Methane production from *in vitro* incubation of kikuyu grass, lucerne and forages containing condensed tannins

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Abstract
A series of *in vitro* incubations with kikuyu grass (*Pennisetum clandestinum*), lucerne and six legumes containing condensed tannins (CT) were undertaken to evaluate this technique against *in vivo* trials in New Zealand, measuring methane emissions. Published trials have demonstrated a reduction in methane emissions associated with CT and in one instance from kikuyu. The incubations used fresh minced forage (equivalent to 0.5 g dry matter (DM)) and were carried out in 50 ml sealed bottles containing buffer and rumen inoculum. Gas was sampled through a septum to monitor volume and composition throughout the 24h incubation. Incubation for 24 h resulted in 2.4–6.6 % conversion of DM to methane, and suggested CT concentrations below about 8% of the DM can reduce methane production without inhibiting fermentation rate. Higher concentrations of CT (> 8%) were associated with a lower rate of digestion. In common with *in vivo* trials, CT concentration in forage DM was inversely related to methane (adjusted $R^2 = 0.49$; $P = 0.01$) and volatile fatty acid (adjusted $R^2=0.86$; $P<0.001$) production. Ash concentration in forage DM was proportionally related to methane (adjusted $R^2=0.56$; $P=0.005$).
Keywords: ash, condensed tannins, *in vitro* incubation, methane, rumen

Introduction
Methane losses from ruminant digestion account for about 33% of NZ's greenhouse gas (GHG) emissions, 6-7% of feed energy eaten, or 9-10% of metabolisable energy (ME) intake. This represents a loss of feed energy that could otherwise contribute to production.

When methane emissions are expressed as g CH_4/kg dry matter intake (DMI), animal trials have shown condensed tannins (CT) in *Lotus* species are able to reduce methane losses by about 15% in sheep (Waghorn *et al.* 2002) and cattle (Woodward *et al.* 2004). Larger reductions have been reported in sheep and cows grazing kikuyu (*Pennisetum clandestinum*) dominant pasture during autumn, but not during summer (Ulyatt *et al.* 2002). In general legumes result in lower emissions than grasses (Waghorn *et al.* 2002; O'Hara *et al.* 2003), although attempts to relate actual emissions with forage composition or digestibility (Johnson and Johnson, 1995) have not produced conclusive relationships.

Animal trials to measure methane are time consuming and expensive. Methane is most often measured using marker dilution with sulphur hexafluoride (SF_6), which is an inert gas released at a known rate from a 'permeation tube' placed in the rumen (Lassey *et al.* 2001). Of equal importance to methane emissions is an accurate measurement of feed intake. This is difficult in grazing animals, so many measurements of methane production are made with animals housed in doors to enable individual feeding (Waghorn *et al.* 2002). Other techniques include indirect calorimetry, which requires measurement of air flow and composition, usually by placing animals in a sealed respiration calorimeter. This technique is suitable for small numbers of animals, is expensive and also restricts animal behaviour.

The importance of methane emissions for global warming, lost feed energy and the considerable expense of animal trials, provide an incentive for alternative techniques to screen forage diets to identify those having low methanogenic potential. The data presented here have been derived from a fresh minced forage preparation that resembled chewed material (Barrell *et al.* 2000), and include measurements of gas production and composition, to evaluate this *in vitro* technique against *in vivo* trials in NZ measuring methane emissions. Ten forage samples were incubated *in vitro* to evaluate repeatability between replicates and to compare methane measurements with published *in vivo* data from sheep and cattle fed kikuyu (Ku), lucerne (*Medicago sativa*, Lu), *Lotus* spp. and sulla (*Hedysarum coronarium*, Hc).

