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Effects of dietary *Sanguisorba minor*, *Plantago lanceolata*, and *Lotus corniculatus* on urinary N excretion of dairy cows

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ABSTRACT

Context. Mitigating urinary nitrogen (N) losses is an important target of sustainable cattle nutrition concepts. One option to achieve this may be dietary inclusion of tanniferous herbs. **Aims.** Aim of the study was to investigate herbs with different profiles of tannins for their efficiency to abate urinary N losses. Small burnet (*Sanguisorba minor*) with high concentrations of total tannins, plantain (*Plantago lanceolata*) with low concentrations and birdsfoot trefoil (*Lotus corniculatus*) with expectedly high concentrations of condensed tannins were included in the treatments. **Methods.** The test plants were mixed in dried form into a grass–maize-silage diet at 80 g/kg of dietary dry matter. They replaced dried perennial ryegrass (control). Twenty-four multiparous dairy cows were randomly allocated to the four diets. Intake, eating time, rumination time, and milk yield were recorded individually, and representative samples of milk and excreta were collected and analysed six times within 14 days, following 10 days of adaptation. The diets with ryegrass, birdsfoot trefoil, plantain or burnet contained, per kilogram of dry matter, 0, 1.8, 1.2 and 1.9 g condensed tannins, 0.1, 1.9, 1.7 and 15.5 g total tannins, and 26.2, 28.5, 27.5 and 26.6 g N. **Key results.** Milk yield and composition were not affected by treatment, apart from a decline in milk protein content when feeding plantain. Milk urea concentration was reduced with burnet by more than 30%, compared with the control and plantain. Birdsfoot trefoil also reduced milk urea concentration, but to a lesser degree. Furthermore, the burnet treatment substantially shifted N excretion from urine to faeces (about 30% lower urine N losses). All treatments lowered the proportion of fine particles of <1.0 mm in faeces, what might be due to high fibre content of the control. **Conclusions.** At dietary proportion of 80 g/kg, burnet is a forage herb with potential to reduce ruminal ammonia generation as indicated by reduced urinary N and milk urea. Plantain and birdsfoot trefoil had no or negligible effects. **Implications.** The study indicated that small burnet could have potential as a feed additive for dairy cows in terms of N-use efficiency, lower emissions to the environment, and reduced animal metabolic stress.

Keywords: ammonia, chewing behaviour, low-input system, milk urea nitrogen, nitrogen emission, organic agriculture, plant secondary compound, polyphenol, rumen.

Introduction

Dairy production systems that are based on low or zero dietary inputs of cereals and oilseeds are increasingly aspired in ecological and organic agriculture (Brito and Silva 2020; Leiber 2022), so as to reduce feeding of livestock with human-edible food (Schader *et al.* 2015). Such systems may experience seasonal alterations in nutrient composition of the forages, which, due to the aforementioned reason, cannot be fully counterbalanced through concentrates. This may become a problem when there is excess of dietary N (Powell *et al.* 2012), such as, for example, in spring and autumn pasture. When high forage-N concentrations are associated with a relative lack of energy in the rumen, this may lead to an increase in ruminal ammonia production, with the consequence of metabolic stress and elevated urinary N excretion (Nousiainen *et al.* 2004; Pacheco and Waghorn 2008; Powell *et al.* 2012). One approach

to minimise ruminal ammonia production and thus N losses could consist of protecting greater proportions of feed protein from ruminal degradation by using plant tannins, which build complexes with proteins at ruminal pH (Mueller-Harvey *et al.* 2019). At abomasal pH, tannin–protein complexes may disintegrate, thus making the protein available for small-intestinal digestion (Mueller-Harvey *et al.* 2019). Even then, urinary N excretion would be reduced because the absorbed amino acids would be available for use in endogenous protein synthesis (Tseu *et al.* 2020), provided sufficient endogenous presence of energy, and the metabolic potential of the genotype. Any undigested protein will be excreted as less easily volatile faecal N, and will thus reduce urinary N losses. Besides condensed tannins (CT; Barry and McNabb 1999; Mueller-Harvey *et al.* 2019), hydrolysable tannins (HT) have also been demonstrated to lower ruminal ammonia production (Jayanegara *et al.* 2011) and milk urea concentration (Ali *et al.* 2017), the latter being considered as an indicator for urinary N excretion (Nousiainen *et al.* 2004).

