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Dose-response effects of woody and herbaceous forage plants on *in vitro* ruminal methane and ammonia formation, and their short-term palatability in lactating cows

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*Plant secondary compounds (**PSC**) are prevalent in many woody, temperate-climate plant species and play a crucial role in dietary attempts to mitigate methane emissions in ruminants. However, their application requires sufficient palatability and feeding value. In the present study, leaves from silver birch (*Betula pendula*), hazel (*Corylus avellana*), blackcurrant (*Ribes nigrum*), green grape vine (*Vitis vinifera*) and the herbs rosebay willow (*Epilobium angustifolium*) and wood avens (*Geum urbanum*) were tested in various doses with the Hohenheim gas test method* in vitro *and their short-term palatability in dairy cows. For the palatability experiment, the plants were pelleted with lucerne in different proportions to obtain the same phenol content, but realised contents differed from expected contents. The pellets were provided separately from a mixed basal ration (0.4 : 0.6) to each cow, in a randomised order, for 3 days per plant. All plants mitigated* in vitro *methane and ammonia formation, often in a linear dose response. These levels of effects differed among plants. Significant effects were observed at 100 (hazel, rosebay willow) to 400 g/kg of plant material. The test plants had a lower feeding value than the high-quality basal diet. This was indicated by* in vitro *organic matter digestibility, short-chain fatty acid formation and calculated contents of net energy of lactation. Simultaneously, the linear depression of ammonia formation indicated a dose-dependent increase of utilisable CP. Only blackcurrant and birch were less preferred to lucerne. However, this aversion subsided on day 3 of offer. The rosebay willow pellets had the highest phenol content but were not the least palatable. Accordingly, PSC may not be the main determinants of palatability for the plants tested. Plants did not differ significantly in their short-term effects on milk yield and composition, and all of the plants substantially reduced milk urea content. Overall, the results suggest that hazel and vine leaves, and rosebay willow and wood avens herbs should be tested for their potential to mitigate methane and N emissions* in vivo*.*

Keywords: phenols, hazel, vine, rosebay willow, wood avens

Implications

Phenol rich plants as feed supplements to ruminant diets should mitigate methane and nitrogen emissions without affecting feed intake. Four of the six plants tested in the present study, the leaves of hazel and green grape vine as well as the herb of rosebay willow and wood avens, dose-dependently reduced methane and ammonia formation *in vitro*. These plants were also highly palatable in the short term when fed as supplements to lactating dairy cows. Thus, their use may assist in mitigating environmentally harmful emissions and simultaneously increase biodiversity of plants used for ruminant production.

Introduction

Plant secondary compounds (**PSC**), such as phenols, have an important role as mitigants in feeding strategies that are aimed at abating methane and nitrogen emissions from ruminants. In this regard, forages from tropical trees, shrubs and herbaceous legumes may be rich in effective PSC and could also possess a comparatively high feeding value. Similar plants from temperate regions are much rarer (Frutos *et al.*, 2004). Nevertheless, an extensive *in vitro* screening,

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of a range of woody plants from temperate regions, identified a number of plants with the potential to mitigate ruminal methane and ammonia emissions without affecting dietary digestibility (Terranova *et al.*, 2018a). The dose of PSC is particularly important for determining whether they will have beneficial or adverse effects on production. According to the meta-analysis by Jayanegara *et al.* (2012), >20 g tannins/kg diet DM are needed to effectively mitigate methane. Aerts *et al.* (1999) found that 20 to 40 g/kg DM of condensed tannins (**CT**) is required to improve protein utilisation. Tannins and particularly CT have the capacity to form rumen undegradable complexes with proteins and therefore increase the protein availability at the duodenum (Waghorn *et al.*, 1994). However, elevated dietary PSC contents may impede intake and digestibility (Kumar and Singh, 1984; Beauchemin *et al.*, 2008; Waghorn, 2008). At just 20 g tannins/kg DM grazing animals rejected the feed (Donnelly and Anthony, 1969), and >50 g tannins/kg DM were frequently found to depress voluntary feed intake (Aerts *et al.*, 1999; Barry and McNabb, 1999). This illustrates that the optimum range in tannin supply is small.

In an extensive *in vitro* screening, Terranova *et al.* (2018a) tested 16 different temperate-climate plants with a dose of 167 g/kg on top of a common dairy basal diet. These authors identified six woody plants which showed the potential to mitigate ruminal ammonia and methane formation without adversely affecting digestibility *in vitro*. These plants were birch (*Betula pendula*), hazel (*Corylus avellana*), blackcurrant (*Ribes nigrum*) and vine (*Vitis vinifera*, green-leaved) leaves as well as rosebay willow (*Epilobium angustifolium*) and wood avens (*Geum urbanum*) herbs. One aim of the present study was, therefore, to test how *in vitro* fermentation parameters respond to increasing doses of these plants. Furthermore, the cows' palatability of these six plants is unknown. So far only the palatability of birch (voluntary intake; Meier *et al.*, 2014) and hazel leaves (pellets with different doses; Wang *et al.*, 2018) in sheep has been studied. A further aim of the study was, therefore, to examine the palatability of the six plants in dairy cows. Consequently, the hypotheses tested in the present study were that (1) there will be a linear response between plant dose and *in vitro* fermentation parameters and (2) plant composition and their palatability will be related.

Materials and methods

Experimental design

The present study consisted of two experiments, an *in vitro* incubation with the Hohenheim gas test (**HGT**, hypothesis 1) and an animal experiment for *in vivo* palatability determination (hypothesis 2). The six plants investigated in both experiments were leaves of birch, hazel, blackcurrant and vine as well as herbs of rosebay willow and wood avens. The leaves of sweet chestnut (*Castanea sativa)* were used as a positive control *in vitro* as they are known to efficiently suppress ruminal methane formation (Jayanegara *et al.*, 2011). The plant materials consisted of four different production lots. Lots A and B were used in the HGT *in vitro*, and lots C and D were used in the *in vivo* palatability experiment. All lots were obtained from Alfred Galke GmbH (Bad Grund, Germany), Dixa AG (St. Gallen, Switzerland), Ried Pharmacy (Konstanz, Germany) and Herbathek Naturheilmittel (Berlin, Germany). The materials were available in dried (maximum 40°C) and cut (4 to 6 mm size) form.