Materials and methods
Lucerne (Lu), kikuyu grasses (Ku; two samples harvested in Northland during the *in vivo* trials of Ulyatt *et al.* (2002) and one sample from Grasslands Research Centre, Palmerston North), four *Lotus* species (*corniculatus*, Lc; *corniculatus rhizomatus*, Lr; *pedunculatus,* Lp*; tenuis*, Lt), Hc (two samples, harvested in different seasons) and erect dorycnium (*Dorycnium rectum*, Dr) were incubated *in vitro.* The forages were harvested between 1100 and 1400 h and placed on ice then transferred to a freezer at –18ºC for storage. All forages were separated into leaf and stem

fractions and only the leaf fractions were used in the incubations. The preparation and incubation of forages has been described by Barrell *et al.* (2000). The cysteine hydrochloride reducing agent used by Barrell *et al.* (2000) was not included in the incubations because of possible methanogenic inhibition. Fresh minced forage samples (0.5g DM equivalent) were incubated with buffered rumen inoculum (5 × replication) and gas production (methane and hydrogen) measured (by gas chromatography) over 24 h according to the method described by Tavendale *et al.* (2005). The rumen inoculum added to each bottle (3 ml) was a composite obtained from four fistulated sheep grazing ryegrass pasture. Lucerne was incubated as a reference forage during the three incubations. Data presented here have been generated from incubated leaf, which compared to stem (data not shown), contains higher concentrations of crude protein (CP), non-structural carbohydrates (NSC) and CT.

The incubations were carried out over three days as follows. A total of 65 incubation bottles were prepared: 5 from each forage sample, except for lucerne where three sets of 5 bottles were prepared and used as a reference forage on each of the three incubation days. All 5 bottles from each forage sample were incubated on the same day, and each bottle represents a replicate.

Measurements included gas production at approximately 2 h intervals over the first 12 h of incubation and at the conclusion at 24h. Head space gas was analysed to determine concentrations of methane and hydrogen by gas chromatography according to Tavendale *et al.* (2005). At the conclusion of incubations (24h), subsamples of rumen liquor were taken to determine concentrations of volatile fatty acids (VFA) according to the method of Attwood *et al.* (1998).

Forage composition was estimated by near infrared reflectance spectroscopy (Corson *et al.* 1999) and concentrations of CT by the butanol – HCl extraction method described by Terrill *et al.* (1992). The gross energy (GE) concentrations in the forages were calculated from feed composition, enabling methane production to be expressed in terms of forage GE content.

Data are presented to show the percentage of methane in the gases of fermentation. Gas production includes that from DM digestion and carbon dioxide (CO_2) released from the buffer as a consequence of declining pH, in response to VFA production. Carbon dioxide release from the buffer was measured in a separate set of bottles over a series of pH values (6.8 to 5.4) by addition of acetic acid to sealed bottles containing 12 ml of saturated buffer solution and relating CO_2 volumes to pH of the solution. The amounts of CO_2 from buffer were subtracted from measured gas volumes, on the basis of media pH at collection, enabling methane to be expressed in terms of gases from fermentation. The presence of significant concentrations of hydrogen may indicate the presence of oxygen, and the inhibition of methanogenesis. Hydrogen concentrations exceeded 2% in nine of the 65 bottles (2 Hc, 5 Lu, 1 Ku, 1 Lc) so these data were not included in the analyses.

The accumulated methane, percent methane, and the total volume of gas produced were analysed using Residual Maximum Likelihood analysis (REML) in GenStat v8.0 (2005). REML allows sources of variation to be included as follows: (a) the day of incubation; (b) the forages; and (c) the replicates. Forages and time and their interaction were fitted as fixed effects. An autoregressive model with an order of 1 (AR1) model was fitted to model the correlation between gas measurement times. In order to make the variance homogeneous as required for a REML analysis, the data was square-root transformed. The relationships between CT concentrations, VFA yield or pH and methane production were analysed using linear regression, as was the relationship between CT concentration and VFA yield. Multiple regression was used to determine which chemical variables were the best predictors of the amount of methane produced.

