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Mature herbs as supplements to ruminant diets: effects on *in vitro* ruminal fermentation and ammonia production

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Abstract

Context. High concentrations of crude protein in ruminant diets may lead to excessive production of ruminal ammonia, which may stress the animal's metabolism and impact nitrogen efficiency. This may become a problem in zero-concentrate feeding systems when pasture grass is rich in crude protein. Polyphenols such as tannins may protect part of dietary protein from ruminal degradation and thus inhibit ammonia formation.

Aims. The present study screened mature herbs for their potential to mitigate ruminal ammonia formation in cattle, when provided as a supplement to a forage diet.

Methods. Thirty-five temperate-climate, herbaceous meadow plant species (including three legumes) that appear in biodiverse natural and sown pastures were investigated for their effects on ruminal ammonia production. Aboveground material was harvested during ripening of the seeds and analysed for nutrient and phenol concentrations. Net energy and protein absorbable at the duodenum were calculated. Incubations (24 h) with cattle rumen fluid following the *in vitro* Hohenheim Gas Test protocol were performed to compare the effects of the test plants on ruminal gas and ammonia formation. Test plants replaced one-third of a basal mixture consisting of 57% *Lolium perenne* L. and 43% *Medicago sativa* L. (air-dry-matter basis). Results were compared with those obtained with the basal mixture alone.

Key results. According to regression analysis, ammonia concentration after incubation was negatively related to concentrations of total extractable phenols and total tannins in feed mixtures, whereas the relationship was weakly positive with dietary crude protein. In 23 and 19 of the test diets, respectively, *in vitro* gas production (indicating ruminal organic matter digestibility) and ammonia concentrations in the incubation medium after 24 h were significantly lower than with the basal mixture alone. Incubations containing *Galium verum* L., *Leontodon hispidus* L., *Lotus corniculatus* L., *Onobrychis viciifolia* Scop., *Plantago lanceolata* L., *Sanguisorba minor* Scop. and *Scabiosa columbaria* L. maintained gas production and estimated *in vitro* organic matter digestibility while at the same time lowering ammonia concentrations.

Conclusions. Seven mature herbs of a screening of 35 proved to have potential for positive effects on ruminal protein utilisation without impairing fermentation.

Implications. These herbs are of particular interest as dietary supplements for dairy cows grazing protein-rich pastures.

Keywords: condensed tannins, forbs, hydrolysable tannins, plant secondary compounds, protein efficiency.

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Introduction

When cattle consume diets excessive in rumen-degradable crude protein (CP), the net ammonia production in the rumen may rise because the fermentable carbohydrate and thus energy supply from these diets limit rumen microbial protein synthesis. Excessive ammonia is absorbed from the rumen, leading to a burden for the ruminant's metabolism as well as environmental pollution, with easily soluble nitrogen (N)-containing compounds excreted via their urine (Reynolds and Kristensen 2008; Sinz *et al*. 2019*a*). Excess dietary rumen-degradable CP supply occurs, for example, in low-input ruminant systems during autumn, when grasses with high CP concentrations but limited energy content are abundant (Pacheco and Waghorn 2008). An alternative approach to compensate for high-protein pasture and conserved forage

builds on including plants with elevated concentrations of secondary compounds such as tannins in the diet of ruminants. Tannins bind to feed proteins, with bond formation depending on factors such as pH of the medium, type of protein and other plant compounds (Perez-Maldonado *et al*. 1995). Accordingly, tannin–protein complexes are formed under ruminal pH conditions. These complexes hamper rumen microbial CP degradation and thus decelerate ammonia formation. Part of these tannin-protected proteins may be released in the abomasum and digested there and in the small intestine (Piluzza *et al*. 2014). In this way, they can contribute to covering the ruminant's amino acid requirements. Alternatively, the complexes remain, or are re-established under small intestinal pH conditions, and the protein is excreted with the faeces (Dschaak *et al*. 2011). Based on these mechanisms, cattle grazing on herb-rich pastures or receiving herbaceous supplements may increase their protein utilisation (Totty *et al*. 2013). Together with further implications for animal health and welfare (Leiber *et al*. 2020), this should encourage farmers trying to establish herbs as supplements to cows grazing young grass in spring or autumn. However, it is important to know first the extent to which suitable herb species would contribute to improving protein digestion in ruminants.

Several screenings of herbal species for their nutrient and phenolic contents have been made. Examples are the studies by Macheboeuf *et al*. (2014), who analysed a large collection of wild plants grown in the French Massif Central Area, Jayanegara *et al*. (2011), who investigated several alpine forages, and Terranova *et al*. (2018), who screened woody plants grown in temperate climatic conditions. Still, data on temperate-climate meadow herbs and herbaceous legumes are scarce. These species typically occur on swards established to generate a high biodiversity and are harvested late, often accomplished during seed ripening. In order to assess the suitability of mature, herb-rich swards as supplements that influence protein digestion, plants need to be investigated specifically at this mature stage, because concentrations of phenols can either increase or decrease during plant development, depending on species and other factors (Kälber *et al*. 2014; Stewart *et al*. 2019).

The present study was conducted to assess a large number of temperate climate herbs and legumes harvested during the seed-ripening stage. They were compared with respect to their nutrient and phenolic concentrations and their efficiency to reduce *in vitro* the ammonia concentration in rumen fluid while maintaining organic matter digestibility (OMD) when added to a standard diet. The overall aim was to identify plant species particularly promising at late harvest. Herbs with low rumen ammonia production and high ruminal fermentation rates could then be used as supplements for cattle in periods of dietary CP excess.

Materials and methods

Swiss ecotypes of 35 plant species from 13 plant families (as listed in Table 1) were used. They had been grown as pure cultures in Lenggenwil, Switzerland (47°28′31.34″N, 9°11′11.67″E; 580 m a.m.s.l.). Harvest took place from July to August 2016, when the seeds of the plants ripened. For each plant species, 5–10 kg wet weight was sampled by cutting 1 cm aboveground, divided into three batches, dried at 48°C for 24 h, and milled through a 0.5-mm sieve (SK100; Retsch, Haan, Germany). Pure stands of *Lolium perenne* L. and *Medicago sativa* L. were also harvested during the ripening period (first cut from seed-production fields) and treated the same way to form the basal mixture.