Dose-response experiment in vitro *(testing hypothesis 1)*

The HGT was operated according to Menke and Steingass (1988) and Soliva and Hess (2007). Rumen fluid was obtained after morning feeding from a cannulated Brown Swiss cow in mid lactation fed first- and second-cut grass hay and a mineral supplement (UFA 195, UFA-AG, Herzogenbuchsee, Switzerland). After being strained through four layers of gauze (1 mm pore size), the rumen fluid was added to the Menke buffer solution (ratio 1 : 2). The buffered rumen fluid (30 ml) and the substrate (200 mg DM, 1 mm size) were incubated in double-outlet syringes. Test plants (with equal mixtures of lots A and B) were incubated in doses of 100, 200, 300, 400, 500 and 1000 g/kg. These amounts replaced equal amounts of the mixed ration, which was composed of maize silage, grass silage (mixed sward, ryegrass dominated), grass hay (mixed sward, balanced) and concentrate (50 : 25 : 10 : 15). The concentrate consisted (g/kg DM) of rapeseed cake, 500; soybean meal 170; maize gluten, 150; wheat, 130; calcium phosphate, 30; calcium carbonate, 20. Each of the 42 treatments (seven plants × six doses) was repeated in six independent HGT runs, each with fresh rumen fluid and over a period of 7 weeks. In addition, each run included duplicates of no substrate (blank), basal diet only, standard hay and concentrate and an utilisable CP standard (obtained from the University of Hohenheim, Stuttgart, Germany).

After 24 h of incubation at 39°C, the total gas volume was recorded. The incubation fluid was analysed for pH and ammonia with a potentiometer (pH: model 632; ammonia: model 713; Metrohm, Herisau, Switzerland). Methane and carbon dioxide ($\mathbf{CO_2}$) concentrations were measured in the total gas using a gas chromatograph (6890N, Agilent Technologies, Wilmington, NC, USA) equipped with a thermal conductivity detector. Protozoal and bacterial counts were obtained after fixation in formaldehyde using counting chambers (Blau Brand, Wertheim, Germany) with 0.1 and 0.02 mm depth, respectively. Short-chain fatty acids (**SCFA**) were analysed with high-performance liquid chromatography (La Chrom, L-7000 series, Hitachi Ltd, Tokyo, Japan) equipped with an UV detector.

Palatability experiment in vivo *(testing hypothesis 2)*

Six late-lactation Brown Swiss cows were selected from the herd of the ETH Research Station Chamau (Hünenberg, Switzerland). These cows were in first to fifth lactation and weighed 609 ± 37 kg. They were kept in individual boxes to allow measurement of individual feed intake and had permanent access to water.

Test plant pellets (**TPP**) were produced from the six cut woody plants (specified earlier), lucerne (*Medicago sativa*; cut to 3 mm particle size with a Sigma 5.2 hammer mill, Kuhn AG, Bottighofen, Switzerland) and 20 g of molasses/kg DM. The ratio of test plant material to lucerne (low in total extractable phenols (**TEP**), Table 1), was determined in a way that all pellets produced contained about 60 g TEP/kg DM, based on the analyses of lots A and B (used *in vitro*). The C and D lots of each plant were used to produce different pellet batches. Control pellets consisted (g/kg DM) of lucerne, 980, and molasses, 20. Lucerne, test plant materials and molasses were mixed with a Speedmix DFML-1000 (Bühler AG, Uzwil, Switzerland). Pellets (4.5 mm in diameter) were produced with a Kahl 40P pellet press at <60°C (Amandus Kahl GmbH, Reinbek, Germany) with the help of steam (Installation Bühler AG).

Diets were composed of TPP (7.2 kg DM) and a mixed basal ration (**MBR**) (40 : 60). They were provided separately three times per day. The MBR consisted of, in g/kg DM, grass silage (mixed sward, ryegrass dominated), 495; maize silage, 267; hay (mixed sward, ryegrass dominated), 91; soybean meal, 87; sugar beet pulp, 48; mineral supplement, 9 and feed-grade urea, 3. In addition, 100 g/day of a mineral-vitamin mix (KRONI; Kroni Locher, Altstätten, Switzerland) and 50 g salt (390 g NaCl/kg, Agrisal, Matile GmbH, Rubigen, Switzerland) were provided. Cows were adapted to lucerne pellet additions to their regular MBR for 5 days. This was followed by 3 days of quantifying individual lucerne pellet intake as a reference value (control). Thereafter, all six types of TPP were fed in a randomised order to each cow for 3 days (see Supplementary Material Table S1; each plant lot was fed to three cows). Intakes of TPP and MBR were measured daily. Separately, TPP intake was recorded during the first 5 h of feeding. Milk yield was recorded at each milking. Milk samples were taken on day 3, conserved with Bronopol® and analysed for fat, protein, lactose and urea content with a MilkoScan FT6000 (Foss, Hillerød, Denmark).

Laboratory analyses

Dietary components were analysed for contents of DM, ash, nitrogen, ether extract (**EE**), NDF and ADF and ADL according to standard procedures (AOAC, 1997; more details in Terranova *et al.*, 2018a). Concentrations of TEP, non-tannin phenols (**NTP**) and CT were determined according to Makkar (2003). Total tannins (**TT**) and hydrolysable tannins (**HT**) were calculated from these results. Details of procedures are described by Wang *et al.* (2018).

