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In vitro rumen methane output of forb species sampled in spring and summer

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The chemical composition, *in vitro* rumen fermentation variables and methane (CH_4) output of a range of common forb species sampled in spring and summer, and grass silage (14 treatments in total; triplicate replication), were determined in this study. Dried, milled herbage samples were incubated in an *in vitro* rumen batch culture with rumen microbial inoculum (rumen fluid) and buffered mineral solution (artificial saliva) at 39 °C for 24 hours. All herbage chemical composition and *in vitro* rumen fermentation variables were affected by treatment, and more specifically by season, species, and season by species ($p<0.05$), except the acetic acid to propionic acid ratio for which there was no season by species interaction ($p>0.05$). *Rumex obtusifolius* (spring, summer), *Urtica dioica* (summer) and *Senecio jacobaea* (summer) had lower ($p<0.05$) CH_4 outputs per unit of feed dry matter incubated (CH_4i) compared with grass silage, and *Stellaria media, U. dioica* and *S. jacobaea* had lower ($p<0.05$) CH_4i in summer than in spring, reflecting their lower extent of *in vitro* rumen fermentation. Although *Ranunculus repens* (spring) and *Cirsium arvense* (spring) had lower ($p<0.001$) CH_4 output than grass silage when expressed per unit of total volatile fatty acids and total gas produced, respectively, these outcomes were not congruent with the more methanogenic volatile fatty acid profile for the grass silage *versus* all forb treatments. Thus, further study is required to determine whether these forb species would reduce CH_4 output relative to animal product if assessed *in vivo*.

Key words: greenhouse gas, grassland, weeds, fermentation

Introduction

Grassland is the dominant (approximately 0.9) crop on agricultural land in Ireland (O'Riordan and O'Kiely 1996), and any enteric methane (CH_4) mitigation strategies for ruminants must be effective within the predominantly grass-based production systems that prevail.

Previous studies have found that altering grazing management strategy by making specific changes to herbage mass and sward allowance, and choosing among specific perennial ryegrass varieties and perennial grass species, had little impact on *in vitro* rumen CH_4 output (Purcell et al. 2011a, 2012). However, some forb species occurring naturally in grassland can have a high apparent nutritive value for grazing livestock (Fairbairn and Thomas 1959), and this could impact on enteric methanogenesis. However, the latter has not been confirmed.

The objective of this study was therefore to quantify the chemical composition, and *in vitro* rumen fermentation variables and CH_4 output using a batch culture gas production technique, of a range of common forb species sampled in spring and summer, and of grass silage (GS).

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Materials and methods

Treatments

All herbage samples were collected at Teagasc Grange, Dunsany, Co. Meath, Ireland (53° 30' N, 6° 40' W, 83 m above sea level), identified with reference to Webb (1977), and named according to the International Plant Names Index (2011). Except for the GS, which was made from a *Lolium perenne* (L.) dominant permanent grassland sward, samples were collected on both 30 April (2009; spring) and 29 August (2009; summer), or on 30 April only (*Ranunculus repens* only), from three separate field plots within permanent grass pastures. Samples were cut to a stubble height of 20 mm. The 14 treatments included in this experiment were *Stellaria media* (L.) Vill. (spring, summer), *Taraxacum officinale* F.H. Wigg (spring, summer), *Rumex obtusifolius* (L.) (spring, summer), *Urtica dioica* (L.) (spring, summer), *Cirsium arvense* (L.) Scop. (spring, summer), *Senecio jacobaea* (L.) (spring, summer), *Ranunculus repens* (L.) (spring), and GS.

Herbage chemical composition analyses

Each herbage sample was initially stored at –18 °C before being thawed at 4 °C for 24 hours, bowl-chopped, and individually mixed. Subsamples of each herbage were thermally dried in a ventilated oven with forced air-circulation at 40 °C for 48 hours prior to milling through a sieve with 1 mm apertures. Determination of *in vitro* dry matter (DM) digestibility (DMD) was carried out using the Tilley and Terry (1963) technique, and the final residue was isolated by filtration rather than centrifugation. The NDF (neutral detergent fibre assayed with a heat stable amylase and sodium sulphite, and expressed exclusive of residual ash), ADFsep (acid detergent fibre analysed on a sample separate from that analysed during the NDF assay and expressed exclusive of residual ash) and ADFseq (sequential analysis of acid detergent fibre on the residue from the NDF assay and expressed exclusive of residual ash; carried out for *R. obtusifolius* [spring, summer] only) concentrations were determined using the filter bag techniques (ANKOM 2006 a and b), and ash content was determined by complete combustion in a muffle furnace at 550 °C for 5 hours. The crude protein (CP; N × 6.25) concentration was determined using a Leco FP 528 N analyser based on method 990-03 of the Association of Official Analytical Chemistry (AOAC, 1990), and the concentration of water soluble carbohydrate (WSC) was determined using the anthrone method (Thomas 1977).