Still, it remains a challenge to verify and implement the effects of tanniferous feed components in farm practice. One straightforward way could be the integration of forages with elevated tannin concentrations that grow under temperate climate into pasture swards (Cheng *et al.* 2017) or into mixed-feed rations. Until now, a focus of this approach was put on integrating tanniferous legumes such as sainfoin (*Onobrychis viciifolia*), birdsfoot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*) and sulla (*Hedysarum coronarium*) (e.g. Woodward *et al.* 2001; Min *et al.* 2003; Grosse Brinkhaus *et al.* 2016; Leiber *et al.* 2020; Kapp-Bitter *et al.* 2021*a*). For instance, birdsfoot trefoil was shown before to reduce ruminal protein degradation (Molan *et al.* 2001) and urinary N excretion (Ghelichkhan *et al.* 2018). As legumes have inherently high N concentration, which counteracts the purpose of the tannin supplementation, we put, with the current study, an emphasis on tanniferous herbs. We chose plantain (*Plantago lanceolata*) and small burnet (*Sanguisorba minor* Scop.; in the following called 'burnet'). Like legumes, the two herb species have been successfully established in grasslands (Hamacher *et al.* 2012). Plantain was already investigated on pasture or in mixed rations, but its effects on urine N losses and milk yield were not consistent (Cheng *et al.* 2017; Minneé *et al.* 2017; Bryant *et al.* 2018; Ineichen *et al.* 2019). Burnet, present in natural pastures, is characterised by a high content of secondary plant compounds (Hamacher *et al.* 2012) and has been shown to reduce ruminal ammonia concentration in sheep (Meissner *et al.* 1993) and urine N excretion in cattle (Stewart *et al.* 2019). *In vitro*, incubating a forage mixture containing burnet has been shown to result in markedly lower ammonia concentrations in the incubation liquid than those for the control, this at unchanged *in vitro* organic-matter digestibility (Kapp-Bitter *et al.* 2021*b*). In a recent study on the effects of multispecies forage mixtures so as to maintain milk yield and mitigate methane emissions of cows (Loza *et al.* 2021*a*), burnet was also included, but its specific contribution to the effect could not be evaluated with this approach. The aim of the present study was to test the hypothesis that the mitigation of urinary N losses would be overall more efficient with dietary supplementation of tanniferous herbs than with tanniferous legumes, owing to their concomitantly lower N concentration. A second hypothesis tested was that burnet, which is rich in dietary HT, would be more efficient than plants containing mainly CT (Kapp-Bitter *et al.* 2021*b*). Therefore, we included birdsfoot trefoil and plantain, both containing CT, and burnet, rich in TT, but with low concentrations of CT (which is assumed to be indicative for HT), in a standard forage-based basal diet for dairy cows at 80 g/kg dry matter (DM) and evaluated the effects on intake behaviour, digestibility, milk yield and quality, in addition to N excretion in faeces and urine. For the present study, we selected ryegras hay as the control.

Materials and methods

Ethical statement

This experiment was conducted in spring 2019 at the Research Station AgroVet-Strickhof, Eschikon-Lindau, Switzerland (47°26′54.613″N, 8°40′50.173″E). It was approved by the cantonal veterinary office of Aargau, Switzerland (AG75689).

Dietary treatments, cows and housing

The basal diet consisted of grass silage (ryegrass-dominated sward, harvested at panicle formation stage), maize silage, ryegrass hay (similar sward type and harvest stage as for the grass silage), wheat straw, and a protein-rich concentrate mixed on farm (Tables 1 and 2). The concentrate was composed of soybean meal, maize gluten, rapeseed meal, rapeseed cake, triticale, sugar beet molasses, sunflower meal, wheat starch, and minerals. This forage-based diet was designed to be slightly excessive in dietary protein so as to allow effects of tanniferous forages on urine N excretion. The experimental plant material was always supplemented at 80 g/kg DM. Owing to the limited vigour and growth performance of the test plants, we considered scenarios of establishment of such forages in pastures and diets on farm at a maximum of 80–100 g/kg biomass DM as realistic. In the control treatment, dried perennial ryegrass (*Lolium perenne*; purchased from Urs Knecht, Brütten, Switzerland) was used as non-tanniferous plant material. The test materials were dried birdsfoot trefoil, plantain and burnet (all purchased from Phyzolaboratoire, 26400 Aouste-sur-Sye, France; collected from natural swards in France, Albania and China respectively). We used these collected materials, because our investigation aimed also at the idea of introducing these herbs into pastures. To minimise selection of single components, all diets were offered as total mixed rations. For this purpose, a mixer

Table 1. Composition of the diet components (means ± s.d.).