Table 1 *Analysed composition (g/kg DM) of experimental plants and pellets used* in vitro *(Hohenheim gas test experiment) and* in vivo *(dairy cow experiment)*

Plant species	Lot[1]		OM	CP	EE	NDF	ADF	ADL	TEP	NTP	TT	CT	HT
Plant material used *in vitro*													
Birch	A+B		944	140	45.7	438	257	134	63.2	37.0	26.2	15.2	11.0
Sweet chestnut	A+B		956	107	55.6	406	266	99	152.6	24.7	127.9	4.3	124.0
Hazel	A+B		925	112	28.6	414	276	116	105.3	38.4	67.0	61.3	5.7
Rosebay willow	A+B		933	123	17.2	438	372	101	79.0	14.9	64.1	0.7	63.4
Wood avens	A+B		910	123	12.6	361	297	54	103.8	21.5	82.3	5.8	76.5
Blackcurrant	A+B		900	162	31.6	291	260	150	78.4	29.5	48.9	33.6	15.3
Vine	A+B		908	130	35.8	316	211	96	87.1	20.8	66.3	27.0	39.4
Basal diet used *in vitro*			927	146	31.0	426	275	36	16.5	14.9	1.6	0.1	1.5
Feeds used *in vivo*		g/kg[2]											
Mixed basal ration			920	152	29.1	419	268	38	18.5	15.5	3.0	0.2	2.9
Lucerne pellets		980	858	198	17.3	432	334	87	13.4	11.7	1.7	0.1	1.5
Test plant pellets													
Birch	C	980	949	148	65.3	424	254	137	57.1	26.9	30.2	25.2	5.1
	D	980	955	152	65.9	408	241	128	67.6	25.1	42.5	30.3	12.1
Hazel	C	540	913	158	27.4	388	229	77	45.3	21.2	24.0	18.2	5.8
	D	540	900	183	20.0	426	158	102	50.4	22.8	27.6	21.7	5.9
Rosebay willow	C	800	916	159	23.5	326	242	68	97.7	16.2	81.6	3.4	78.1
	D	800	916	139	25.0	302	251	64	92.6	15.6	76.9	0.4	76.5
Wood avens	C	600	893	160	17.0	365	274	79	76.5	15.9	60.5	8.1	52.5
	D	600	903	178	30.3	337	222	58	62.1	12.6	49.5	4.3	45.3
Blackcurrant	C	800	887	183	34.3	320	260	138	67.2	28.6	38.7	34.7	4.0
	D	800	891	178	38.2	272	240	118	72.4	27.8	44.6	37.0	7.7
Vine	C	720	897	144	37.7	333	214	91	70.2	15.9	54.3	17.5	36.8
	D	720	897	164	35.1	361	248	114	67.8	19.2	48.7	32.2	16.4

OM = organic matter; EE = ether extract; TEP = total extractable phenols; NTP = non tannin phenols; TT = total tannins; CT = condensed tannins; HT = hydrolysed tannins.
[1]Average values of lots A and B depicted because 1 : 1 mixtures were incubated *in vitro*. Harvest times Lot A and B, see Terranova *et al.* (2018a); Lot C/D: birch, June to July/June; blackcurrant, May to August/July; hazel, May to June/August; rosebay willow, June/June; vine, August to September/September; wood avens, September/June.
[2]Values indicate proportion of plant material in pellet. Difference to 1000 g/kg consisted of lucerne and 20 g/kg molasses.

Calculations and statistical analysis

Total gas amounts produced, *in vitro* organic matter digestibility (**IVOMD**) and net energy of lactation contents (**NEL**) were calculated after the adjustment of gas production measured in the blank fermentations, and hay and concentrate standards, using equations by Menke and Steingass (1988). The uCP content was calculated as outlined by Edmunds et al. (2012), and values were adjusted by the Hohenheim standard containing 183 mg uCP/g DM. Palatability was determined by the palatability index (**PAL**) following Ben Salem *et al.* (1994), with MBR or lucerne pellet intakes as the reference for TPP intake. Accordingly, $PAL_{72\ h}$ (%) = (test plant pellet intake (kg)/test plant pellet offered (kg))/(MBR or lucerne pellet intake (kg)/(MBR or lucerne pellet offered (kg)) × 100. The PAL was calculated individually, for each day, in relation to the lucerne-only pellets.

Data were analysed with the MIXED procedure of SAS (version 9.4, SAS Institute, Carry NC, USA) with Tukey-Kramer adjustment. Model 1 included 42 treatments (seven plants × six doses), test plant, dose and interaction as fixed factors and run as random factor. Model 2 compared the plant's effect within the dose considering run as random factor. Linear and non-linear effects of dose within test plants were evaluated by orthogonal polynomial contrasts. Model 3 (*in vivo* data) considered the TPP and lactation stage as fixed, and lactation number and animal as random factors. Individual animal data recorded during the control feeding were used as a covariate for milk yield and composition analysis. Significance level was set to $P < 0.05$. Pearson correlation coefficients between variables and their significance were calculated using the correlation procedure of SAS.

Results

Chemical composition of experimental feeds

Organic matter (**OM**) contents varied between 858 and 956 g/kg DM, but were similar among lots of each plant species (Table 1). Lucerne pellets were highest in CP. Birch and rosebay willow were highest, and blackcurrant TPP (lot D) was the lowest in NDF. In all plant materials, except hazel (lot D), ADF was >210 g/kg DM. The ADL was lowest in the basal diet and highest in blackcurrant. All five phenol fractions were low in the basal diet, lucerne pellets and MBR. Chestnut leaves were richest in TP, TT and HT. Among the TPP, rosebay willow pellets contained the most TP, TT and HT. The highest NTP and CT contents were found in hazel leaves. Birch, hazel and blackcurrant TPP contained >20 g NTP/kg DM. The CT content varied from 0.4 (rosebay willow TPP, lot D) to 37 g/kg DM (blackcurrant TPP, lot D).

In vitro *experiment*

After 24 h of fermentation, the pH of the incubation fluid ranged between 6.8 and 6.9 (data not shown). All plants reduced the IVOMD linearly ($P < 0.05$; Table 2). Values differed significantly from 0 g/kg at doses of either 200 or 300 g/kg. As the dose of sweet chestnut increased, the decline in IVOMD became larger (quadratic contrast, $P < 0.05$). The different responses to the test plants were manifested in the plant × dose interaction in the IVOMD ($P < 0.001$). Total SCFA concentration of the incubation fluid responded similarly as IVOMD with respect to plants and doses. With increasing plant doses, the acetate proportion increased linearly ($P < 0.05$). Only with sweet chestnut the acetate proportion decreased ($P < 0.05$). The proportions of the other SCFA declined with most plants depending on the dose (Table 2, Supplementary Material Table S2). This induced large changes in the acetate-to-propionate ratio (Supplementary Material Table S3). Plant × dose interactions were observed in all SCFA-related variables ($P < 0.001$).