Sample analyses for nutrient and phenolic concentrations

All analyses were repeated three times per individual plant species by using the different batches of the harvested material (i.e. $n = 3$ per test plant species). In the dried material, concentrations of dry matter (DM), total ash, CP, crude fibre, and neutral (NDF) and acid (ADF) detergent fibre were determined with near-infrared reflectance (NIR) spectroscopy (NIRFlex N-500; Büchi, Flawil, Switzerland). This equipment had been previously calibrated with 180 samples from different mixed grass–herb swards analysed in parallel by wet chemistry. For each variable and plant species, the arithmetic mean of three NIR determinations was used. The OM was calculated as DM minus total ash. Using compositional data, net energy for lactation (NEL) and absorbable protein at the duodenum were estimated using regressions of Agroscope (2020). The absorbable protein at the duodenum is limited to either the sum of microbial protein from energy supply and rumen-undegradable protein (APDE) or the sum of microbial protein from rumen-degradable CP and rumen-undegradable protein (APDN) (Colin-Schoellen *et al*. 2000; Agroscope 2020).

For analysing total extractable phenols (TEP), 60 mg ground plant material was extracted with 6 mL 70% aqueous acetone (v/v). The supernatant was filtered (Cameo syringe filter, non-sterile, pore size 1.2 µm; GVS, Bologna, Italy). From this extract, 0.02 mL was taken to determine the amount of TEP and 1.0 mL the amount non-tannin phenols (NTP), using the Folin-Ciocâlteu method described in detail by Makkar (2003). Absorption was measured at 725 nm with a spectrophotometer (BioSpectrometer D30; Eppendorf, Hamburg, Germany). Total tannins (TT) were calculated as the difference between TEP and NTP, and given as equivalents of tannic acid. Condensed tannins (CT) were analysed following the protocol for the butanol–HCl assay (Makkar 2003). Hydrolysable tannins (HT) are equivalent to the difference between TT and CT. Absorption was measured at 550 nm and given as leucocyanidin equivalents.

In vitro *study of ammonia production and organic matter digestibility*

As a control, the basal diet, consisting of *L. perenne* and *M. sativa*, was incubated at a ratio of 0.57 : 0.43 (114 mg and 86 mg DM) according to the method of Menke and Steingass (1988). Each of the 35 test plants was incubated together with the basal diet at a ratio of 0.30 : 0.70 (test plant : basal diet). For each of the 36 combinations, 200 mg was incubated in a Hohenheim Gas Test apparatus on two different days (two runs) in three syringes each, giving six observations per plant.

Table 1. Chemical composition of the collected plants (n = 3)

Values in bold differ ($P < 0.05$) from the basal mixture of *Lolium perenne* and *Medicago sativa* in a ratio of 0.57 : 0.43 on a dry matter (DM) basis. Within columns, means followed by the same letter are not significantly different at $P = 0.05$. s.e.m., standard error of the mean. ADF, acid detergent fibre; APDE, absorbable protein at the duodenum calculated as the sum of microbial protein from energy supply and rumen undegradable protein; APDN, absorbable protein at the duodenum calculated as the sum of microbial protein from rumen degradable protein and rumen undegradable protein; CF, crude fibre; CP, crude protein; NDF, neutral detergent fibre; NEL, net energy lactation; OM, organic matter

No.	Plant species	Plant family	OM	CP	CF	NDF	ADF	NEL	APDE	APDN
			(g/kg DM)					(MJ/kg DM)	(g/kg DM)	
01	Basal mixture		898m–o	85q	353c	612b	443f–i	3.44hi	57.2l–o	57.1no
02	*Achillea millefolium* L.	Asteraceae	**932e–g**	107l–n	**346d**	**491g–k**	**469de**	**3.00l–o**	58.7l–n	**67.7l**
03	*Anthyllis vulneraria* subsp. *carpatica* (Pant.) Nyman	Fabaceae	**952b**	160d	**268qr**	**438m–o**	**367mn**	**4.13ef**	**78.0cd**	**102.3d**
04	*Aquilegia vulgaris* L.	Ranunculaceae	**910j–m**	196b	**251t**	**295r**	**253u**	**4.83b**	**90.0a**	**125.7b**
05	*Campanula rapunculoides* L.	Campanulaceae	**836q**	204a	**175y**	**115t**	**94x**	**5.10a**	**92.0a**	**131.0a**
06	*Campanula rapunculus* L.	Campanulaceae	**940b–e**	86q	**314hi**	**488h–k**	440g–i	3.27i-k	57.0m–o	54.0op
07	*Carum carvi* L.	Apiaceae	**948bc**	**160d**	**182x**	**337q**	**308r–t**	**4.57c**	**80.3bc**	**103.0d**
08	*Clinopodium vulgare* L.	Lamiaceae	909k–m	**101mn**	**334e**	**503f–j**	449e–i	**2.80o**	55.3o	**63.7m**
09	*Crepis biennis* L.	Asteraceae	895no	**120ij**	**329ef**	**508f–h**	**461d–g**	**3.10k–m**	**61.3k**	**76.7j**
10	*Daucus carota* L.	Apiaceae	**928e–h**	**129gh**	**263rs**	**423n–p**	**390kl**	**4.10e–g**	**73.0f–h**	**82.3hi**
11	*Galium mollugo* L.	Rubiaceae	**919h–k**	**73s**	**301kl**	**505f–i**	**491c**	**3.13k–m**	**52.3p**	**45.0q**
12	*Galium verum* L.	Rubiaceae	**917h–k**	**119j**	**318gh**	**448l–o**	447f–i	3.57h	**66.3ij**	**75.3j**
13	*Hieracium pilosella* L.	Asteraceae	**855p**	**112kl**	**307j**	616b	329o–r	**2.97m–o**	58.3l–n	**71.3k**
14	*Knautia arvensis* (L.) Coult.	Caprifoliaceae	**916h–k**	**108lm**	**334e**	**554de**	**84x**	**3.03l–n**	59.0k–m	**68.3kl**
15	*Leontodon autumnalis* (L.) Moench	Asteraceae	899l–n	**164d**	**298lm**	**390p**	**346no**	**4.00fg**	**77.7de**	**105.0d**
16	*Leontodon hispidus* L.	Asteraceae	**920g–k**	**100no**	**307jk**	**524e–g**	449e–i	**3.20j-l**	59.7kl	**62.7m**
17	*Leucanthemum vulgare* Lam.	Asteraceae	**945b–d**	**72s**	**422a**	**480h–l**	428ij	**1.47q**	**34.7r**	**45.0q**
18	*Lotus corniculatus* L.	Fabaceae	**919h–k**	**136fg**	**279p**	**347q**	**322p-s**	**4.10e–g**	**74.0fg**	**86.3fg**
19	*Onobrychis viciifolia* Scop.	Fabaceae	887no	**130gh**	**306jk**	**444m–o**	**409jk**	3.60h	**68.7i**	**82.7hi**
20	*Origanum vulgare* L.	Lamiaceae	**922f–j**	**133f-h**	**318g–h**	**336q**	**290t**	3.57h	**68.7i**	**84.3gh**
21	*Picris hieracioides* L.	Asteraceae	**913i–k**	85q	**361b**	**744a**	**555a**	**2.23p**	**46.0q**	**53.3n–p**
22	*Plantago atrata* Hoppe	Plantaginaceae	**923f–i**	**127hi**	**229v**	**278rs**	**255u**	**4.37cd**	**75.3ef**	**80.7i**
23	*Plantago lanceolata* L.	Plantaginaceae	**920g–k**	**101mn**	**226vw**	**260s**	**190w**	**4.53c**	**72.3gh**	**63.0m**
24	*Primula elatior* Hill	Primulaceae	**933d–f**	**137f**	**292no**	**423op**	**370m**	**3.90g**	**72.7gh**	**87.0fg**
25	*Prunella vulgaris* L.	Lamiaceae	898m–o	**121ij**	**312ij**	**440m–o**	**381lm**	3.40h–j	**65.0j**	**76.7j**
26	*Rumex acetosa* L.	Polygonaceae	**938c–e**	84qr	**297l–n**	**443m–o**	440hi	3.40h–j	57.3l–o	**52.3p**
27	*Salvia pratensis* L.	Lamiaceae	896no	**180c**	**260s**	**336q**	**335o–q**	**4.37cd**	**82.7b**	**115.7c**
28	*Sanguisorba minor* Scop.	Rosaceae	**922f–j**	**128h**	**293m–o**	**469j–m**	**308r–t**	3.47hi	**66.7ij**	**81.3hi**
29	*Scabiosa columbaria* L.	Caprifoliaceae	**929e–h**	**94op**	**343d**	590bc	**500bc**	**3.00l–o**	56.3no	58.7n
30	*Silene dioica* (L.) Clairv.	Caryophyllaceae	**936c–e**	**139f**	**221w**	**457k–n**	**342op**	**4.30de**	**75.3ef**	**88.7f**
31	*Silene flos-cuculi* (L.) Greuter & Burdet	Caryophyllaceae	886o	**128h**	**272q**	**470i–m**	**320q–s**	**3.97fg**	**71.7gh**	**81.3hi**
32	*Silene nutans* L.	Caryophyllaceae	**832q**	**148e**	**238u**	**330q**	**231v**	**4.30de**	**77.3de**	**94.3e**
33	*Silene vulgaris* (Moench) Garcke	Caryophyllaceae	**913i–k**	**118jk**	**341d**	**534ef**	463d–f	**2.87no**	58.7l–n	**75.0j**
34	*Stachys officinalis* (L.) Trevis.	Lamiaceae	**973a**	89pq	**346d**	585bd	**480cd**	**3.20j-l**	57.7l–o	55.3n–p
35	*Thymus pulegioides* L.	Lamiaceae	**911i–l**	**109l**	**328f**	**434no**	**383lm**	**3.10k–m**	59.7kl	69.3kl
36	*Tragopogon pratensis* subsp. *orientalis* (L.) Čelak	Asteraceae	**968a**	**77rs**	**288o**	**570cd**	**512b**	**3.00l–o**	**52.0p**	48.3q
	s.e.m.		3	3	5	12	11	0.07	1.2	2.1
	P-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Along with the test mixtures, in each run, six blank syringes and three syringes each filled with either hay or concentrate as Hohenheim Gas Test standards were incubated for later adjustment of the different runs (Menke and Steingass 1988).