In vitro rumen incubation

The *in vitro* rumen gas production technique of Purcell et al. (2011a) was employed in this study. Replication (× 3) for the *in vitro* rumen incubation was provided by the three independent field replicates. Approximately 0.5 g of each dried, milled feed sample was weighed into 160 ml *in vitro* rumen fermentation bottles. Buffered mineral solution (artificial saliva) was prepared according to McDougall (1948). To obtain a source of rumen microbial inoculum, solid and liquid phase samples of rumen fluid (RF) were collected from different parts of the rumens of four rumen fistulated steers one hour prior to feeding. Each steer was individually offered a restricted allowance (0.9 of *ad libitum* intake) of a 60:40 (DM basis) grass silage plus concentrate diet, with fresh feed offered at 10:30 h daily. The RF was pooled across steers, after which the RF and buffered mineral solution were maintained at 39 °C and under a constant stream of carbon dioxide (CO_2) at all times. The RF was added to the buffered mineral solution at a ratio of 1:4 (RF:buffered mineral solution) after which 50 ml of this buffered RF was dispensed into each fermentation bottle.

All inoculated fermentation bottles were flushed with CO_2 before being sealed and incubated at 39 °C for 24 hours. The gas headspace pressure inside each of the fermentation bottles was recorded at the end of the 24 hour incubation period using a detachable pressure transducer. The total gas produced (TGP) in

each bottle was estimated using the equation:

$$TGP\ (ml) = \left(\frac{bottle\ \ headspace\ \ volume\ \ [ml]}{atmospheri\ \ c\ \ pressure\ \ [hPa]} \right) \times bottle\ \ headspace\ \ \ pressure\ \ (hPa)$$

A 0.8 ml sample of gas was then transferred to a pre-evacuated 2 ml screw-top glass vial for determination of CH_4 concentration. A 0.8 ml sample of liquid medium was obtained from each bottle, placed in 2 ml Eppendorf tubes with 20 µl of a 9 M H_2SO_4 solution, and stored at –18 °C prior to volatile fatty acid (VFA) analysis. The CH_4 and VFA concentrations were measured by gas chromatography using a Shimadzu GC-17A with a flame ionisation detector using iso-caproic acid (0.04 M) as an internal standard for the VFA as described by Ranfft (1973). Temperatures were 150 °C in the column, 150 °C in the injector and 180 °C in the detector. The TGP for each sample was corrected by inclusion of blank fermentation bottles containing buffered RF only, and the mmol of total VFA per gram of feed DM incubated (tVFAi) were corrected using the total VFA concentrations of the pre-incubation buffered RF.

Statistical analysis

The experimental unit in this study was the independent field replicate of each species within each season. Data for the 14 independent treatments were analysed, and a contrast analysis between GS and the mean of all forbs was carried out, using the Proc MIXED procedure of the Statistical Analysis System (SAS, 2003). Differences between treatments were tested using a multiple comparison procedure (Tukey). Data for *S. media, T. officinale, R. obtusifolius, U. dioica, C. arvense* and *S. jacobaea* were also analysed using a model that accounted for season, species, and their interaction with the Proc MIXED procedure of the Statistical Analysis System (SAS, 2003). Where season by species interactions occurred, differences between seasons for individual species were tested using a multiple comparison procedure (Tukey).

Results and discussion

The forb species evaluated in this study are commonly found in permanent grassland in Ireland and other temperate regions, and were deemed to be of potentially high apparent nutritive value by Fairbairn and Thomas (1959). The GS was included as a reference feed for comparative purposes.

Herbage chemical composition

When analysed as 14 independent treatments, all herbage chemical composition variables were affected (p<0.001) by treatment (Table 1). The GS had a higher (p<0.05) DMD than *R. obtusifolius* (summer) and *S. jacobaea* (summer) but did not differ (p>0.05) from all other treatments. The GS had a higher (p<0.05) NDF concentration than all other treatments, and ADFsep concentration was higher (p<0.05) for GS than for all spring forb treatments except *R. obtusifolius* and *C. arvense*, but did not differ (p>0.05) from any summer forb treatment except *T. officinale*, which was lower than GS (p<0.05).

