non-specifically affected every bacterial group in the same way without affecting their abundance. The lower abundance of *Butyrivibrio* detected when feeding RG (the low CP diet) might be explained by the primary proteolytic activity commonly attributed to this genus (Attwood and Reilly, 1996; Wallace, 1996). However, other taxa, such as *Prevotella* and *Streptococcus*, which are also involved in proteolytic activity (Attwood and Reilly, 1996; Wallace, 1996), did not react. In the case of *Prevotella*, which is also an abundant fiber digester, the similar intake of fiber with all silage diets might have masked differences among treatments.

Methanosphaera (more abundant in CL and SF), unlike *Methanobrevibacter* (more abundant in RG), are methylotrophic strains (Garcia et al., 2000). Methyl groups are produced in the catabolism of pectins (Saengkerdsub and Ricke, 2014), which are known to be more abundant in legumes (clover and sainfoin) than in grasses (ryegrass). Moreover, some species of *Methanosphaera* are dependent on acetate as the main carbon source for growth (Saengkerdsub and Ricke, 2014), and this substrate was more abundant in CL and SF than in RG.

5. Conclusion

This study shows that it can be useful to combine sainfoin and *Acacia mearnsii* extract for the purpose of reducing urinary N excretion and NH_3 volatilization from slurry of dairy cows. The effects of sainfoin and Acacia were widely additive in this respect, mostly confirming hypotheses i and iii. However, when implementing these feeding strategies in dairy farming, possible adverse effects on animal productivity, such as a lower ECM yield (Acacia) or lower N utilization (sainfoin), must be taken into account (contradicting hypothesis ii). The utility of MUN and BUN as indicators of NH_3 volatilization from manure were more reliable in the case of dietary CP reduction than in the case of CT supplementation. Further studies should focus on the additive effects of sainfoin and Acacia at a practical scale, where NH_3 emissions are affected by critical environmental factors and slurry is often stored for a longer time before soil application. In addition, it should be determined whether both sources are also additive in terms of methane mitigation.

CRediT authorship contribution statement

G. Lazzari: Methodology, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **A. Münger**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **D. Heimo**: Methodology, Writing – original draft. **J. Seifert:** Methodology, Validation, Writing – original draft. **A. Camarinha-Silva**: Methodology, Data curation, Validation, Writing – original draft. **D. Borda-Molina:** Data curation, Writing – original draft. **M. Zähner:** Funding acquisition, Writing – original draft. **S. Schrade**: Conceptualization, Supervision, Project administration, Writing – original draft, Funding acquisition. **M. Kreuzer**: Supervision, Writing – original draft, Writing – review & editing. **F. Dohme-Meier**: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115577.

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