Item	Basal-diet component					Test plant			
	Grass silage[A]	Maize silage[A]	Concentrate[B]	Grass hay[A]	Wheat straw[A]	Perennial ryegrass[B]	Birdsfoot trefoil[B]	Plantain[B]	Burnet[B]
Analysed variables (g/kg dry matter)									
Dry matter (g/kg wet weight)	378 ± 25	321 ± 28	918 ± 1	918 ± 1	920 ± 2	940	901	908	848
Organic matter	782 ± 19	895 ± 6	872 ± 3	843 ± 4	851 ± 4	853	823 ± 4	743 ± 1	840
Nitrogen	29.6 ± 1.4	12.6 ± 0.6	63.8 ± 0.5	12.8 ± 0	7.7 ± 0.2	7.5	34.4	21.3 ± 0	9.4
Neutral detergent fibre	373 ± 40	360 ± 44	183 ± 8	412 ± 10	790 ± 25	744 ± 1	347 ± 3	308 ± 15	441 ± 2
Acid detergent fibre	274 ± 17	206 ± 8	104 ± 6	214 ± 3	421 ± 7	482 ± 1	277 ± 4	274 ± 1	246 ± 1
Crude fibre	255 ± 16	220 ± 6	117 ± 7	363 ± 1	439 ± 3	375 ± 0	242 ± 3	252 ± 0	256 ± 2
Total extractable phenols[C,D]	18.2 ± 0.3	15.0 ± 0.2	7.5 ± 1.4	10.1 ± 1.4	7.5 ± 0.3	7.1 ± 1.2	26.0 ± 1.8	45.7 ± 1.2	243.6 ± 24.8
Non-tannin phenols[C,D]	18.2 ± 1.1	15.0 ± 0.3	7.2 ± 0.7	9.8 ± 0.3	7.5 ± 0.2	6.7 ± 1.0	3.6 ± 0.3	26 ± 1.0	40 ± 1.6
Total tannins[C,D,E]	0	0	0.3 ± 1.3	0.3 ± 0.9	0	0.4 ± 1.2	22.4 ± 1.8	20.1 ± 1.2	203.5 ± 24.9
Condensed tannins[C,F]	ND	ND	ND	ND	ND	ND	22.4 ± 0.9	14.6 ± 0.7	25.2 ± 1.2
Calculated variables[G]									
NEL (MJ/kg dry matter)	5.61 ± 0.14	6.54 ± 0.08	7.27 ± 0.00	4.18 ± 0.04	3.21 ± 0.07	3.55 ± 0.05	5.95 ± 0.05	5.25 ± 0.05	5.50 ± 0.00
APDE (g/kg dry matter)	76 ± 1	68 ± 1	221 ± 9	65 ± 1	48 ± 1	51 ± 1	106 ± 1	86	72
APDN (g/kg dry matter)	116 ± 5	49 ± 3	294 ± 19	49 ± 1	29 ± 1	29	138 ± 1	85	36

APDE, absorbable protein at the duodenum, based on rumen-undegradable nitrogen compounds plus microbial protein calculated on the basis of fermentable energy; APDN, absorbable protein at the duodenum based on rumen-degradable nitrogen compounds; NEL, net energy for lactation; ND, not detected.
[A]Mean of 10 samples with three replicates per sample.
[B]Mean of two samples with three replicates per sample.
[C]Samples pooled before analysis to one sample per run.
[D]Tannic acid equivalents.
[E]Difference between total extractable phenols and non-tannin phenols.
[F]Leucocyanidin equivalents.
[G]For calculation, the mean of the dry-matter content of supplements was taken.

wagon (Rovibec 542, Robivec Agrisolutions Inc., Québec, Canada) was used. Feed was offered *ad libitum*. Fresh portions were provided twice daily, after refusals were weighed and removed.

Twenty-four multiparous dairy cows were subjected to the experiment. These were about half of Brown Swiss (11) and Holstein (13) breed due to the limited size of the herd, from which cows of the same stage of lactation were selected. Half of the cows assigned to each treatment were from each breed, except for birdsfoot trefoil where four Holstein and two Brown Swiss cows were employed. Within breed, assignment was based on baseline data ensuring balanced means of milk yield (mean ± s.d.; 26.4 ± 5.7 kg/day;) days in milk (263 ± 89), protein content (3.84 ± 0.29%), and milk urea (25.0 ± 4.9 mg/dL). Since only 12 animals could be assessed at once, cows were divided into two consecutive runs. Per run, three cows per dietary treatment were assessed for 25 days each. The total of six cows per treatment was considered sufficient for the experimental purpose of digestibility and N-excretion studies (Südekum *et al.* 2006; Kälber *et al.* 2012).

The schedule of both runs is shown in Fig. 1. During Days 1–4, cows were kept in a loose housing system and received the basal diet as described in Table 1. Thereafter, cows were kept in a tied stall with individually separated feeding troughs during Days 5–25. Cows were randomly allocated to their stands. From Day 5 to Day 25, the treatment diets, as defined in Table 2, were fed. Days 5–14 were considered as adaptation phase. Subsequently, during Days 15–18 and 22–25, two sampling periods were conducted (Mondays–Thursdays). Over 72 h (Monday 1700 hours to Thursday 1700 hours) in each of the two sampling periods, sampling was performed from every animal. Feeds were renewed twice daily at 0500 hours and 1600 hours, and milking at the stands took place at the same time.

Samples from all diet components were taken 10 times (five times equally distributed over each run). Due to lower variation, concentrate and test-plant meals were pooled to one sample per run. After collection, the roughages employed in the basal diet were dried at 40°C for 48 h. The low drying temperature was chosen to minimise N and tannin losses. Subsequently, all feeds were milled through a 0.5-mm sieve (Retsch SK 100, Retsch GmbH, Haan, Germany). Feed intake was registered with balances mounted below the individual feed-weighing plates at each stand (Mettler-Toledo, 8606 Greifensee, Switzerland). Cows wore RumiWatch® halter sensors (Itin + Hoch GmbH, Liestal, Switzerland) for

Table 2. Composition and nutrient concentrations of the complete diets.