Results

The legume leaves contained over 21% CP in the DM, less than 20% NDF (neutral detergent fibre) (except for Lu) so the majority of the DM (Table 1) should have been highly digestible, unless affected by the CT concentration. The kikuyu had a lower CP and much higher fibre concentrations than the legumes. The concentration of CT in the DM of forages, other than Lu and Ku, ranged from 1.6 to 18.6% of the DM (Table 1). When concentrations of CT exceeded 3.3% of the DM, the majority was not bound with forage protein or fibre and was free to bind with components of the *in vitro* incubation.

Incubations of 0.5g DM yielded 80 – 108 ml of gas after 12 h of incubation and a further 4 – 12 ml between 13 and 24 h (Figure 1). However at 24 h, between 11 and 26 ml of the gas was derived from non-fermentation CO_2 (Table 2), released from the buffer as the pH declined over the incubation. The average methane concentration in gases from fermentation ranged from 7% (Lp, Lr) to 16% (Lu) at 24 h.

The rate of methane production over the 24h *in vitro* incubation varied amongst feed types, with very rapid initial rates of production from Lu and Hc, decreasing after 12h (Figure 1). Kikuyu had a slow initial rate of methane production, after which it increased rapidly to achieve a yield similar to Hc at 24h. Rates and total methane production were lowest for Lp, Lr and Dr, with

Table 1 Chemical composition of forages used for *in vitro* incubations. Data are g/100 g DM. Rhiz. refers to rhizomatous strain of *L. corniculatus.*

Species	Condensed tannin Total	Unbound	CP	Lipid	Ash	NDF	NSC	GE MJ/kg DM
Lotus								
pedunculatus	11.2	8.6	21.5	4.4	6.9	16.1	39.9	18.4
cornic. rhiz.*	9.6	7.5	21.1	4.5	6.6	17.0	41.2	18.4
corniculatus	3.3	1.7	28.2	4.5	7.4	15.1	41.5	18.7
tenuis	1.6	0.4	22.3	4.6	8	19.2	44.3	18.3
Sulla	6.6	4.8	25.2	3.3	11.7	15.6	37.6	17.5
D. rectum	18.6	15.2	21.3	3.7	6.9	11.8	37.4	18.2
Kikuyu	0	0	20.7	3.6	11.5	44.7	19.5	17.3
Lucerne	0	0	24.3	3.3	10.3	31.2	30.9	17.7

Figure 1 Cumulative gas (A) and methane (B) production (ml) from 0.5g DM over 24 h from fresh forages incubated *in vitro.* Abbreviations see text.