Rumen fluid was taken before the morning feeding from four cannulated lactating Jersey cows (mixture from two cows per run). These cows had been fed with a total mixed ration containing grass and maize silage, grass hay, barley straw and a concentrate mixture at a forage : concentrate ratio of 0.68 : 0.32 (on DM basis). Housing of, and rumen fluid collection from, the rumen-cannulated cows were approved by the Regierungspräsidium Stuttgart, Germany (licence no. A401/14 TE).

After filtration of the rumen fluid, a buffer solution was added to stabilise the pH. A 30-mL sample of this mixture was placed in the pre-warmed syringes and the air was removed. Subsequently, the syringes were placed in a water bath at 39°C for 24 h. The syringes were gently shaken hourly during the first 6 h of incubation. When the gas production exceeded 70 mL after 8 h, syringes were reset to 35 mL by releasing part of the gas produced. After 24 h, the total amount of gas produced was recorded and the incubation fluid was placed in sediment tubes for ammonia analysis by a pH meter (Model 713; Metrohm, Herisau, Switzerland) equipped with an ion-selective electrode. The ammonia value was corrected for the concentration in the blanks. To calculate the net gas production, the mean gas volume produced from the syringes containing only buffered rumen fluid (i.e. blanks) was subtracted from the volumes measured with the respective test diets. This result was adjusted by using a correction factor

calculated from the observed and expected net gas productions from the standard hay and standard concentrate. *In vitro* OMD (IVOMD) was calculated based on the compositional data and the net gas production (Menke and Steingass 1988) as: IVOMD (mg/g OM) = 148.8 + 8.893 × gas production (mL/200 mg DM) + 0.448 × dietary CP (mg/g DM) + 0.651 × dietary total ash (mg/g DM).

Statistical analyses

SPSS Statistics version 24 (IBM, Armonk, NY, USA) was used for statistical analysis. For the *in vitro* data, the 35 diets containing the test plants were treated as fixed effects and the two incubation runs in the Hohenheim Gas Test as random effects (n = 6 per test-plant-based diet). For assessing differences between the basal mixture and the means obtained with the test plants, Tukey's procedure was used, and $P < 0.05$ was considered to be significant. In addition, a principal component analysis (PCA) was performed including all available data from the 35 test diets (total $n = 3 \times 35$ for nutrient and phenol concentrations and IVOMD, and $n = 6 \times 35$ for gas production and ammonia concentration). For this purpose, the compositional data were calculated for the respective diets from composition and proportions of test plants and basal mixture. The first two principal components (PCs) were used to calculate the factor scores for each plant. Finally, based on the data averaged per test-plant-based diet, a regression analysis was performed with gas production and ammonia concentration of the inoculum after 24 h of incubation as dependent variables, and CP, TEP and TT as independent variables. The analyses were first performed with linear, quadratic, exponential and logarithmic terms. The best fitting model is presented, considering $P < 0.05$ as significant. If none of the models was significant, the linear regression is indicated.