Diet	Control	Birdsfoot trefoil	Plantain	Burnet
Components (g/kg dry matter)				
Perennial ryegrass	80	–	–	–
Birdsfoot trefoil	–	80	–	–
Plantain	–	–	80	–
Burnet	–	–	–	80
Grass silage	377	377	377	377
Maize silage	327	327	327	327
Mineralised concentrate	154	154	154	154
Grass hay	41	41	41	41
Wheat straw	21	21	21	21
Variables analysed or calculated from analysed values (g/kg dry matter)				
Dry matter (g/kg wet weight)	421	420	421	418
Organic matter	842	840	833	841
Nitrogen	26.2	28.5	27.5	26.6
Neutral detergent fibre	380	347	344	355
Acid detergent fibre	244	227	226	224
Crude fibre	241	230	230	231
Total extractable phenols	14.0	15.6	17.2	32.1
Non-tannin phenols	14.0	13.7	15.5	16.6
Total tannins	0.1	1.9	1.7	15.5
Condensed tannins	0.0	1.8	1.2	1.9
Calculated variables[A]				
NEL (MJ/kg dry matter)	5.89	6.09	6.03	6.06
APDE (g/kg dry matter)	92	97	95	94
APDN (g/kg dry matter)	110	119	114	111

APDE, absorbable protein at the duodenum, based on rumen-undegradable nitrogen compounds plus microbial protein either from fermentable energy; APDN, absorbable protein at the duodenum based on rumen-degradable nitrogen compounds; NEL, net energy for lactation; ND, not detected.
[A]Calculated from dietary proportions and determined composition (see Table 1) of the ingredients.

Fig. 1. Time schedule of the experiment. The schedule was repeated in two separate runs.

recording of jaw movements during the two 72-h sampling periods. Data from the sensors were resolved to eating, ruminating, and idling with the RumiWatch converter® V0.7.3.2 from Itin + Hoch GmbH, Liestal, Switzerland, https://www.rumiwatch.com/index.html (Rombach *et al.* 2018). Frequencies of changes between the different activities (eating, ruminating, and idling) were calculated within validation ranges according to Leiber *et al.* (2022). Data obtained between 1200 hours on Monday and 1159 hours on Thursday in the respective sampling periods were taken to calculate means per hour. Chewing data were analysed on aggregated averages across 24 h.

During the 72 h sampling periods, milk, urine and faeces amounts were recorded and samples were collected from each individual cow daily, according to Leiber *et al.* (2004). Evening and morning milk samples were pooled corresponding to milk amounts obtained and conserved with Bronopol®. Complete faeces volumes were individually collected in trays arranged below a grid mounted at the rear end of the stands and weighed every 24 h. Representative samples from the total faeces were collected daily and stored at 4°C. Complete urine volumes were collected in urinals attached to the skin around the vulva with Velcro straps. The urine was directed via tubes into 20 L canisters and weighed every 24 h. A subsample of approximately 10% of the urine stream was continuously directed into smaller canisters, containing 20 mL of 5 M sulfuric acid adjusted to keep the pH always below 3, so as to prevent gaseous N losses (Kälber *et al.* 2012). From each subsample canister, one sample per day was taken and frozen at −20°C. Later, faeces and urine samples each were pooled to one sample per cow and sampling week. An aliquot part of the faeces was dried at 60°C for 48 h and milled to 0.5 mm diameter. The remainder was frozen at −20°C.

Laboratory analyses

The contents of DM, total ash and N of all feed items and faeces were analysed by standard methods (AOAC International 2005). DM and total ash were determined with a thermogravimetric device model TGA 701 (Leco Corporation, St Joseph, MI, USA) and N was analysed on a C–N analyser (TruMac CN, Leco Corporation, St Joseph, Michigan, USA, AOAC Official Method No. 968.06). The contents of neutral (NDF) and acid detergent fibre (ADF) of the concentrate were determined on a Fibretherm analyser (Gerhardt, Königswinter, Germany; Methods 6.5.1 and 6.5.2 respectively, VDLUFA 2012). Fibre fractions were expressed without residual ash, NDF was assayed with heat-stable amylase and without sodium sulfite. The contents of fibre fractions in all other feed items and faeces were determined with near-infrared spectroscopy (NIRFlex N-500, Büchi, Flawil, Switzerland). This device had been calibrated with parallel chemical analysis of 180 forage samples (from different grass–herb swards and silages) and 45 faeces samples (from five different farms).

Total extractable phenols (TEP), non-tannin phenols (NTP) and CT of the feed items were analysed. In the first step, 60 mg of feed material were extracted in 6 mL acetone (70%) during 20 min (2 × 10 min, 5 min break between sonication) and

filtrated (syringe filter). From this extract, 1 mL was further incubated with polyvinylpolypyrrolidone (PVPP, 77627-100G, Sigma-Aldrich, Buchs, Switzeland) for 15 min, and subsequently centrifuged at 3000*g* for 10 min at 4°C, so as to precipitate the tannins from total phenolics. The supernatant and the acetone extract were stored at 4°C. After reaction with Folin–Ciocalteu-solution, TEP (from acetone extract) and NTP (from PVPP-extract) were measured at 725 nm on a spectrophotometer (Bio Spectrometer Eppendorf D30). The results were expressed against gallic acid standard. For CT, 250 μL of acetone extract were incubated with 100 μL ammonium iron (III) sulfate and 1500 μL butanol–HCl at 100°C for 1 h. After reaction, the samples were measured at 550 nm on a spectrophotometer (Bio Spectrometer Eppendorf D30) and calculated as leucocyanidin equivalents. TT were calculated as TEP minus NTP. The difference between TT and CT is assumed to contain mainly hydrolysable tannins (HT; Jayanegara *et al.* 2011). However, due to different standards in the measurements, it was not possible to quantify HT.