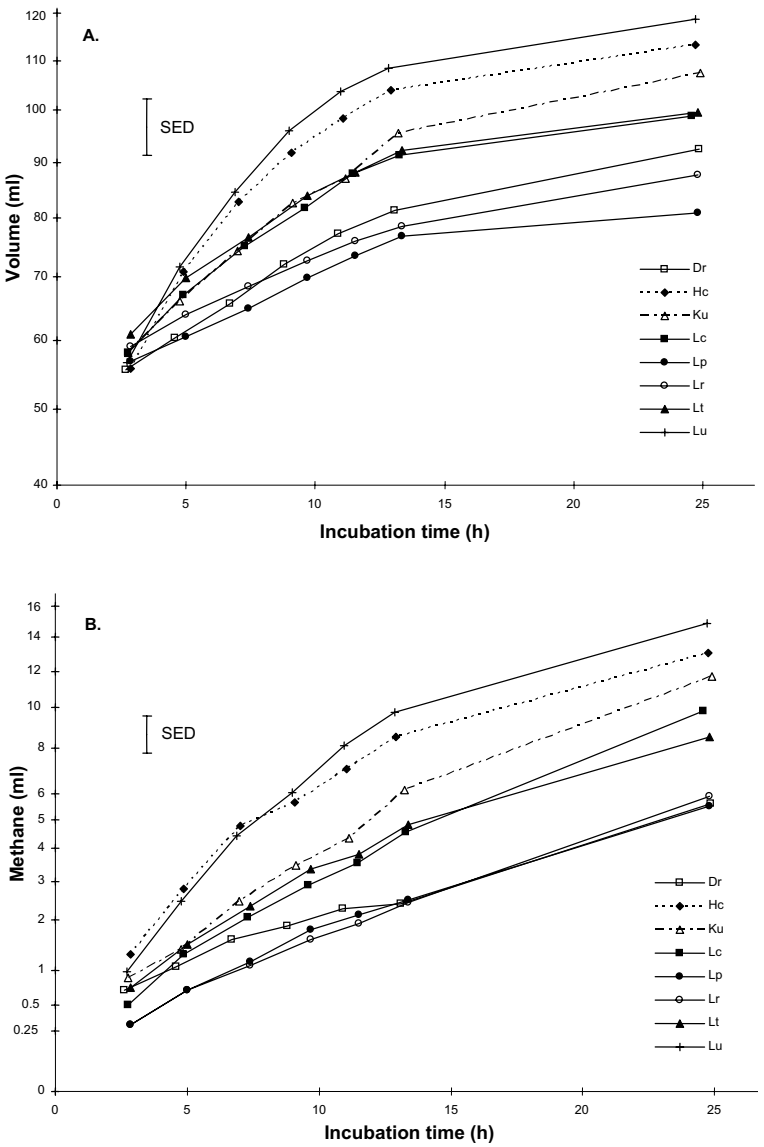
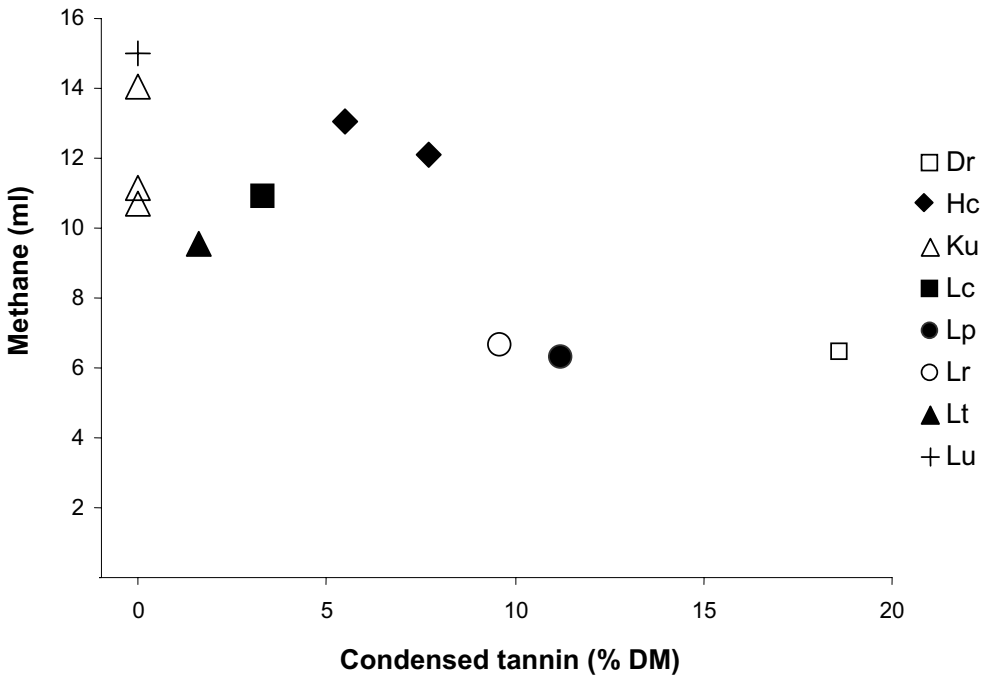


Table 2 Total methane produced from *in vitro* incubation of 0.5g forage DM after 3, 13 and 24 h of incubation, with pH and carbon dioxide emissions at 24 h. The approximate least significant difference (LSD) for the total methane data is calculated using the average standard error of difference and 2.89 degrees of freedom (as estimated by REML). As the total methane data required square-root transforming, the LSD relates to this. Back-transformed values on the original scale are also given in parentheses.