The ADFsep concentrations of *R. obtusifolius* in spring (265 g kg^{-1} DM) and summer (361 g kg^{-1} DM) were higher than the corresponding NDF (169 and 291 g kg^{-1} DM) and ADFseq (118 and 199 g kg^{-1} DM) values. The apparent anomaly of the higher ADFsep concentrations can be explained by the high condensed tannin (CT) concentration of *R. obtusifolius* (Waghorn and Jones 1989) forming insoluble complexes with plant protein during oven drying (Pagán et al. 2009) and thus remaining in the ADFsep residue. In contrast, the sodium

sulphite used in the NDF assay likely removed much of the CT-protein complexes, thereby preventing a similar anomaly with NDF or ADFseq. Thus, this study confirms the conclusion of Terrill et al. (2010) that sequential NDF-ADF analysis provides more realistic ANKOM fibre estimates of ADF for oven-dried plant material containing condensed tannins compared with separate ADF analysis.

The GS had a lower (p<0.05) CP concentration than *R. obtusifolius* (spring) and *U. dioica* (spring) but did not differ (p>0.05) from the other treatments. The WSC concentration for GS was either lower (p<0.05) than or did not differ (p>0.05) from all forb treatments. Ash concentration was lower (p<0.05) for GS than for nine of the 13 other treatments (Table 1).

Table 1. *In vitro* dry matter (DM) digestibility (DMD; g kg^{-1}) and chemical composition of the forb species in spring and summer and of grass silage (GS)

Treatment	DMD	NDF	ADFsep	CP	WSC	Ash (g kg^{-1} DM)
Spring						
Stellaria media (L.) Vill.	817	329	210	169	88	121
Taraxacum officinale F.H. Wigg	810	225	189	205	152	91
Rumex obtusifolius (L.)	646	169	265	296	85	92
Urtica dioica (L.)	810	259	165	278	69	162
Cirsium arvense (L.) Scop.	763	348	254	221	155	146
Senecio jacobaea (L.)	852	194	155	154	52	125
Ranunculus repens (L.)	716	276	222	187	126	109
Summer						
Stellaria media (L.) Vill.	707	419	266	119	97	166
Taraxacum officinale F.H. Wigg	725	308	197	207	63	173
Rumex obtusifolius (L.)	514	291	361	228	61	119
Urtica dioica (L.)	686	390	293	182	41	182
Cirsium arvense (L.) Scop.	708	357	283	91	75	175
Senecio jacobaea (L.)	571	444	348	123	144	93
GS	739	521	332	145	17	82
SEM	27.8	12.5	21.2	16.6	13.5	6.2
Sig	***	***	***	***	***	***
GS *versus* forbs						
SEM	17.1	7.93	13.2	10.4	8.5	3.9
Sig	NS	***	***	*	***	***
Species						
SEM	11.4	9.80	16.2	12.8	10.4	4.8
Sig	***	***	**	***	**	***
Season						
SEM	6.6	5.67	9.4	7.4	6.0	2.8
Sig	***	***	***	***	*	***
Species × season						
SEM	16.2	13.85	22.9	18.1	14.7	6.7
Sig	***	***	**	*	***	***

Abbreviations: Spring, 30 April; Summer, 29 August; NDF, neutral detergent fibre assayed with a heat stable amylase and sodium sulphite, and expressed exclusive of residual ash (g kg^{-1} DM); ADFsep, acid detergent fibre analysed on a sample separate from that analysed during the NDF assay and expressed exclusive of residual ash (g kg^{-1} DM); CP, crude protein (g kg^{-1} DM); WSC, water soluble carbohydrate (g kg^{-1} DM); Sig, significance; ***, p<0.001; **, p<0.01; *, p<0.05; NS,

not significant.

Overall, the GS did not differ (p>0.05) in DMD from the mean of all forbs, but had higher (p<0.001) NDF and ADFsep concentrations, and lower (p<0.05) CP, WSC and ash concentrations (Table 1).