Particle-size distribution in the faeces was quantified by a sieve washing method (Leiber *et al.* 2015). Exactly 100 g of sample was put on top of four stapled sieves with mesh sizes of 4, 2, 1 and 0.3 mm. Each sieve was rinsed for 10 s with water. Subsequently, the residues were dried for 12 h at 105°C and weighed.

Urine N was analysed in the acidified samples with the Dumas method (Trumac CN, Leco Corporation, St Joseph, MI, USA). Milk was analysed at Suisselab (Zollikofen, Switzerland) for fat, true protein, lactose and urea by using Fourier-transform infrared spectroscopy (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark).

Calculations and statistical analyses

Contents of net energy for lactation (NEL) and of absorbable protein at the duodenum (APD), on the basis of rumen-undegradable protein plus microbial protein either from fermentable energy (APDE) or from rumen-degradable protein (APDN), were estimated applying the equations of the Swiss feed-evaluation system (Agroscope 2022) on the basis of the measured contents of DM, total ash, N and ADF. According to Agroscope (2022), energy-corrected milk (ECM) was calculated as follows:

$$\mathrm{ECM} = \text{Milk yield [kg]} \times (0.38 \times \text{Fat [\%]} + 0.24 \times \text{Protein [\%]} + 0.17 \times \text{Lactose [\%]})/3.14$$

Data were analysed with SPSS® ver. 24 (IBM Research Europe, Zurich, Switzerland; https://www.ibm.com/de-de/products/spss-statistics), applying a general linear model, with treatment, run and their interaction as fixed effects, and animal as the experimental unit. As sampling week within run was not significant in a first model run, the repeated measurement effect was omitted from the model, and the data were averaged across weeks. Milk-related data obtained 14 days before the experiment were considered as baseline and statistically used as a covariate. Multiple comparisons among treatment means were performed with Tukey's procedure, considering $P < 0.05$ as significant and $P < 0.10$ as tendency. All milk-related variables are least-square means, corrected for the baseline as explained above. All other variables are shown as arithmetic treatment means with standard errors of the mean.

Results

Feed characteristics, intake and eating behaviour

The dried ryegrass used for the control treatment was of unexpectedly poor quality (low in N, high in fibre, confirmed by repeated analyses; Table 1). By chance, the ryegrass thus represented a good control to the burnet in terms of N concentration, whereas plantain and birdsfoot trefoil were richer in N. However, the ryegrass was inferior in NEL content to all other test forages. The differences in N concentration among the complete diets were far smaller (Table 2). There was a decreasing gradient in TEP and TT contents from burnet to plantain and birdsfoot trefoil (Table 1). Overall, the burnet diet contained most TEP and TT. The variation among treatments regarding CT was less pronounced. The high TT content of burnet is assumed to indicate mainly high HT concentrations, although HT was not directly quantified.

Intakes of DM and organic matter (OM) were similar in all groups (Table 3). Differences in nutrient intake therefore resulted from compositional differences. Accordingly, there were tendencies for higher N and APDN intakes with birdsfoot trefoil than in the control ($P < 0.10$). The APDN: APDE ratio was 1.19, 1.23, 1.20 and 1.18 for control, birdsfoot trefoil, plantain and burnet respectively. The TEP intake was twice as high and that of TT 10 times as high with burnet than with birdsfoot trefoil and plantain ($P < 0.05$). The burnet group also consumed most TT and NTP (the latter not being significantly different from plantain). Intake of TEP with plantain was different ($P < 0.05$) from control, whereas TT intake was significantly ($P < 0.05$) higher with plantain and birdsfoot trefoil than in the control.

Eating and rumination times did not differ among groups (Table 3).

Amounts and composition of faeces and urine, and digestibility

No differences were found for faeces DM amounts, whereas urine amounts were lower with burnet than in all other groups (Table 4, $P < 0.05$). There was no treatment effect on faecal DM content, while faecal N concentration was higher ($P < 0.05$) in the burnet group than in all other groups. Further, birdsfoot trefoil resulted in a higher ($P < 0.05$) faecal OM content than did plantain. Faeces of the burnet-fed cows had the lowest NDF (compared with control and the plantain

Table 3. Intake, eating and ruminating time ($n = 6$ per treatment; calculated from 2 × 72 h recording time per cow).