Results

Chemical composition of the test plants

Concentrations of OM covered a range from 832 g/kg DM (*Silene nutans*) to 973 g/kg DM (*Stachys officinalis*) (Table 1). The median was 919 g/kg DM. The CP concentrations ranged from 72 g/kg DM for *Leucanthemum vulgare* to 204 g/kg DM for *Campanula rapunculoides* with its median was 120 g/kg DM. The concentration of crude fibre was highest in *L. vulgare* (422 g/kg DM) and lowest in *C. rapunculoides* (175 g/kg DM). The median was 304 g/kg DM. NDF content was very low in *C. rapunculoides* at 115 g/kg DM. The highest NDF content of 744 g/kg DM was found in *Picris hieracioides*, and the median was 452 g/kg DM. The median of ADF was 382 g/kg DM, with the lowest ADF content of 84 g/kg DM found in *Knautia arvensis*, and the highest (555 g/kg DM) in *P. hieracioides*. The highest calculated contents of NEL (5.10 MJ/kg DM), APDE (92 g/kg DM) and APDN (131 g/kg DM) were found in *C. rapunculoides*, and the lowest NEL (1.47 MJ/kg DM) and APDE values (35 g/kg DM) were calculated for *L. vulgare*. APDN was lowest in *L. vulgare* and *Galium mollugo* (both 45 g/kg DM). The medians for these variables were 3.45 MJ NEL/kg, 66 g APDE/kg DM and 76 g APDN/kg DM. A large number of individual plants differed in composition ($P < 0.05$) from the basal mixture (values in bold in Table 1).

The highest concentrations of TEP and NTP were detected in *Origanum vulgare* at 192 and 94 g/kg DM, respectively, and the lowest in *Tragopogon pratensis* subsp. *orientalis* at 6 and 6 g/kg DM, respectively, with medians at 48 and 33 g/kg DM (Table 2). The concentration of TT was highest in *Sanguisorba minor* at 135 g/kg DM and lowest in *T. pratensis* subsp. *orientalis*, for which no tannins were detected. The median for TT was 15 g/kg DM. Eight plants contained CT: *Campanula rapunculus*, *L. corniculatus*, *O. viciifolia*, *Primula elatior*, *S. minor*, *Silene vulgaris*, *S. officinalis* and *Thymus pulegioides*. Among these plants, *O. viciifolia* had the highest CT concentration at 38 g/kg DM. The median CT concentrations of these eight plants was 4 g/kg DM. The differences between TT and CT show that most test plants were mainly characterised by HT (data for HT not presented because not directly analysed). Except for *Anthyllis vulneraria* subsp. *carpatica*, *Leucanthemum vulgare* and *Silene flos-cuculi*, all test plants differed ($P < 0.05$) in at least one of the concentrations of TEP, NTP and TT from those of the basal mixture (values in bold in Table 2).

In vitro organic matter digestibility, gas production and ammonia concentration after incubation

The pH of the incubation fluid after 24 h of incubation was 6.8–6.9 (data not shown). The highest net gas production numerically during 24 h was realised with the basal mixture, at 57 mL/200 mg DM (Table 2). When one-third of the basal mixture was replaced by the test plants, the diet containing *S. pratensis* resulted in the highest gas production per 200 mg DM (53 mL/24 h), and the diet with *O. vulgare* the lowest (24 mL/24 h). The median was 43 mL/200 mg DM during 24 h. Compared with the basal mixture, gas production was decreased ($P < 0.05$) with 23 of the 35 test diets. The median of IVOMD was 541 g/kg OM, the lowest value was 336 g/kg OM (with *O. vulgare*) and the highest value 684 g/kg OM with *P. lanceolata*. Compared with the basal mixture, IVOMD was decreased ($P < 0.05$) with 18 of the 35 test diets. The median ruminal ammonia concentration of the incubation fluid after 24 h of incubation was 16 mmol/L. The lowest value was 11 mmol/L (with *P. hieracioides*) and the highest was 22 mmol/L (with *S. flos-cuculi*). Nineteen test diets led to lower ($P < 0.05$) ammonia concentrations after 24 h of incubation than the basal mixture alone.

Results of principal component and regression analyses

A close relationship among variables of plant composition and *in vitro* ruminal fermentation variables was shown by PCA, with the first PC explaining 38.8% and the second 21.3% of the variation, adding up to 60.1% (Fig. 1). Antagonistic relationships were observed for the concentrations of TEP, NTP or TT with net gas production or ammonia concentration, and for fibre contents (NDF and ADF) on the one side with CP, APDN, APDE and NEL on the other. Separation of ammonium formation from gas production and IVOMD by PCA was not possible.

Table 2. Phenolic contents of the collected plants ($n = 3$) and *in vitro* fermentation characteristics after 24 h of incubation of 200 mg dry matter (DM) mixtures of test plants with a basal mixture (0.3 : 0.7; $n = 6$)

Values in bold differ ($P < 0.05$) from the basal mixture of *Lolium perenne* and *Medicago sativa* in a ratio of 0.57 : 0.43 on DM basis. Within columns, means followed by the same letter are not significantly different at $P = 0.05$. s.e.m., standard error of the mean; n.d., ot detected. CT, condensed tannins; IVOMD, *in vitro* organic matter digestibility; NTP, non-tannin phenols; TEP, total extractable phenols; TT, total tannins