When analysed as a two-factor model, all herbage chemical composition variables were affected by species, season, and had species by season interactions (p<0.05; Table 1). The Tukey adjusted comparisons indicate that the effect of season on individual species was either in agreement with or did not contradict the overall effect of season for all herbage chemical composition variables, except for WSC concentration where *S. jacobaea* was lower (p<0.05) in spring than in summer. The DMD for *S. media*, *R. obtusifolius*, *U. dioica* and *S. jacobaea* were higher (p<0.01) in spring than in summer, and NDF concentration was lower (p<0.01) in spring for all species except *T. officinale* and *C. arvense*. Both *U. dioica* and *S. jacobaea* had lower (p<0.05) ADFsep concentrations in spring, and *U. dioica* and *C. arvense* had higher (p<0.05) CP concentrations in spring compared to in summer. The WSC concentrations for *T. officinale* and *C. arvense* were higher (p<0.05), while *S. jacobaea* was lower (p<0.05), in spring, and *S. media* and *T. officinale* had lower (p<0.01) ash concentration in spring *versus* summer.

In vitro rumen fermentation variables

When analysed as 14 independent treatments, all *in vitro* rumen fermentation variables were affected by treatment (p<0.001; Table 2). The lower (p<0.05) ml of CH_4 output per gram of feed DM incubated (CH_4i) for *R. obtusifolius* compared to GS in both spring and summer reflected its lower extent of *in vitro* rumen fermentation as evidenced by its lower (p<0.05) ml of TGP per gram of feed DM incubated (TGPi) in spring, and its lower (p<0.05) TGPi and tVFAi in summer. Furthermore, the likely presence of CT in *R. obtusifolius* (Waghorn and Jones 1989), which can reduce methanogenesis (Min et al. 2005, Tavendale et al. 2005, Puchala et al. 2005), may have also contributed to its lower CH_4i. Similar to *R. obtusifolius*, the lower (p<0.05) CH_4i for *U. dioica* and *S. jacobaea* compared to GS in summer reflects their lower extent of fermentation as evidenced by their lower (p<0.05) TGPi values, and also the lower (p<0.05) tVFAi for *U. dioica* (summer), compared with GS. The GS did not differ (p>0.05) in mmol of CH_4 output per mmol of tVFA output (CH_4/tVFA) from other treatments except for *R. repens* (spring) and *S. media* (summer), which were lower (p<0.001) and higher (p<0.05), respectively, than the GS. For mmol of CH_4 output per mmol of TGP (CH_4/TGP), the GS was lower (p<0.001) and higher (p<0.05), respectively, than *U. dioica* (spring) and *C. arvense* (spring), but did not differ (p>0.05) from any other treatments.

The less methanogenic direction of the fermentation of the GS compared to the forb treatments, as evidenced by its lower (p<0.001) acetic acid to propionic acid ratio (A:P) and non-glucogenic to glucogenic VFA ratio (NGGR; (acetic acid + [butyric acid × 2]) / propionic acid), most likely reflects the higher NDF concentration of the GS, and would be expected to lead to a reduction in CH_4 output relative to total VFA and TGP due to a reduction in H_2 production per unit of feed digested (Janssen 2010). Considering this, the finding that the GS did not differ (p>0.05) in CH_4/tVFA or CH_4/TGP from all but two of the 13 other treatments for both variables was surprising.

Overall, there were no differences (p>0.05) in CH_4 output between the GS and the mean of all forbs, whether expressed relative to feed DM incubated (CH_4i), TGP (CH_4/TGP), or tVFA (CH_4/tVFA). There was also no difference (p>0.05) in tVFAi between GS and the mean of all forbs. However, the GS had a higher (p<0.05) TGPi, and a lower (p<0.001) A:P and NGGR, than the mean of all forbs.

Table 2. *In vitro* rumen fermentation variables of forb species in spring and summer and grass silage (GS) after 24 hours of batch culture fermentation.