Item	Treatment				s.e.m.	*P*-value
	Control	Birdsfoot trefoil	Plantain	Burnet		
Daily intake per cow						
Dry matter (kg)	18.9	19.9	19.8	19.8	0.36	0.765
Organic matter (kg)	16.0	16.7	16.5	16.7	0.30	0.815
Nitrogen (kg)	0.499(a)	0.566(b)	0.545(ab)	0.526(ab)	0.0097	0.100
Neutral detergent fibre (kg)	7.20	6.90	6.83	7.02	0.133	0.782
Acid detergent fibre (kg)	4.61	4.50	4.49	4.43	0.086	0.904
NEL (MJ)	112	121	120	120	2.2	0.399
APDE (kg)	1.75	1.92	1.88	1.86	0.034	0.299
APDN (kg)	2.08(a)	2.36(b)	2.27(ab)	2.19(ab)	0.040	0.109
Total extractable phenols (g)	266a	310ab	341b	635c	6.5	<0.001
Non-tannin phenols (g)	265a	273ab	308bc	328c	5.2	<0.001
Total tannins (g)	2a	37b	33b	308c	2.1	<0.001
Condensed tannins (g)	0a	36c	23b	38c	0.4	<0.001
Eating time (min/day)	417	421	378	412	8.7	0.292
Rumination time (min/day)	495	498	501	501	6.3	0.981

APDE, absorbable protein at the duodenum, based on rumen-undegradable nitrogen compounds plus microbial protein either from fermentable energy; APDN, absorbable protein at the duodenum, based on rumen-degradable nitrogen compounds; NEL, net energy for lactation.
Means within a row with different letters (a–c) differ significantly ($P < 0.05$), according to the Tukey test.
Means within a row followed by different letters in parentheses (a and b) tend to differ ($P < 0.10$), according to the Tukey test.
P-values provided in the last column provide the overall significance of the ANOVA.

group, $P < 0.05$) and, concomitantly, the highest ADF content (compared with the birdsfoot trefoil group, $P < 0.05$). Urine N concentration was not affected by the treatment. Apparent N digestibility was higher ($P < 0.05$) and apparent ADF digestibility tended to be higher ($P < 0.10$; general treatment effect $P < 0.05$) with plantain than with burnet, but treatments did not differ in apparent OM and NDF digestibility. The sum of faecal particles >0.3 mm was lower with plantain and burnet than in the control ($P < 0.05$). This was most pronounced in the fractions of particles >0.3 and ≤1 mm in length ($P < 0.05$).

Milk yield and composition

Yields of milk, ECM, fat, protein, and lactose did not differ among groups (Table 5). Milk protein content was highest in the control and with birdsfoot trefoil, lowest with plantain and intermediate with burnet ($P < 0.05$). No effects on milk fat content were found. Lactose concentration was higher with plantain than with birdsfoot trefoil ($P < 0.05$). Milk urea concentration was up to 30% lower with burnet than in the other treatments ($P < 0.05$).

Nitrogen losses with faeces and urine

Nitrogen excretion with faeces was higher with burnet than with plantain, but did not differ from control or birdsfoot trefoil ($P < 0.05$). The same effects ($P < 0.05$) were found when faeces N was related to total N losses with faeces and urine. The urinary N excretion was significantly lower with burnet, by about 30% on average, than in all other groups (Table 6, $P < 0.05$). Even relative to N intake, the urinary N losses declined by 85 g/kg, on average, with burnet, compared with the other treatments ($P < 0.05$). This affected the urine-N proportion of total faeces- and urine-N excretion accordingly ($P < 0.05$).

Discussion

Experimental design and feeds

The goal of the present investigation was to extend the list of alternatives for farm practice to abate urine N excretion in dairy cows in low-concentrate feeding systems during periods of dietary N excess. Therefore, we studied cows fed a common forage-based diet with N excess (26–28 g N/kg DM), as indicated by a certain surplus of APDN over APDE. According to calculation equations behind the APD system (Agroscope 2022), this implies that the available protein exceeds the necessary energy levels at the rumen needed to use the degraded feed protein in the rumen completely for microbial synthesis. From this, excessive ruminal ammonia can be deduced. The aim of the current study was to counteract this excess by replacing ryegrass (control) with tanniferous plant meals of either birdsfoot trefoil or plantain or burnet,

Table 4. Characteristics of faecal and urinary excretion as well as apparent total tract digestibility ($n = 6$ per treatment; calculated from 2 × 72 h individual total collection per cow).

Item	Treatment				s.e.m.	*P*-value
	Control	B. trefoil	Plantain	Burnet		
Daily amounts (kg/cow)						
Faeces (DM)	4.95	5.28	4.65	5.49	0.148	0.283
Urine	29.1a	32.3a	29.4a	23.1b	0.80	0.002
Faeces composition (g/kg)						
Dry matter (DM)	126	127	119	113	2.9	0.291
Organic matter in DM	752ab	757b	737a	743ab	2.0	0.005
Nitrogen in DM	27.5a	28.5a	28.1a	30.4b	0.19	<0.001
Neutral detergent fibre in DM	495a	482ab	497a	473b	0.2	0.004
Acid detergent fibre in DM	428ab	414b	431ab	437a	0.3	0.025
Fractions (g/kg total DM in faeces)						
$\sum \geq 0.3$ mm	360a	348ab	311b	317b	5.2	0.004
>0.3, <1 mm	185a	163ab	151b	145b	3.0	<0.001
>1, <2 mm	109(a)	100(ab)	93(b)	94(ab)	2.3	0.078
>2, <4 mm	47.1	60.7	49.0	48.4	2.26	0.134
>4 mm	20.2	25.0	17.2	29.8	2.02	0.149
Urine N (g/kg urine)	5.75	5.25	5.25	4.94	0.116	0.113
Apparent total tract nutrient digestibility (%)						
Organic matter	76.5	75.8	79.4	75.6	0.65	0.154
Nitrogen	72.3ab	73.1ab	76.1b	68.7a	0.75	0.012
Neutral detergent fibre	65.5	62.7	66.4	63.2	1.01	0.509
Acid detergent fibre	53.4(ab)	51.1(ab)	56.0(a)	46.2(b)	1.31	0.033