No.	Plant species	TEP	NTP	TT	CT	Total gas	IVOMD	Ammonia
		(g/kg DM)				(mL/200 mg DM)	(g/kg OM)	(mmol/L)
01	Basal mixture	14.5r–t	12.4pq	2.2st	n.d.	57.3a	701a	21.3ab
02	*Achillea millefolium*	**88.1ef**	**36.1h–l**	**52.0d–f**	n.d.	**41.5e–l**	**524c–h**	15.5c–j
03	*Anthyllis vulneraria* subsp. *carpatica*	24.0q–r	18.3n–p	5.7n–t	n.d.	**44.9b–k**	562a–g	12.9h–j
04	*Aquilegia vulgaris*	**42.9l–n**	**40.6f–i**	2.3r–t	n.d.	**44.1b–k**	564a–g	19.6a–d
05	*Campanula rapunculoides*	**46.7k–n**	**37.8g–k**	8.9m–t	n.d.	**41.5f–l**	**531b–h**	19.1a–e
06	*Campanula rapunculus*	**49.7j–l**	**35.2l–m**	**14.5k–n**	1.9e	**42.7d–l**	**535b–h**	16.1k–l
07	*Carum carvi*	**32.8n–p**	**31.9k–m**	0.9o–t	n.d.	50.1a–i	620a–e	17.1a–i
08	*Clinopodium vulgare*	**144.9c**	**80.9b**	**64.1c**	n.d.	**35.5k–m**	**458f–i**	**11.6ij**
09	*Crepis biennis*	**75.5gh**	**43.8eg**	**31.7ij**	n.d.	**42.2d–l**	**534b–h**	**14.5d–j**
10	*Daucus carota*	**48.4k–m**	**22.2n**	**26.2jk**	n.d.	49.2a–i	611a–f	17.7a–h
11	*Galium mollugo*	**43.9k–n**	**32.5k–m**	**11.3l–q**	n.d.	**42.2d–l**	**531b–h**	**13.3f–j**
12	*Galium verum*	**50.0j–l**	**33.0k–m**	**17.1k–m**	n.d.	50.7a–g	649a–d	**14.7d–j**
13	*Hieracium pilosella*	**43.9k–n**	**33.8j–m**	10.1l–s	n.d.	**37.8j–m**	**487e–i**	**14.4d–j**
14	*Knautia arvensis*	**44.4k–n**	**31.8k–m**	**12.6l–r**	n.d.	**32.3l–n**	**422g–i**	**14.5d–j**
15	*Leontodon autumnalis*	**36.1n–p**	21.6n	**14.5l–p**	n.d.	**40.2h–l**	**513c–h**	**15.7c–j**
16	*Leontodon hispidus*	**37.6m–o**	22.2n	**15.4k–o**	n.d.	52.0a–d	661a–c	**12.8h–j**
17	*Leucanthemum vulgare*	21.1qr	13.4op	7.6m–t	n.d.	48.5a–i	570a–g	16.4a–j
18	*Lotus corniculatus*	**65.5hi**	30.7lm	**34.9hi**	34.5b	51.9a–e	615a–f	**15.3c–j**
19	*Onobrychis viciifolia*	**78.0fg**	29.4m	**48.6ef**	37.8a	50.4a–h	622a–e	**14.5d–j**
20	*Origanum vulgare*	**192.2a**	**93.5a**	**98.7b**	n.d.	**24.5n**	**336i**	**12.9h–j**
21	*Picris hieracioides*	**40.4l–n**	**30.7lm**	9.8m–t	n.d.	**40.4g–l**	**512c–h**	**11.2j**
22	*Plantago atrata*	**60.7ij**	**49.3de**	11.4m–t	n.d.	**45.8b–k**	572a–g	17.0a–i
23	*Plantago lanceolata*	**89.9e**	13.1op	**76.9c**	n.d.	53.4a–c	684ab	**12.3h–j**
24	*Primula elatior*	**97.4de**	**42.5f–h**	**54.9ef**	22.1c	51.0a–f	631a–e	17.0a–i
25	*Prunella vulgaris*	**103.3d**	**54.6d**	**48.7ef**	n.d.	**42.4d–l**	**536b–h**	16.9a–i
26	*Rumex acetosa*	**74.6gh**	**39.7g–j**	**34.9gh**	n.d.	**43.1c–k**	**540b–g**	**13.2g–j**
27	*Salvia pratensis*	**108.4d**	**46.4ef**	**62.0de**	n.d.	53.4a–b	661a–d	16.9a–i
28	*Sanguisorba minor*	**170.8b**	**35.4l–m**	**135.3a**	4.1d	47.1a–j	588a–f	**13.2g–j**
29	*Scabiosa columbaria*	**43.5l–n**	**33.2k–m**	10.3n–t	n.d.	48.4a–i	576a–g	**14.8d–j**
30	*Silene dioica*	25.5p–r	**18.9n–o**	6.6n–t	n.d.	**44.8b–k**	557a–g	20.7a–c
31	*Silene flos-cuculi*	17.8q–s	13.6op	4.2q–t	n.d.	**42.1d–l**	**534b–h**	21.7a
32	*Silene nutans*	**26.6o–q**	**21.7n**	4.9p–t	n.d.	**42.5d–l**	542a–g	17.0a–i
33	*Silene vulgaris*	**28.6o–q**	**19.5no**	9.1m–t	3.8d	**32.3l–n**	**423g–i**	**15.8c–j**
34	*Stachys officinalis*	**55.0i–k**	**38.0g–k**	**17.0kl**	1.7e	**39.8i–m**	**501d–h**	**13.8e–j**
35	*Thymus pulegioides*	**135.3c**	**65.8c**	**69.6cd**	2.4e	**41.3f–l**	**523c–h**	18.7a–g
36	*Tragopogon pratensis* subsp. *orientalis*	5.5t	**5.8r**	n.d.	n.d.	**29.8mn**	**380hi**	**15.6c–j**
	s.e.m.	4.2	1.8	3.0	3.1	0.6	9	0.3
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

The plot of the first two PC scores in Fig. 2 describes the association of each plant to the factor scores. In general, those test plants situated in the upper half of the plot were rich in phenols, and those in the lower half had high gas production and ammonia concentrations after 24 h when incubated in combination with the basal mixture. The test-plant species arranged at the right side of the plot had high CP concentration and high nutritive values concerning APDE, APDN and NEL, whereas the ones on the left side were characterised by high fibre concentrations. The test plants allocated to the upper right quadrant of the plot were relatively rich in phenols and had a high nutritional value (i.e. rich in CP, NEL, APDE and APDN). When incubated together with the basal mixture, they had the potential to reduce ammonia concentrations *in vitro* while improving the nutritional value of the mixed diet concerning NEL, APDE and APDN compared with the basal mixture alone. The plants found in this quadrant included *C. rapunculoides*, *O. viciifolia*, *O. vulgare*, *P. atrata*, *P. lanceolata*, *P. elatior*, *P. vulgaris*, *S. pratensis* and *S. minor*. However, no plant reached the centre or even the right upper corner of this quadrant, which would have meant a clear association of low ammonia production and high nutritional quality. On the other hand, in particular *C. rapunculus*, *G. mollugo*, *G. verum*, *H. pilosella*, *L. hispidus*, *L. vulgare*, *P. hieracioides*, *S. columbaria*, *S. vulgaris* and *T. pratensis* subsp. *orientalis* had low concentrations of TEP, similar to those of the basal diet, and resulted in high gas production and ammonia concentrations when incubated together with the basal mixture.

Regression analyses served to identify relationships of CP, TEP and TT concentrations of the test-plant based diets on the one hand with *in vitro* gas production and ammonia concentrations on the other hand. As expected, CP concentration was positively related ($P < 0.05$) to ammonia concentration after 24 h of incubation (Fig. 3*b*), whereas the relationship of CP and total gas production was not significant (Fig. 3*a*). There was a quadratic relationship ($P < 0.05$) between TEP and gas production, with an increase followed by a decrease at higher TEP concentrations of the diet (Fig. 4*a*). Incubating test plants with the basal mixture reduced ammonia concentrations in a linear relation ($P < 0.05$) with increasing contents of TEP (Fig. 4*b*) and TT (Fig. 5*b*). No relationship was found between TT and gas production (Fig. 5*a*).