Amounts of NEL, total gas and CO_2 decreased linearly ($P < 0.05$) with increasing dose of each test plant material and differed from the basal diet alone with doses of at least 200 to 300 g/kg ($P < 0.05$; Table 3, Supplementary Material Table S4). In comparison to the non-supplemented control, the calculated uCP content was positively related to each plant dose in the incubation substrate ($P < 0.05$). The relationship was linear for birch and wood avens, non-linear for sweet chestnut and rosebay willow and linear and non-linear for hazel, blackcurrant and vine leaves. In NEL and uCP contents, there were plant × dose interactions ($P < 0.001$). The ammonia concentration decreased ($P < 0.05$) with all seven plants, but the effective dose differed among plants. There was no plant × dose interaction.

The methane produced per unit of DM, CO_2, SCFA and digestible OM (**dOM**) decreased linearly with increasing dose of plant material ($P < 0.05$; Table 4). Only with sweet chestnut, birch and wood avens the decrease also had a non-linear component ($P < 0.05$). The dose-dependent methane mitigation was the highest in methane per unit of DM. The level of absolute and relative methane mitigation differed greatly between plants and dose (interactions generally $P < 0.001$; Supplementary Material Table S4). Among plants, sweet chestnut was most effective at reducing methane (declines of 88% to 96% of control), followed by hazel (32% to 76%), birch (39% to 72%), rosebay willow (22% to 60%), blackcurrant (19% to 52%), vine (26% to 51%) and wood avens (22% to 41%).

Dairy cow experiment

During the control feeding period, 91% of lucerne pellets offered (7.2 kg DM) were consumed. The MBR offered (~11 kg DM) was always completely consumed (Table 5). Hazel, rosebay willow, wood avens and vine TPP (7.2 kg offered) were consumed equally well as the lucerne control pellets. Therefore the PAL for these test plants did not differ in the first 5 h and across all 3 days. However, TPP intake and PAL were reduced with birch and blackcurrant on days 1 and 2 but not on day 3 ($P < 0.05$). Milk yield was lower with all TPP, except wood avens, when compared with lucerne pellets ($P < 0.05$). The milk yield per kilogram of DM intake was reduced with hazel pellets but not with other TPP ($P < 0.05$). Contents of milk fat, protein and lactose did not differ between TTP and lucerne pellets. Milk urea

Table 2 *IVOMD, total SCFA and proportions of acetate (C_2) and propionate (C_3) in incubation fluid as affected by experimental plants in the Hohenheim gas test experiment (further results on individual SCFA in Supplementary Material Tables S2 and S3)*

Variable	Dose (g/kg DM)	Basal diet	Birch	Sweet chestnut	Hazel	Rosebay willow	Wood avens	Blackcurrant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dose	Plant × dose
IVOMD (%)	0	67.7								0.99		<0.001	<0.001	<0.001
	100		66.7	65.7	65.7	64.8	64.4	64.7	65.6		0.815			
	200		62.8*^{b}	61.8*^{b}	63.4^{ab}	62.6*^{b}	64.3^{ab}	66.2^{a}	65.1^{ab}		0.004			
	300		61.6*^{a}	56.9*^{b}	58.9*^{ab}	60.2*^{ab}	62.9*^{a}	62.5*^{a}	61.5*^{a}		0.001			
	400		59.8*^{abc}	49.0*^{d}	57.2*^{bc}	56.0*^{c}	61.9*^{a}	62.0*^{a}	60.5*^{ab}		<0.001			
	500		56.7*^{bc}	46.0*^{d}	53.9*^{c}	55.1*^{c}	60.7*^{a}	60.1*^{ab}	59.2*^{ab}		<0.001			
	1000		43.1*^{c}	32.9*^{e}	39.6*^{d}	45.9*^{c}	57.0*^{a}	53.3*^{b}	53.6*^{b}		<0.001			
	Contrast[1]		L	L Q	L	L	L	L	L					
Total	0	72.5								2.78		<0.001	<0.001	<0.001
SCFA (mmol/l)	100		74.9	73.9	73.4	73.8	72.9	72.5	73.1		0.318			
	200		71.4^{a}	69.9^{ab}	70.4^{ab}	68.9^{b}	70.8^{ab}	70.2^{ab}	71.1^{ab}		0.031			
	300		71.4^{ab}	64.7*^{c}	66.7*^{bc}	67.0*^{bc}	69.8^{a}	66.7*^{bc}	68.2*^{ab}		<0.001			
	400		66.8*^{ab}	61.5*^{b}	65.3*^{ab}	66.7*^{ab}	70.1^{a}	66.4*^{ab}	69.5^{a}		0.002			
	500		66.6*^{bc}	57.8*^{e}	63.2*^{d}	64.7*^{cd}	68.9^{a}	66.0*^{bc}	67.2*^{ab}		<0.001			
	1000		54.7*^{c}	49.2*^{d}	50.0*^{d}	54.9*^{c}	65.7*^{a}	59.5*^{b}	61.9*^{b}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
C_2 (mmol/mol SCFA)	0	66.7								0.36		<0.001	<0.001	<0.001
	100		65.8^{cd}	65.7^{d}	66.3^{ab}	66.1^{bc}	66.1^{bc}	66.3^{ab}	66.5^{a}		<0.001			
	200		66.5^{b}	65.9^{b}	67.3^{a}	67.5^{a}	67.4^{a}	67.5^{a}	67.9^{a}		<0.001			
	300		67.6^{b}	65.3*^{c}	68.3*^{ab}	68.5*^{ab}	68.6*^{ab}	68.9*^{a}	69.4*^{a}		<0.001			
	400		68.7*^{ab}	65.0*^{c}	69.6*^{a}	68.2*^{ab}	67.8^{b}	68.0*^{b}	69.0*^{ab}		<0.001			
	500		67.3^{c}	64.0*^{d}	69.2*^{ab}	69.3*^{ab}	68.6*^{b}	69.0*^{b}	70.1*^{a}		<0.001			
	1000		69.3*^{d}	66.7^{e}	73.8*^{b}	74.6*^{ab}	71.3*^{c}	73.6*^{b}	74.9*^{a}		<0.001			
	Contrast		L	L	L	L	L	L	L					
C_3 (mmol/mol SCFA)	0	18.9								0.40		<0.001	<0.001	<0.001
	100		19.3^{ab}	19.3^{a}	18.9^{c}	19.0^{c}	19.0^{bc}	18.8^{cd}	18.6^{d}		<0.001			
	200		18.8^{b}	19.2^{c}	18.3^{c}	18.2^{c}	18.4^{bc}	18.2^{c}	17.8*^{d}		<0.001			
	300		18.5*^{b}	19.4^{a}	17.8*^{bc}	17.6*^{cd}	18.0*^{bc}	17.7*^{cd}	17.1*^{d}		<0.001			
	400		18.0^{b}	19.5^{a}	17.6*^{bc}	17.5*^{bc}	18.1*^{b}	17.8*^{bc}	17.0*^{c}		<0.001			
	500		19.0^{b}	19.6^{a}	17.4*^{cd}	17.1*^{d}	17.7*	17.3*^{cd}	16.4*^{e}		<0.001			
	1000		19.8*^{a}	19.7^{a}	16.1*^{b}	14.6*^{c}	16.3*^{b}	15.6*^{b}	14.4*^{c}		<0.001			
	Contrast		L Q	L	L	L	L	L	L Q					