Species	Cumulative methane (ml)			pH	24 h		
	3 h	13 h	24 h		ml CO_2 from buffer (ml)	forage	% CH_4 in ferment gas
Lotus							
pedunculatus	0.6 (*0.3*)	1.6 (*2.5*)	2.4 (*5.5*)	6.4	11.0	69.0	8.0
cornic. rhiz.	0.5 (*0.3*)	1.6 (*2.4*)	2.4 (*5.9*)	6.4	10.3	76.3	7.7
corniculatus	0.7 (*0.5*)	2.1 (*4.6*)	3.1 (*9.8*)	6.1	21.7	72.6	13.5
tenuis	0.9 (*0.7*)	2.2 (*4.8*)	2.9 (*8.5*)	6.3	13.6	82.8	10.3
Sulla	1.1 (*1.3*)	2.9 (*8.5*)	3.6 (*13.0*)	5.9	25.0	75.3	17.5
D. rectum	0.8 (*0.7*)	1.5 (*2.4*)	2.4 (*5.6*)	6.2	19.7	67.6	8.5
Kikuyu	0.9 (*0.9*)	2.5 (*6.2*)	3.4 (*11.7*)	6.1	18.4	79.5	14.9
Lucerne	1.0 (*1.0*)	3.1 (*9.7*)	3.9 (*14.9*)	5.8	26.3	77.2	19.3
Approximate LSD	1.0	1.0	1.0				

Figure 2 Methane yield (ml/24 h/0.5g DM) and CT concentrations in the DM of fresh forages incubated *in vitro*. Abbreviations see text.



total yields less than half that for Lu, Ku and Hc (Table 2, Figure 1). Hourly methane yields (ml/0.5g DM fermented) from forages with less than 7% CT in the DM (Lu, Ku, Lt, Lc and Hc) averaged 0.31 (0-3 h), 0.57 (3-13 h) and declined to 0.41 from 13 – 24 h. Comparable values for forages containing higher CT concentrations were 0.16, 0.19 and 0.28 for the respective times.

Although there appeared to be a general inverse relationship between CT concentration and methane production at 24 h (Figure 2) the high values for Hc suggest other factors could have a significant impact on methanogenesis.

Volatile fatty acid concentrations were measured at 24h from incubations of *Lotus* species and Lu. The total yield (Table 3) ranged from 122.4 (Lp) to 347.6 (Lu) mg/g DM. There was a positive correlation between methane and VFA yields for the five forages, suggesting low methane production was accompanied by a low rate of digestion. The molar proportions of VFA were similar for all five forages after 24 h of incubation, with 0.67 acetate, 0.19 propionate, 0.10 butyrate and 0.04

Table 3 Methane yields during *in vitro* incubations of 0.5g forage DM and products of fermentation after 24 h.

Species	ml CH_4/h			24 h			*in vivo*
	0-3 h	3-13 h	13-24 h	CH_4 (mg)	CH_4 (% of GE)	net VFA (mg)	CH_4 (g/kg DMI)
Lotus							
pedunculatus	0.11	0.21	0.26	3.9	2.4	62	11.5[a]
cornic. rhiz.	0.10	0.20	0.30	4.2	2.5	82	*
corniculatus	0.18	0.39	0.45	7	4.2	169	17.9[b]
tenuis	0.26	0.40	0.31	6.1	3.7	138	*
Sulla	0.47	0.71	0.39	9.4	6.0	*	17.5-19.9[a,c]
D. rectum	0.27	0.16	0.27	4.1	2.5	*	*
Kikuyu	0.32	0.52	0.48	8.4	5.4	*	20.7-23.4[d]
Lucerne	0.34	0.85	0.44	10.6	6.6	174	20.6[a]

Cornic rhiz, Rhizomatous strain of *L. corniculatus*. * no data available.
[a] Waghorn *et al.* (2002), [b] Woodward *et al.* 2004, [c] Woodward *et al.* (2002), [d] Ulyatt *et al.* (2002).

minor VFA (isobutyrate, valerate and isovalerate). The proportion of propionate was higher when Lp was incubated (0.24) compared to other forages (0.18).