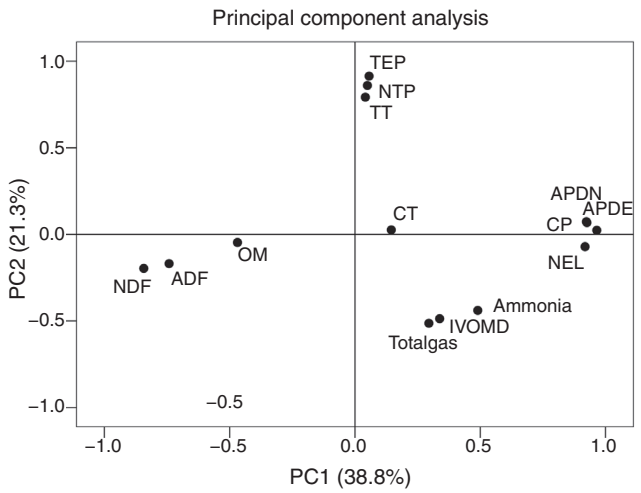

Fig. 1. Plot of the first two principal components (PCs) describing the relationships among variables of plant composition and *in vitro* ruminal fermentation. ADF, Acid detergent fibre; APDE, absorbable protein at the duodenum calculated as the sum of microbial protein from energy supply and rumen undegradable protein; APDN, absorbable protein at the duodenum calculated as the sum of microbial protein from rumen degradable protein and rumen undegradable protein; CP, crude protein; CT, condensed tannins; IVOMD, *in vitro* organic matter digestibility; NEL, net energy for lactation; NDF, neutral detergent fibre; NTP, non-tannin phenols; OM, organic matter; TEP, total extractable phenols; TT, total tannins.

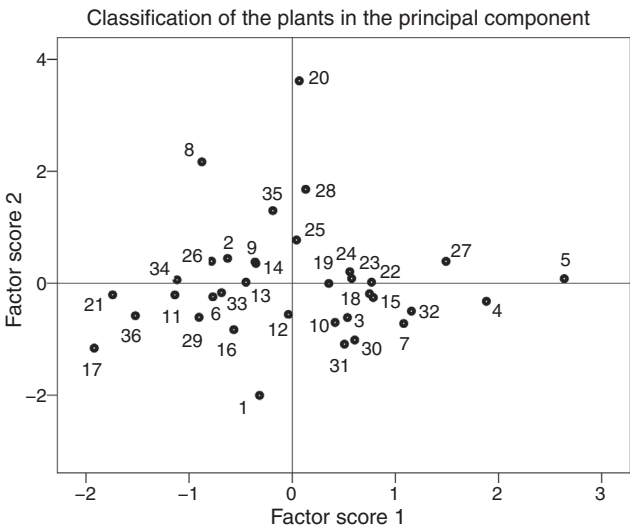

Fig. 2. Plot of the first two factor scores describing the classification of each plant within the principal component loading (Fig. 1). For number codes of plant species, see Table 1.

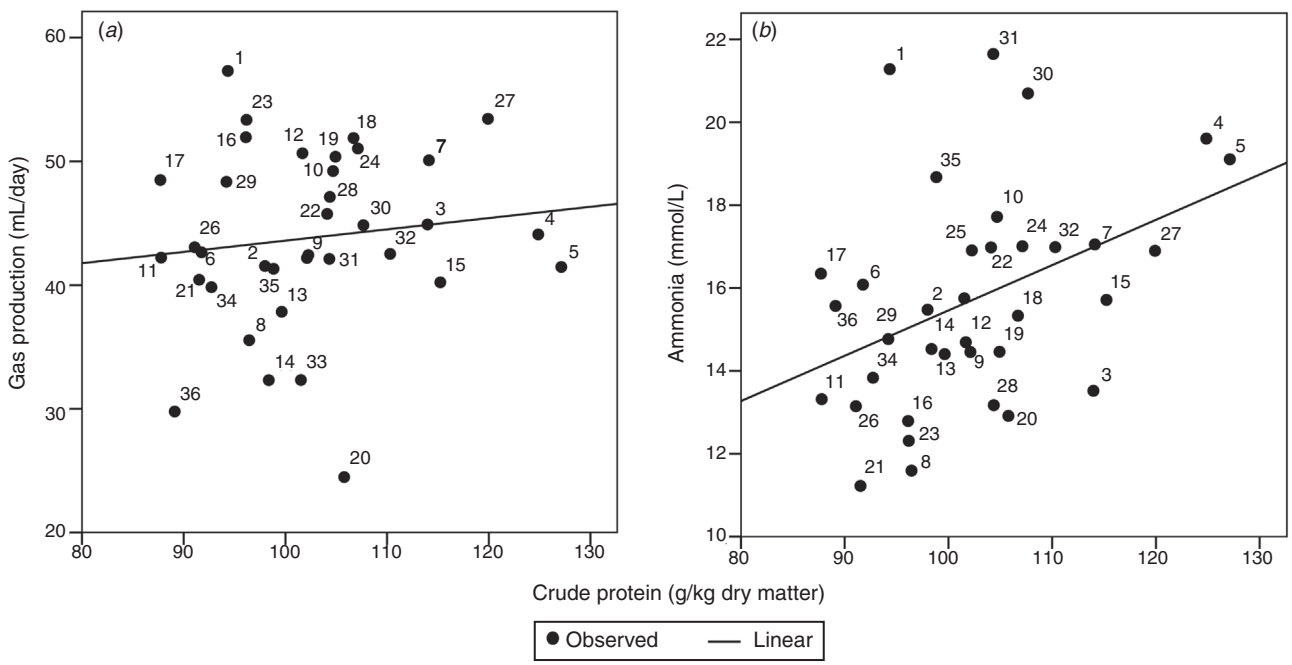

Fig. 3. (*a*) Gas production and (*b*) ammonia concentration in relation to dietary crude protein. Equations: (*a*) $y = 34.5 + 0.091x$ ($R^2 = 0.016$; not significant); (*b*) $y = 4.53 + 0.109x$ ($R^2 = 0.168$; $P < 0.05$). For number codes of plant species, see Table 1.