IVOMD = *in vitro* organic matter digestibility; SCFA = short-chain fatty acid.
[a,b,c,d,e]Least-square means within a row with no common superscript differ ($P < 0.05$).
*Values differ ($P < 0.05$) from those of basal diet alone.
[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 3 *Calculated contents of NEL and uCP per unit of DM as well as measured ammonia (NH_3) concentration in incubation fluid as affected by experimental plants in the Hohenheim gas test experiment*

Variable	Dose (g/kg)	Basal diet	Birch	Sweet chestnut	Hazel	Rosebay willow	Wood avens	Blackcurrant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dose	Plant × dose
NEL (kJ/g DM)	0	5.23								0.115		<0.001	<0.001	<0.001
	100		5.14	5.05	5.01	4.90	4.81	4.83	4.97		0.677			
	200		4.66*	4.59*	4.72	4.61*	4.80	4.99	4.89		0.059			
	300		4.53*^{ab}	4.06*^{b}	4.19*^{ab}	4.34*^{ab}	4.62*^{a}	4.48*^{ab}	4.42*^{ab}		0.022			
	400		4.42*^{a}	3.35*^{d}	4.01*^{bc}	3.87*^{c}	4.49*^{a}	4.38*^{a}	4.29*^{ab}		<0.001			
	500		4.00*^{abc}	3.05*^{d}	3.66*^{c}	3.79*^{bc}	4.33*^{a}	4.14*^{ab}	4.14*^{ab}		<0.001			
	1000		2.77*^{cd}	2.23*^{e}	2.47*^{de}	2.95*^{c}	3.87*^{a}	3.28*^{b}	3.49*^{b}		<0.001			
	Contrast[1]		L	L	L	L	L	L	L					
uCP (mg/g DM)	0	162								7.5		<0.001	0.002	<0.001
	100		171^{b}	182*^{a}	180^{ab}	184*^{a}	177^{ab}	180^{ab}	178^{ab}		0.024			
	200		177^{ab}	183*^{ab}	184*^{a}	175^{ab}	172^{ab}	181^{ab}	168^{b}		0.025			
	300		180^{b}	182*^{ab}	181^{ab}	177^{b}	179^{b}	195*^{a}	185*^{ab}		0.010			
	400		182*^{b}	174^{b}	183*^{b}	179^{b}	185*^{b}	200*^{a}	186*^{b}		<0.001			
	500		189*^{ab}	177^{c}	180^{bc}	168^{c}	179^{bc}	194*^{a}	175^{c}		<0.001			
	1000		197*^{b}	172^{c}	175^{c}	169^{c}	175^{c}	216*^{a}	190*^{b}		<0.001			
	Contrast		L	Q	L Q	Q	L	L Q	L Q					
NH_3 (mmol/l)	0	12.8								0.57		<0.001	<0.001	0.226
	100		11.9^{a}	10.8*^{b}	11.1*^{b}	10.9*^{b}	11.5^{ab}	11.4^{ab}	11.4^{ab}		<0.001			
	200		11.7^{ab}	10.7*^{b}	10.7*^{b}	11.5^{ab}	11.9^{a}	11.9^{a}	12.1^{a}		<0.001			
	300		11.8	10.7*	11.0*	11.2*	11.1*	10.8*	10.8*		0.076			
	400		11.0*	10.4*	10.0*	10.9*	10.4*	10.5*	10.5*		0.087			
	500		10.4*^{abc}	10.1*^{bc}	9.8*^{c}	11.1*^{a}	10.4*^{abc}	10.7*^{ab}	10.9*^{ab}		<0.001			
	1000		9.4*^{bc}	9.1*^{c}	9.1*^{c}	10.4*^{a}	10.0*^{ab}	10.0*^{ab}	9.4*^{bc}		<0.001			
	Contrast		L	L Q	L	L Q	L	L Q	L					

NEL = net energy for lactation; uCP = utilisable crude protein.
[a,b,c,d,e]Least-square means within a row with no common superscript differ ($P < 0.05$).
*Values differ ($P < 0.05$) from those of basal diet alone.
[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 4 *CH_4 formation per unit of DM supply, CO_2, SCFA and dOM as affected by experimental plants in the Hohenheim gas test experiment (further results on gas production in Supplementary Material Tables S4 and S5)*