Means within a row with different letters (a and b) are significantly ($P < 0.05$) different, according to the Tukey test.
Means within a row with different letters within parentheses (a and b) tend to differ ($P < 0.10$), according to the Tukey test.
P-values provided in the last column provide the overall significance of the ANOVA.

provided at dietary proportions of 80 g/kg DM. The level of the test-plant supplements in the present study was chosen to operate within a practicable range. We assumed that up to 80–100 g/kg of such plants in pastures might be realistic for production. Reasons are the likely lower vigour against the main species growing on pastures. Alternatively, such plants could be cultivated in monoculture, with yields of up to 10 t/ha for plantain (Elgersma *et al.* 2015; Pol *et al.* 2021) and birdsfoot trefoil (Bullard and Crawford 1995; Elgersma *et al.* 2015), and between 1.5 and 5 t/ha for burnet (Peel *et al.* 2009; Elgersma *et al.* 2015). However, also for potential systems that would produce tanniferous fodder plants in monoculture, we assumed dietary inclusions of >100 g/kg to be unrealistic.

The ryegrass used as the control was of unexpectedly low quality; especially N concentration and net-energy content were low. As balancing for similar N concentration would have needed to add extra N in a form of concentrate-based protein sources, meaning differences in the component amounts of basal feed, we decided to tolerate the rather small resulting differences in N concentration of the total diets among the treatments. Although the control diet provided less APDN, differences in APDE were not significant among all diets. Since supply with APDE was clearly lower than that with APDN, the former was the limiting factor for all diets. Generally, the phenol and tannin concentrations found in birdsfoot trefoil, plantain and burnet were high, but of the same scale as in other references (Hamacher *et al.* 2012; Ghelichkhan *et al.* 2018; Kara *et al.* 2018; Kara 2019; Stewart *et al.* 2019; Hamacher *et al.* 2021). We found especially high values for CT in plantain (1.4% of DM), where the values in the literature range from 0 (Loza *et al.* 2021*b*) to 1.0% of DM (Kara 2019).

Effects of the test plants on feed intake, digestibility and performance

The unaffected DM intake suggests that all diets were of similar palatability. This is in line with other studies where intake was not impaired by substantial dietary inclusion of birdsfoot trefoil (Broderick *et al.* 2017; Stewart *et al.* 2019), plantain (Cheng *et al.* 2017; Ineichen *et al.* 2019) or burnet

Table 5. Milk yield and milk composition (LS means; $n = 6$ per treatment; each pooled from two samples in each of two periods per cow).

Item	Treatment				s.e.m.	*P*-value
	Control	Birdsfoot trefoil	Plantain	Burnet		
Daily yield						
Total milk (kg)	20.0	17.6	19.6	20.6	0.91	0.673
Energy-corrected milk (kg)	22.8	20.5	21.8	22.5	1.05	0.864
Fat (g)	993	902	951	959	48.6	0.929
Protein (g)	764	696	692	766	32.0	0.747
Lactose (g)	919	788	930	934	43.5	0.585
Urea (g)	5.46	4.43	5.16	4.09	0.260	0.229
Milk composition						
Fat (%)	4.97	5.06	4.90	4.61	0.077	0.204
Protein (%)	3.90a	3.94a	3.61b	3.71ab	0.046	0.046
Lactose (%)	4.61ab	4.43b	4.71a	4.50ab	0.030	0.010
Urea (mg/dL)	27.8a	24.5b	26.7ab	19.4c	0.64	0.001

Means within a row with different letters (a–c) differ significantly ($P < 0.05$), according to the Tukey test.
P-values provided in the last column provide overall significance of the ANOVA.

Table 6. N losses with faeces and urine ($n = 6$ per treatment; calculated from 2 × 72 h individual total amount sampling/recording).

Item	Treatment				s.e.m.	*P*-value
	Control	B. trefoil	Plantain	Burnet		
g N/day per cow						
Faeces	135ab	151ab	131a	163b	4.1	0.034
Urine	166a	167a	152a	114b	4.2	<0.001
g N/kg of N intake						
Faeces	277ab	269ab	239a	313b	7.5	0.012
Urine	335a	297a	281a	219b	7.2	<0.001
Urine N (% of total N excretion)	54.9a	52.3a	54.4a	41.1b	1.10	<0.001

Means within a row with different letters (a and b) differ significantly ($P < 0.05$), according to the Tukey test.
P-values provided in the last column provide overall significance of the ANOVA.

(Stewart *et al.* 2019), although some refusal had been expected due to the bitter and astringent taste properties of tannins (Kapp-Bitter *et al.* 2020).