	CH_4i	CH_4/ tVFA	CH_4/ TGP	TGPi	tVFAi	A:P	NGGR
Treatment							
Spring							
Stellaria media (L.) Vill.	32.9	0.270	0.162	184	4.80	2.99[c]	3.17
Taraxacum officinale F.H. Wigg	30.9	0.230	0.142	196	5.29	2.98	3.16
Rumex obtusifolius (L.)	20.5	0.216	0.137	131	3.71	3.36	3.55
Urtica dioica (L.)	30.3	0.260	0.174	156	4.59	3.53	3.76
Cirsium arvense (L.) Scop.	22.1	0.191	0.127	156	5.14	3.07	3.26
Senecio jacobaea (L.)	29.6	0.226	0.145	184	4.54	3.11	3.33
Ranunculus repens (L.)	29.7	0.168	0.145	187	5.16	3.16	3.39
Summer							
Stellaria media (L.) Vill.	28.2	0.285	0.163	155	3.90	3.35	3.56
Taraxacum officinale F.H. Wigg	30.5	0.252	0.160	171	4.77	3.52	3.74
Rumex obtusifolius (L.)	20.3	0.263	0.161	108	3.04	3.60	3.82
Urtica dioica (L.)	22.1	0.241	0.155	125	3.62	3.52	3.76
Cirsium arvense (L.) Scop.	25.3	0.238	0.141	162	3.34	3.13	3.34
Senecio jacobaea (L.)	19.4	0.231	0.139	122	4.20	3.53	3.74
GS	27.6	0.236	0.147	169	4.60	2.17	2.33
SEM	0.8	0.0090	0.0033	4.9	0.18	0.09	0.084
Sig	***	***	***	***	***	***	***
GS *versus* forbs							
SEM	0.51	0.0058	0.0021	3.11	0.115	0.058	0.054
Sig	NS	NS	NS	*	NS	***	***
Species							
SEM	0.55	0.0067	0.0022	3.7	0.137	0.068	0.062
Sig	***	***	***	***	***	***	***
Season							
SEM	0.32	0.0039	0.0013	1.94	0.073	0.039	0.036
Sig	***	**	**	***	***	***	***
Species × season							
SEM	0.77	0.0095	0.0031	4.76	0.179	0.096	0.088
Sig	***	*	***	***	**	NS	*

Abbreviations: Spring, 30 April; Summer, 29 August; CH_4i, ml of methane output per gram of feed dry matter (DM) incubated; CH_4/tVFA, mmol of CH_4 output per mmol of tVFA output; CH_4/TGP, mmol of CH_4 output per mmol of TGP; TGPi, ml of total gas produced (TGP) per gram of feed DM incubated; tVFAi, mmol of total volatile fatty acid (tVFA) per gram of feed DM incubated; A:P, the acetic acid to propionic acid ratio; NGGR, the non-glucogenic to glucogenic volatile fatty acid ratio ((acetic acid + (butyric acid × 2)) / propionic acid); Sig, significance; ***, p<0.001; **, p<0.01; *, p<0.05; NS, not significant.

When analysed using a two-factor model, all *in vitro* rumen fermentation variables were affected by season and species, and there were season by species interactions for all *in vitro* rumen fermentation variables (p<0.05), except for the A:P ratio (p=0.059). When contrasted using Tukey adjusted comparisons, the effect of season on individual species was either in agreement with or did not contradict the overall effect of season for all *in vitro* rumen fermentation variables, except for CH_4/TGP where *U. dioica* was higher

(p<0.05) in spring *versus* summer. *S. media, U. dioica* and *S. jacobaea* had higher (p<0.05) CH_4i in spring than in summer, which reflects their generally more extensive *in vitro* rumen fermentations, namely their higher (p<0.05) TGPi, and the higher tVFAi for *U. dioica* and *S. jacobaea* (p<0.05), in spring compared to summer. This apparent relationship between the extent of *in vitro* rumen fermentation and CH_4 output expressed relative to feed DM incubated is in accord with Purcell et al. (2011b) and Navarro-Villa et al. (2011), and was most likely due to the higher DMD of these species in spring than in summer.

Despite the overall lower (p<0.01) CH_4/tVFA and CH_4/TGP in spring than in summer, there were no differences (p>0.05) in CH_4/tVFA between spring and summer for any individual species, and no differences (p>0.05) in CH_4/TGP between spring and summer for *S. media, C. arvense* or *S. jacobaea*. These outcomes are in partial agreement with the relative proportions of the main VFA produced, and thus with the direction of the *in vitro* rumen fermentation, namely the lack of differences (p>0.05) in NGGR between these species.

In conclusion, this study established that some forb species had lower *in vitro* rumen CH_4 output compared to GS, and some were lower in summer compared to in spring, when expressed per unit of feed DM incubated. However, these lower CH_4i outputs appear to have been achieved mainly through a reduction in the extent of *in vitro* rumen fermentation, as supported by the correspondingly lower TGPi and/or tVFAi values. Although *R. repens* (spring) and *C. arvense* (spring) had lower CH_4 output than GS when expressed relative to tVFA and TGP, respectively, these outcomes were not congruent with the more methanogenic *in vitro* rumen VFA profile of the GS *versus* the forbs, namely its higher A:P and NGGR. Thus, further evaluation is required to determine whether these forb species would reduce CH_4 output relative to animal product if assessed *in vivo*.