Variable	Dose (g/kg)	Basal diet	Birch	Sweet chestnut	Hazel	Rosebay willow	Wood avens	Blackcurrant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dose	Plant × dose
CH_4/DM (ml/g)	0	34.2								0.80		<0.001	<0.001	<0.001
	100		33.3	31.4	31.6	30.8*	31.0	31.2	31.8		0.258			
	200		29.6*^{a}	26.4*^{b}	29.0*^{a}	28.7*^{a}	29.6*^{a}	30.7*^{a}	30.3*^{a}		<0.001			
	300		27.9*^{a}	18.6*^{b}	25.0*^{a}	26.8*^{a}	24.7*^{a}	27.9*^{a}	27.9*^{a}		<0.001			
	400		25.8*^{a}	11.7*^{d}	23.1*^{c}	23.2*^{bc}	26.7*^{a}	26.2*^{a}	25.6*^{ab}		<0.001			
	500		22.8*^{abc}	6.1*^{d}	20.4*^{c}	22.1*^{bc}	25.2*^{a}	24.1*^{ab}	24.7*^{a}		<0.001			
	1000		9.5*^{d}	1.2*^{e}	8.3*^{d}	13.7*^{c}	20.2*^{a}	16.3*^{b}	16.7*^{b}		<0.001			
	Contrast[1]		L	L Q	L	L	L Q	L	L					
CH_4/CO_2 (ml/l)	0	179								4.5		<0.001	<0.001	<0.001
	100		176	170	172	174	175	177	174		0.511			
	200		175^{a}	155*^{b}	167^{ab}	168^{ab}	169^{a}	167^{ab}	170^{a}		0.004			
	300		167^{a}	122*^{b}	165^{a}	167^{a}	158*^{a}	169^{a}	172^{a}		<0.001			
	400		158*^{a}	94.8*^{b}	156*^{a}	166^{a}	163^{a}	162^{a}	164		<0.001			
	500		155*^{a}	56.6*^{b}	153*^{a}	161*^{a}	156*^{a}	159*^{a}	162^{a}		<0.001			
	1000		109*^{c}	22.0*^{d}	121*^{bc}	139*^{a}	140*^{a}	145*^{a}	132*^{ab}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
CH_4/SCFA (mmol/mol)	0	137								4.9		<0.001	<0.001	<0.001
	100		133^{a}	126^{ab}	125^{ab}	122^{b}	126^{ab}	131^{ab}	127^{ab}		0.043			
	200		123^{a}	111*^{b}	121*^{a}	122^{a}	122^{a}	128^{a}	123^{a}		<0.001			
	300		119*^{a}	84*^{b}	110*^{a}	116*^{a}	104*^{ab}	122^{a}	118*^{a}		<0.001			
	400		113*^{a}	56*^{b}	105*^{a}	102*^{a}	113*^{a}	116*^{a}	108*^{a}		<0.001			
	500		99*^{a}	30*^{b}	94*^{a}	98*^{a}	106*^{a}	106*^{a}	105*^{a}		<0.001			
	1000		50*^{c}	7*^{d}	48*^{c}	72*^{b}	90*^{a}	81*^{ab}	78*^{b}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
CH_4/dOM (ml/g)	0	50.6								1.08		<0.001	<0.001	<0.001
	100		49.5^{a}	47.3^{b}	47.8^{ab}	47.3^{b}	47.9^{ab}	48.1^{ab}	48.3^{ab}		0.012			
	200		47.0^{a}	42.5*^{b}	45.5*^{a}	45.5*^{a}	45.9*^{a}	46.3^{a}	46.5^{a}		<0.001			
	300		45.0*^{a}	32.4*^{b}	42.1*^{a}	44.2*^{a}	39.6*^{ab}	44.8*^{a}	45.2*^{a}		<0.001			
	400		42.7*^{a}	23.3*^{b}	40.2*^{a}	41.2*^{a}	43.1*^{a}	42.6*^{a}	42.3*^{a}		<0.001			
	500		39.6*^{ab}	12.9*^{c}	37.5*^{b}	39.6*^{ab}	41.5*^{a}	40.6*^{ab}	41.6*^{a}		<0.001			
	1000		21.6*^{c}	3.42*^{d}	20.8*^{c}	29.4*^{b}	35.6*^{a}	31.3*^{b}	31.1*^{b}		<0.001			
	Contrast		L Q	L Q	L	L	L	L	L					

CH_4 = Methane; CO_2 = carbon dioxide, SCFA = short-chain fatty acid, dOM = digestible OM.
[a,b,c,d,e]Least-square means within a row with no common superscript differ ($P < 0.05$).
*Values differ ($P < 0.05$) from those of basal diet alone.
[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 5 *Intake, palatability, milk yield and performance in the dairy cows fed mixed basal ration and lucerne (control) or test plant pellets*