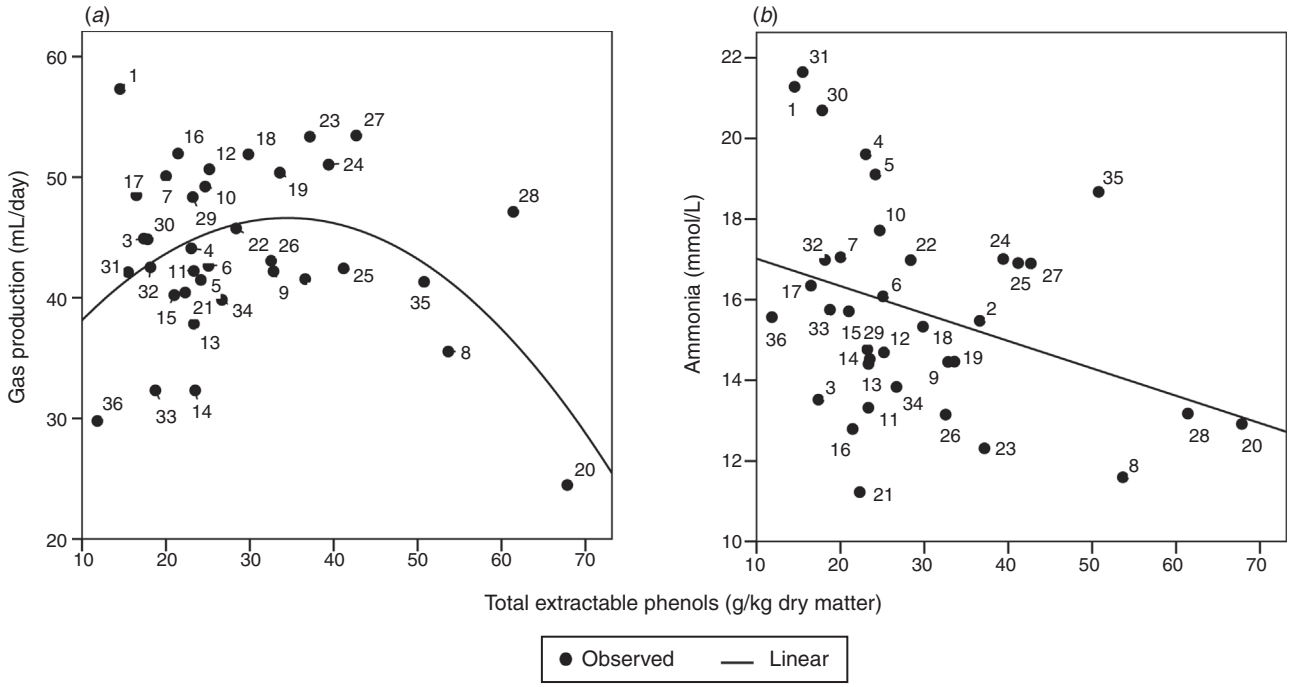

Fig. 4. (*a*) Gas production and (*b*) ammonia concentration in relation to dietary contents of total extractable phenols. Equations: (*a*) $y = 29.8 + 0.976x - 0.0141x^2$ ($R^2 = 0.198$; $P < 0.05$); (*b*) $y = 17.7 - 0.068x$ ($R^2 = 0.116$; $P < 0.05$). For number codes of plant species see Table 1.

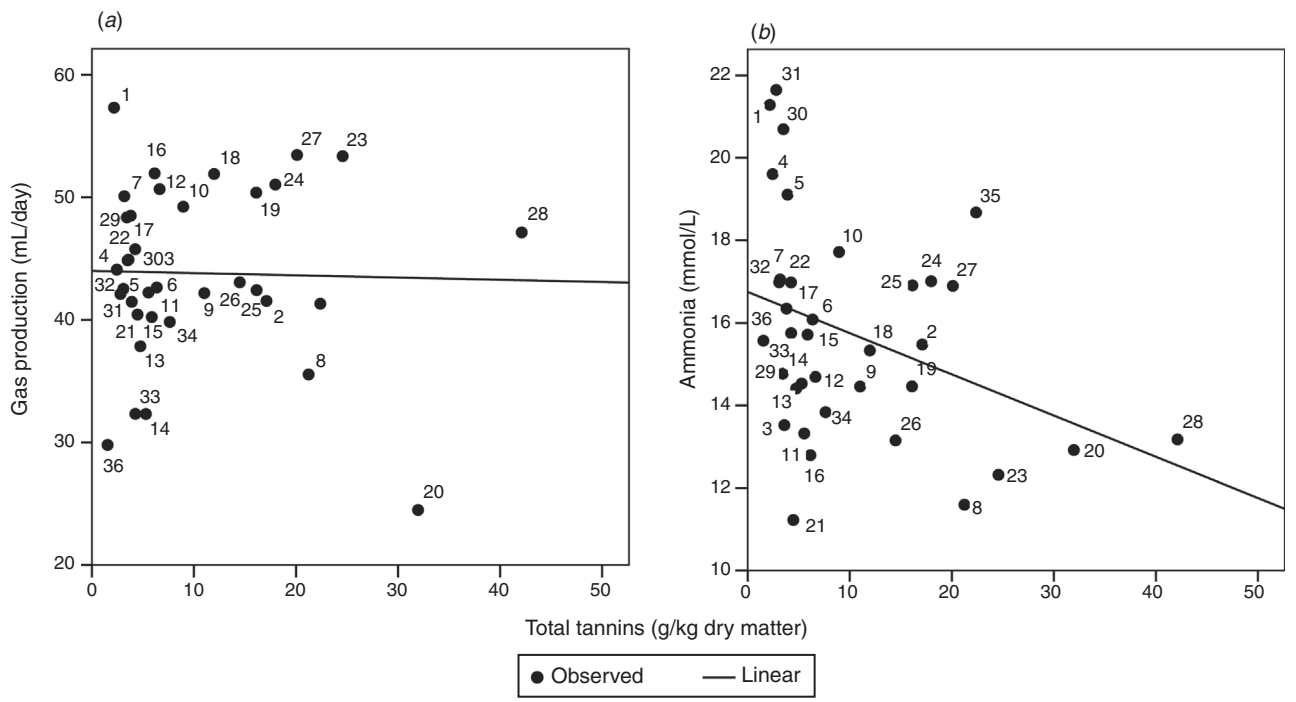

Fig. 5. (*a*) Gas production and (*b*) ammonia concentration in relation to dietary contents of total tannins. Equations: (*a*) $y = 44.0 - 0.0181x$ ($R^2 = 0.001$; not significant); (*b*) $y = 16.8 - 0.0999x$ ($R^2 = 0.128$; $P < 0.05$). For number codes of plant species see Table 1.

Discussion

The goal of the present study was to compare different temperate herb species harvested at a late growth stage with respect to their nutrient and phenol concentrations and their influence on gas production and ammonia formation during 24 h of *in vitro* incubation in rumen fluid together with a basal diet. One objective was to identify herbs that have a high digestibility (estimated as IVOMD from nutrient composition and gas volume) and yet, at the same time, are able to lower ammonia formation in rumen. Such plants would be of value for further investigation into developing forage-based feed additives for improving protein utilisation by dairy cows. A second objective was to establish relationships among compositional and fermentation variables across a large number of plant species for this late harvest stage. All 35 plants tested were grown at the same time and at the same site, which enhanced comparability among plants but restricted the applicability of the results to these conditions. The dietary inclusion of 30% was high with respect to practical feasibility; however, levels of tannin supplementation that are too low may fail to achieve effects (Kapp-Bitter *et al*. 2020). The voluntary intake of plants containing high phenolic contents may be reduced or, in certain metabolic states of the animal, unchanged or even increased (Villalba *et al*. 2015). However, these effects should be the subject of future investigations and of targeted on-farm management.