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References

ANKOM 2006a. *Acid detergent fiber in feeds filter bag technique*. ANKOM Technology, Macedon, NY.

ANKOM 2006b. *Neutral detergent fiber in feeds filter bag technique*. ANKOM Technology, Macedon, NY.

Association of Official Analytical Chemistry 1990. Official methods of analysis. *Association of Official Analytical Chemistry* 15: 3–4.

Fairbairn, C.B. & Thomas, B. 1959. The potential nutritive value of some weeds common to north-eastern England. *Journal of the British Grassland Society* 14: 36–46.

International Plant Names Index. Updated 3 November 2011. Cited 25 January 2012. Available on the internet: http://www.ipni.org/index.html

Janssen, P.H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160: 1–22.

McDougall, E.I. 1948. Studies on ruminant saliva i. The composition and output of sheep's saliva. *Biochemical Journal* 43: 99–109.

Min, B.R., Pinchak, W.E., Fulford, J.D. & Puchala, R. 2005. Wheat pasture bloat dynamics, *in vitro* ruminal gas production, and potential bloat mitigation with condensed tannins. *Journal of Animal Science* 83: 1322–1331.

Navarro-Villa, A., O'Brien, M., López, S., Boland, T. & O'Kiely, P. 2011. In vitro rumen methane output of red clover

and perennial ryegrass assayed using the gas production technique (GPT). Animal Feed Science and Technology 168: 152–164.

O' Riordan, E. & O'Kiely, P. 1996. Potential for beef production systems based on grass. *Irish Grassland and Animal Production Association Journal* 30: 185–217.

Pagán, S., Wolfe, R.M., Terrill, T.H. & Muir, J.P. 2009. Effect of drying method and assay methodology on detergent fibre analysis in plants containing condensed tannins. *Animal Feed Science a*nd Technology 154: 119–124.

Puchala, R., Min, B.R., Goetsch, A.L. & Sahlu, T. 2005. The effect of a condensed tannin-containing forage on methane emission by goats. Journal of Animal Science 83: 182–186.

Purcell, P.J., O'Brien, M., Boland, T.M., O'Donovan, M. & O'Kiely, P. 2011a. Impacts of herbage mass and sward allowance of perennial ryegrass sampled throughout the growing season on in vitro rumen methane production. Animal Feed Science and Technology 166–167: 405–411.

Purcell, P.J., O'Brien, M., Boland, T.M. & O'Kiely, P. 2011b. In vitro rumen methane output of perennial ryegrass samples prepared by freeze drying or thermal drying (40°C). Animal Feed Science and Technology 166–167: 175–182.

Purcell, P.J., O'Brien, M., Navarro-Villa, A., Boland, T.M., McEvoy, M., Grogan, D. & O'Kiely, P. 2012. *In vitro* rumen methane output of perennial ryegrass varieties and perennial grass species harvested throughout the growing season. *Grass and Forage Science* Article published online at DOI: 10.1111/j.1365-2494.2011.00845.x

Ranfft, K. (1973) Gas-chromatography determination of short-chain volatile fatty acids in the ruminal fluid. *Arch Tierernahr* 23: 343–352.

SAS, 2003. Statistical Analysis System. SAS Inc., Cary, NC.

Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T. & Sivakumaran, S. 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology* 123–124: 403 – 419.

Terrill, T.H., Wolfe, R.M. & Muir, J.P. 2010. Factors affecting ANKOM™ fiber analysis of forage and browse varying in condensed tannin concentration. *Journal of the Science of Food and Agriculture* 90: 2723–2726.

Thomas, T.A. 1977. An automated procedure for the determination of soluble carbohydrates in herbage. *Journal of the Science of Food and Agriculture* 28: 639–642.

Tilley, J.M.A. & Terry, R.A. 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Journal of the British Grassland Society* 18: 104–111.

Waghorn, G.C. & Jones, W.T. 1989. Bloat in cattle. Potential of dock (*Rumex obtusifolius*) as an antibloat agent for cattle. *New Zealand Journal of Agricultural Research* 32: 227–235.

Webb, D.A. 1977. *An Irish flora. 6th revised edition*. Dundalgan Press, Dundalk, Co. Louth. 277 p.