Variable	Lucerne[1]	Birch	Hazel	Rosebay willow	Wood avens	Blackcurrant	Vine	SEM	*P*-value
Intake (kg DM)									
Total (per day)	17.3[a]	14.3[b]	18.0[a]	17.5[a]	17.8[a]	15.2[b]	17.5[a]	0.31	<0.001
Mixed basal ration (per day)	10.8	10.7	10.8	10.8	10.8	10.8	10.8	0.06	0.459
Pellets (per day)	6.58[a]	3.58[b]	7.19[a]	6.68[a]	6.95[a]	4.34[b]	6.64[a]	0.31	<0.001
Pellets (first 5 h)	–	1.67	2.88	2.55	2.53	1.72	2.60	0.47	0.366
Palatability index (%)									
Mixed basal ration[2]	92.6[a]	50.8[b]	100.0[a]	92.7[a]	96.5[a]	60.3[b]	92.5[a]	4.30	<0.001
Pellets[3]	–	54.4[b]	110.5[a]	102.5[a]	106.9[a]	66.6[b]	102.0[a]	4.92	<0.001
Day 1[3]	–	62.3[b]	110.8[a]	102.3[a]	110.4[a]	72.6[b]	106.0[a]	4.77	<0.001
Day 2[3]	–	30.6[b]	110.1[a]	106.4[a]	110.6[a]	54.6[b]	102.7[a]	8.10	<0.001
Day 3[3]	–	70.4	110.5	98.9	99.8	72.6	97.2	11.50	0.115
Milk yield									
kg/day	19.2[a]	16.1[d]	17.1[cd]	17.7[bc]	18.5[ab]	17.1[cd]	17.7[bc]	0.28	<0.001
kg/kg DM intake	1.11[ab]	1.16[a]	0.95[c]	1.01[bc]	1.04[abc]	1.15[a]	1.02[bc]	0.028	<0.001
Milk fat (%)	4.71	4.76	4.47	4.50	4.71	4.73	4.82	0.178	0.754
Milk protein (%)	4.07	4.05	4.06	3.97	4.00	3.98	4.02	0.056	0.812
Milk lactose (%)	4.59	4.48	4.60	4.62	4.56	4.59	4.59	0.042	0.296
Milk urea (mg/dl)	27.5[a]	21.5[b]	16.4[bc]	14.4[c]	17.1[bc]	17.8[bc]	17.6[bc]	1.17	<0.001

[a,b,c,d]Least-square means within a row with no common superscript differ ($P < 0.05$).
[1]Lucerne pellets without plant additives were fed during control feeding (Supplementary Material Table S1).
[2]Test pellet intake related to mixed basal ration intake.
[3]Test plant pellet intake related to average lucerne pellet intake (control feeding; Supplementary Material Table S1).

Table 6 *Pearson correlation coefficients between variables describing intake or palatability and composition of the test plant pellets fed to the dairy cows (n = 6)*

Variable	OM	CP	EE	NDF	ADF	ADL	TEP	NTP	TT	CT	HT
Intake (DM)											
Total	−0.353***	0.136	−0.602***	0.073	−0.029	−0.533***	−0.099	−0.510***	0.027	−0.450***	0.248**
MBR	−0.108	0.011	−0.149†	−0.115	−0.050	−0.092	0.047	−0.038	0.058	−0.042	0.071
Pellets	−0.344***	0.137	−0.591***	0.088	−0.023	−0.529***	−0.106	−0.513***	0.021	−0.451***	0.242**
Pellets first 5 h	−0.136	0.061	−0.272	0.042	−0.323†	−0.322†	−0.033	−0.318†	0.070	−0.267	0.167
Palatability index											
MBR	−0.345***	0.144	−0.585***	0.102	−0.012	−0.521***	−0.119	−0.514***	0.008	−0.451***	0.231**
Pellets	−0.393**	0.097	−0.651***	0.007	−0.316*	−0.616***	−0.013	−0.629***	0.181	−0.540***	0.316***
Day 1	−0.370*	0.098	−0.606***	−0.022	−0.348*	−0.615***	0.031	−0.633***	0.219	−0.487**	0.364*
Day 2	−0.462**	0.079	−0.728***	−0.054	−0.250	−0.652***	0.062	−0.666***	0.255	−0.579***	0.430**
Day 3	−0.214	0.140	−0.374*	0.152	−0.062	−0.362*	−0.178	−0.357*	−0.093	−0.351*	0.014

OM = organic matter; EE = ether extract; TEP = total extractable phenols; NTP = non tannin phenols; TT = total tannins; CT = condensed tannins; HT = hydrolysed tannins; MBR = mixed basal ration.
†$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

concentration was reduced by all TPP, and the biggest reduction was observed with rosebay willow and the smallest with birch ($P < 0.05$).

There were negative correlations among total intake, daily pellet intake and all PAL variables with concentrations of NTP, CT, ADL, OM (except PAL Day 3) and EE ($P < 0.05$ to <0.001; Table 6). Positive relationships were found between HT and most intake and PAL variables ($P < 0.05$ to <0.01). Weak correlations occurred between ADF content and intake related to PAL variables, and none for NDF, TEP, TT and CP contents.

Discussion

Dose responses in methane and ammonia emissions

In the preceding *in vitro* screening of Terranova *et al.* (2018a), 16 different temperate-climate plants were tested for their ability to mitigate methane and ammonia emissions without affecting nutrient digestibility and six woody plants were identified that showed most promise. As the plants were only screened at one dose, the present study extended that work to test the six promising plants at a range of doses. Our results indicate that there are dose-response relationships with *in vitro* fermentation parameters and optimal doses

have been determined for the different plant species. The methane yields (CH_4/DM) were reduced by 33% to 96% with the positive control (sweet chestnut) and by 10% to 76% with the other test plants when compared to the basal diet without plant additive. Higher plant doses enhanced the mitigating effect, supporting our first hypothesis. However, methane mitigation is useful only if nutrient availability is not concomitantly impaired. The most relevant relationship in this regard, CH_4/dOM, decreased with all plants at levels ≥300 g/kg DM, with sweet chestnut being the most effective. Jayanegara *et al.* (2012) showed that ≥20 g tannins/kg diet is required to mitigate methane. In the present study, hazel, wood avens and rosebay willow reduced methane emission even at lower tannin contents. However it is also possible that NTP, or other PSC, may have contributed to these effects (Beauchemin *et al.*, 2008; Jayanegara *et al.*, 2012). Consistent with the current *in vitro* results, CH_4/dOM was reduced by 25% in sheep with 50% hazel leaves in the diet (Wang *et al.*, 2018). In the present study, the methane mitigation effect at 200 mg plant/g DM was similar to that found with 167 g/kg by Terranova *et al.* (2018a). Typically, methane mitigation is associated with a lower acetate-to-propionate ratio. The opposite was observed in the present study, except in the case of sweet chestnut, indicating that the methane decline was not primarily caused by depressed fibre degradation.