Ammonia-mitigation potential of the test plants

Technically, ammonia could be measured only once, at the end-point of incubation after 24 h. Therefore, this approach does not reflect temporal dynamics of ruminal N metabolism, but it can serve as an indicator of ammonia formation *in vivo*. The *in vitro* ammonia concentration in the inoculum after 24 h of incubation increased with increasing CP concentration of the plants, as expected (Frank and Swensson 2002; Chanthakhoun *et al*. 2014). Ammonia production depends on the rumen-degradable CP concentration of the diet and, simultaneously, on the accessible energy available in the rumen (Reynolds and Kristensen 2008). Absorbed ammonia must be considered as a loss, and is even a metabolic burden for the liver (Parker *et al*. 1995), and it contributes significantly to blood and milk urea concentrations and thus urinary N excretion (Nousiainen *et al*. 2004). It is expected that tannins will protect dietary proteins from ruminal degradation by the formation of complexes (Perez-Maldonado *et al*. 1995), which could reduce the generation of ammonia and improve protein efficiency of dairy cows. There is *in vitro* (Jayanegara *et al*. 2011; Sinz *et al*. 2019*b*) and *in vivo* evidence (Kälber *et al*. 2012; Sinz *et al*. 2019*a*) that tannin-rich forage herbs may have this effect. It is important to identify forage combinations that simultaneously lower ruminal ammonia concentration and maintain fermentation efficiency, including microbial protein synthesis (Jayanegara *et al*. 2013; Sinz *et al*. 2019*b*).

Results of the present study confirm the expected negative relationship between phenol concentrations and ammonia formation during *in vitro* incubation. The reasons for this include especially the protein-binding effects of tannins (McSweeney *et al*. 2001) as discussed above. Although other factors cannot be excluded, the present results suggest that the ammonia production was negatively related with TT concentrations. Among the tannins, CT are likely most efficient for lowering ammonia production in rumen (Naumann *et al*. 2017; Koenig *et al*. 2018). However, because only a few of the test plants contained detectable levels of CT, the relationships with CT established in the present study should be considered with care. Accordingly, the position of CT in the PCA was not clear in one direction, as was also the case in the study by Jayanegara *et al*. (2011). Rather, it is likely that HT played a major role here. Additionally, of the 19 test-plant diets that lowered ammonia concentration, all except those with *P. lanceolata*, *S. vulgaris* and *T. pratensis* subsp. *orientalis* had greater NTP concentration than the basal mixture. Thus, effects of other phenols such as flavonoids (Waghorn and McNabb 2003; Sinz *et al*. 2018) or differences concerning other compounds (e.g. CP content) have to be considered. Further, there were incubation mixtures with greater TEP content in which the ammonia concentration in the inoculum after 24 h was not different from the basal mixture even if the TT content of the test plants was greater than that in the basal mixture. This is consistent with results of previous research, where effects on ammonia formation of phenol-rich plants were found to depend on the plant species, the extracts of which were investigated by Sinz *et al*. (2019*b*). Although not investigated in the present study, plant bioactive lipid compounds may also have affected ammonia formation (Khiaosa-ard and Zebeli 2013).

Relationships between ammonia mitigation and nutritive and digestion characteristics

It was not possible to separate effects on the different incubation variables, meaning that ammonia was mitigated together with gas and, consequently, also IVOMD in most cases. The desired candidate would be a herb that results in incubation variables that are displayed in the upper right corner of the PCA, indicating low ammonia formation at high dietary protein. No test plant completely fulfilled this criterion. However, some approached this goal, among them *C. rapunculoides* and *S. pratensis*, both of which had rather high protein content and still only average ammonia formation. Both were characterised by high proportions of NTP. On the other hand, *O. vulgare* and *S. minor* expressed low ammonia formation at average nutrient concentrations. These plants were both characterised by high TEP concentrations but almost an absence of CT, indicating that HT indeed had a major effect, which is in line with the literature (Jayanegara *et al*. 2011; Aboagye *et al*. 2019; Stewart *et al*. 2019). However, again, the NTP fraction was also clearly present in these plants and might have played a role (Sinz *et al*. 2018). The two herbs with high CT concentrations (*L. corniculatus* and *O. viciifolia*) also had quite clear mitigating effects on ammonia formation, as expected (Mueller Harvey 2006; Dschaak *et al*. 2011; Ghelichkhan *et al*. 2018), at fair dietary protein contents. Among the test plants of the present study, *O. viciifolia* is the most researched with respect to ammonia formation. It was found earlier to have

clear rumen-ammonia-mitigating properties across various cultivars and harvest dates (Scharenberg *et al*. 2009; Azuhnwi *et al*. 2012; Grosse Brinkhaus *et al*. 2016).

In the PCA, negative relationships of concentrations of NEL, APDE and APDN with fibre (NDF and ADF) concentrations were expected because fibrous carbohydrates have a lower digestibility than other carbohydrates. There was a quadratic relationship between dietary TEP concentrations and gas production, indicating that at low and high TEP concentrations adverse effects will occur, whereas intermediate TEP concentrations may even promote ruminal nutrient degradation. This supports the observation that higher TEP concentrations impair certain rumen bacteria and thus, for instance, inhibit cellulolysis (McSweeney *et al*. 2001; Min *et al*. 2002) and, with this, nutrient degradation. It explains why apparent total tract digestibility declined with increasing dietary TEP concentrations in the *in vivo* studies of Yang *et al*. (2017) and Henke *et al*. (2017).

Conclusions

Among 35 mature herbs grown in a temperate climate, we did not find one that would decrease ammonia formation and at the same time improve IVOMD in *in vitro* rumen fermentation. We found indications of a correlation of HT and NTP with ammonia mitigation by this plant type. *Galium verum*, *Leontodon hispidus*, *Lotus corniculatus*, *Onobrychis viciifolia*, *Plantago lanceolata*, *Sanguisorba minor* and *Scabiosa columbaria* mitigated ammonia and moderately maintained IVOMD. These plants might be beneficial for lowering ammonia production when supplemented to grassland-based diets excessive in CP, and this without impairing nutritional quality. Implementation of such plants in farm practice appears realistic because all plants investigated were grown for seed production, thus making them available for cultivation. Further studies should investigate whether these plants will also exhibit the detected effects *in vivo*, and whether this is the case at lower dietary proportions.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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