We observed rumination and eating behaviour with chewing sensors; however, in contrast to the study of Tseu *et al.* (2020) who fed an *Acacia mearnsii* bark extract as a tannin source, intake and duration of eating and ruminating were not affected by the tanniferous test plants in the present study. Thus, we can exclude effects through changed chewing-behaviour patterns for this experiment.

As a potential proxy of fibre degradation in the digestive tract, which could also be applied on commercial farms, we assessed the abundance of particle fractions in faeces. Cows fed plantain and burnet had a lower total content of particles >0.3 mm in faeces DM than did the control cows, which points towards an improved fibre degradation compared with the control (Kornfelt *et al.* 2013). However, the differences could not be explained by a correspondingly different time spent for rumination. Perhaps, differences in fibre structure from the test plants may have led to variation in ruminal degradation and retention times (Owens *et al.* 1998; Kornfelt *et al.* 2013). The fact that there was no congruence of the faecal particle fractions with the measured digestibility is important information, constraining the proposed use of faecal particle fractionation as proxy for fibre digestibility (Leiber *et al.* 2015).

The low apparent ADF digestibility with burnet, which is congruent with comparably high contents of large particles in faeces, may indicate that not only ruminal ammonia formation was reduced with that treatment, but even fermentation in general was affected. Previous *in vitro* studies support that possibility (Jayanegara *et al.* 2011; Kapp-Bitter *et al.* 2021*b*).

Effect of the test plants on N partitioning to urine and faeces, and milk

It needs to be stated that a complete N balance was not calculated, because the total of milk and excreta N did not completely resemble the intake. Since an error could not be identified, N balance could not be established. However, the urine and faeces N-excretion data as well as milk composition are informative in themselves, therefore being displayed in this report.

With increasing dietary contents of tannins, N excretion was expected to shift from urine to faeces, which then would be associated with a higher faecal N concentration and a lower apparent N digestibility (Dschaak *et al.* 2011; Kälber *et al.* 2012; Ghelichkhan *et al.* 2018; Mueller-Harvey *et al.* 2019). A shift such as this was indeed observed, but, similar to the study of Stewart *et al.* (2019), only with burnet. Further, the burnet treatment led to reduced milk urea concentration at unchanged milk protein content. This is an indication that high concentrations of TT, presumably containing high proportions of HT, indeed exhibit a protein-protecting effect in the rumen. Apart from that, it seems that the tannin amounts provided by the supplementation of birdsfoot trefoil and plantain were too small, in the present study, to cause a significant rerouting of diet N from urine to faeces. At 107 g/day CT intake through dietary birdsfoot trefoil, which is four times higher than in the present study, Grosse Brinkhaus *et al.* (2016) found no reduction of ruminal ammonia concentration and urinary N losses in dairy cows. With a plantain-containing diet, which provided about

100 g dietary phenols extra per cow and day, compared with other diets, Ineichen *et al*. (2019) found no effect on milk urea and urinary N. Minneé *et al*. (2017) found clear effects of dietary plantain, when added at 40% DM, which is five times higher than the proportion chosen in the current study. Cheng *et al*. (2017) achieved reduced urinary N excretion with a pasture containing 86% DM of plantain. These results show clearly that the desired effects are achieved only at dietary concentrations far above what was used in the present experiment. In this light, the effect of burnet seems to be mainly related to the distinctly higher overall concentration, and whether it played a role that tannins were hydrolysable, cannot be deduced from our data. Furthermore, it has to be acknowledged that phenolic concentrations in herbage largely vary with the phenological stage (Kälber *et al*. 2014; Stewart *et al*. 2019) and that tannins may differ in their effectiveness due to structural differences among cultivars (Mueller-Harvey *et al*. 2019).

However, the reduced milk urea concentration in the burnet treatment gives a rather strong indication that tannins from this herb indeed protected part of the feed protein from ruminal degradation and thus reduced ammonia formation (Pacheco and Waghorn 2008). Milk urea concentration and urinary N excretion were indicators of the effectiveness of burnet in the present study, not only in principle but also concerning the level of effect, as the average declines in urine N excretion and milk urea secretion were quite similar, being 30% and 26%, respectively, when we fed burnet instead of the control.

Conclusions

The present study demonstrated that burnet is a valuable feed supplement for the reduction of urinary N losses at a dietary proportion of about 8% DM, while plantain and birdsfoot trefoil did not lead to desired effects at this proportion. The main difference among the treatments was the sheer concentration of total tannins, which was 10 times higher in burnet. Whether the structure of tannins (condensed versus hydrolysable) plays a role could not be elucidated with the present study. The rather low dietary proportions had been chosen to be close to practical applicability. In this view, burnet seems to have a clear advantage; however, its production is clearly less advanced than for plantain and birdsfoot trefoil, and the establishment of a targeted introduction of burnet into forage production would still need development. As similar comparative studies including burnet are rare, broader experimental evidence is needed for this particular herb.

It has to be stated that the control was of poor quality. Even the mitigation potential of burnet might have been underestimated and would be expressed more clearly when compared with a higher-quality hay.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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