As hypothesised, all plants reduced ruminal ammonia formation *in vitro* in a dose-dependent manner. Based on the sharp decline in milk urea content it appears as though the reduction in ammonia formation also occurred *in vivo*. Furthermore, Wang *et al.* (2018) found a linear decrease in urinary nitrogen excretion with increasing doses of hazel in diets fed to sheep. Birch, hazel and vine pellets, all containing ≥18 g CT/kg DM, were most effective in reducing milk urea contents in the present *in vivo* palatability experiment. The capacity of PSC, particularly tannins, to form undegradable complexes with proteins is well known (Waghorn *et al.*, 1994). The reduction in ruminal ammonia concentration found in our *in vitro* study at a dose of 200 g/kg plant material was less pronounced than that found by Terranova *et al.* (2018a) at a dose of 167 g/kg DM. Differences in the plant lots used could have caused this different effectiveness.

Nutritional value of the test plants

Feeds suitable for reducing methane and nitrogen emissions also need to be palatable and have a high nutritional value to ensure that productivity is not adversely affected. There have been reports of plants that showed promise for their potential to modulate rumen fermentation and reduce methane and nitrogen emissions, but were not palatable or potentially adverse to productivity (Jayanegara *et al.*, 2011; Meier *et al.*, 2014). In the present study, information about the nutritional value of the plants was obtained from their effects on total gas production, IVOMD, NEL and uCP contents, and on short-term milk yield and composition. Both IVOMD and NEL are largely determined by total gas production, which decreased linearly, like the SCFA, with increasing plant dose. This result confirms our first hypothesis and indicates that the fermentable energy content of the test plants was lower than that of the high-quality basal diet. This observation coincides with the lower diet digestibility reported, in sheep, for diets containing high doses of hazel leaves (Wang *et al.*, 2018). Among test plants, hazel, blackcurrant, vine and wood avens showed an *in vitro* digestibility superior to that of birch, rosebay willow and sweet chestnut, as expected from previous screening (Terranova *et al.*, 2018a). However, in that study birch did not have such a low content of fermentable energy. The NEL content of the test plants was likely limited by two factors, fibre lignification (high in birch) and depression of digestibility by PSC (high in chestnut). The increase in estimated uCP content by all plants is likely to be related to the capacity of plant PSC to diminish CP degradation. However, it remains to be demonstrated that these PSC-protein complexes are indeed cleaved at low abomasal pH in order to allow postruminal amino acid absorption (Waghorn *et al.*, 1994; O'Connell and Fox, 2001).

Together with the limited NEL contents estimated *in vitro*, milk yield declined with all plants (except wood avens) when compared to the high-quality forage, lucerne. This gap may be less pronounced when compared to a control forage of moderate quality. Milk yield is known to respond quickly after dietary changes. However, 3 days of measurement are relatively limited to show the full effects of feeding the test plants on milk yield and milk composition. Therefore, these data must be considered with caution. Responses in milk protein (variation in NEL and uCP) and fat content (variation in SCFA profile) could be expected over longer-term feeding.

Palatability of test plants

Many forages from woody plants rich in PSC are not palatable. However, high palatability is a prerequisite for the implementation of methane and ammonia reducing feeds by farmers. Our intention was to compare palatability at the same TEP content. However, phenol contents in lots A+B used *in vitro* largely differed from lots C and D used *in vivo*, reflecting the presence of natural variation in plant chemical composition caused by factors including season, region and soil type (Palo *et al.*, 1985, Tiemann *et al.*, 2009). The second hypothesis stating that plant composition is related to their palatability was partly supported by our results. No relationship was observed between palatability and TEP contents, suggesting that other factors influenced palatability. Palatability also seems to differ between grazer-type livestock species. Meier *et al.* (2014) reported that sheep prefer birch leaves when they were offered a choice of six different woody plants. In contrast, birch leaves were the least preferred by the cows in our experiment. Concurrently, birch and black currant had the highest content of lignified fibre. According to our results, the ADL content of the plants is highly relevant for their palatability. Four of the TPP were as palatable as the high-quality forage lucerne. These included rosebay willow, the pellets highest in TEP and HT. Rosebay willow and wood avens (lot C) pellets had HT contents of >50 g/kg pellet DM, but were as

palatable as the control pellets. All TPP-containing diets had CT and HT contents well below 50 g/kg dietary DM each. A concentration of 50 g CT and HT/kg dietary DM is considered to have an impact on voluntary feed intake in ruminants due to astringency at that level and a reduction in digestion and digesta flow rate (Waghorn *et al*., 1994; O'Connell and Fox, 2001; Frutos *et al*., 2004). Astringency can take up to 4 h to develop after ingestion (O'Connell and Fox, 2001). However, in the present study, the reduced palatability of birch and blackcurrant TPP was not significant after 5 h of offering. Our results support the claim of Ben Salem *et al*. (1994) that 1-day intake measurements are not sufficient to predict acceptance in the following 15 days. Indeed the cows in our study may have become accustomed to birch and blackcurrant with time as their palatability increased on day 3. The high palatability of the hazel pellets is similar to the findings of Wang *et al*. (2018), where sheep consumed a diet consisting of 50% hazel leaves equally well as the control diet, over 18 days.

Conclusion

The results from our study indicate that a selection of four out of the six forages tested, hazel, rosebay willow, wood avens and vine, which are all woody plants that grow in temperate environments, have the potential to mitigate methane and nitrogen emissions from livestock and are also palatable. It is important to note that the four promising plants mitigated methane formation per unit dOM, despite their lower ruminal fermentability, when compared to the basal diet. This reflects a genuine and truly beneficial suppression of methanogenesis. However, their nutritional value is limited in terms of net energy, particularly at dietary doses needed for substantial mitigation effects. The commercial availability of the plants that show promise is a critical factor that needs to be considered if any of these plants will be used in practice. Further studies are needed to demonstrate the mitigation potential of the four most promising test plants in live ruminants.

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Declaration of interest

The authors declare no conflicts of interest.

Ethics statement

The experimental protocol complied with the Swiss legislation for Animal Welfare and was approved by the Committee on Animal Experimentation of the Cantonal Veterinary Offices Zurich (ZH 38/14, rumen fluid collection) and Zug (ZG 93/16, palatability experiment).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary materials

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119002076

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