Discussion

Gas measurement from *in vitro* incubation was straightforward, repeatable and has demonstrated significant differences between treatments that correspond to differences in ash and CT concentrations. Measurement of methane and hydrogen emissions, expressed in terms of substrate DM or GE content provided realistic values after 24 h of incubation (Table 3), when compared to *in vivo* digestion. For example, emissions (mg/g DM/ 24h) from Lu (21.2), Hc (18.8), and Ku (16.8) correspond with emissions from sheep (g/kg DMI) fed Lu (20.6) and Hc (17.7) reported by Waghorn *et al.* (2002). Woodward *et al.* (2002) reported methane emissions from cows fed Hc to be 19.9 g/kg DMI and Ulyatt *et al.* (2002) suggested 'normal' emissions from sheep and cows grazing kikuyu to be 20.7 and 23.4 g/kg DM, respectively.

In vitro methane emissions from Lc and Lp (14 and 7.8 mg/g DM) were lower than values from animal trials. Woodward *et al.* (2004) reported 19.9 g CH_4/kg DMI from cows fed *L. corniculatus*, whilst Waghorn *et al.* (2002) measured 11.5g CH_4/kg DMI from sheep fed *L. pedunculatus*.

The yield of VFA at 24 h ranged from about 34% of forage DM for Lu and Lc incubations to only 12% of DM for Lp. The VFA yield from Lu and Lc was similar to the 36% of DM reported by Barrell *et al.* (2000) from a 24 h incubation of minced *L. corniculatus* and corresponds to *in vivo* rumen digestion. For example, if *in vivo* DM digestibility for Lu or Lc is about 70% (Waghorn *et al.* 2002) and approximately 65% of DM digestion takes place in the rumen, with about 65% of digested DM appearing as VFA (remainder to CO_2, ammonia etc), then the VFA yield will be about 30% of the DMI. The high *in vitro* VFA yields from Lu and Lc contrast with the very low VFA synthesis from Lp. When *L. pedunculatus* was fed as a sole diet to sheep it decreased the rate of rumen digestion, reduced VFA concentrations by 30% and increased rumen pool size relative to the same diet fed with polyethylene glycol to remove effects of CT (Waghorn *et al.* 1994). This suggests the CT in Lp does lower VFA production relative to diets containing less CT, but not to the extent indicated by the *in vitro* data here.

The lower methane production from forages containing over 9% CT in the DM was probably a consequence of reduced fermentation. There was an inverse relationship between CT concentration and VFA production from *Lotus* spp. and Lu (adjusted $R^2 = 0.88$, $P = 0.017$) and a positive relationship between methane and incubation pH at 24 h (adjusted $R^2 = 0.86$, $P < 0.001$). Relationships between methane production and individual forage constituents were not convincing, in part because concentrations of protein, lipid, NDF and NSC (except for Ku) were within a narrow range. Correlation were not statistically significant (adjusted $R^2 = 0.0, 0.11, 0.16$ and 0.15, respectively). Methane correlations (adjusted R^2) with total CT, unbound CT and ash were 0.49, $P = 0.01$; 0.47, $P = 0.012$; and 0.56, $P = 0.005$, respectively. Multiple regression suggested that total or unbound CT and ash provided and improved prediction of methane emissions (adjusted $R^2 = 0.64$). There is no obvious explanation for the strong coloration between methane and ash, other than possible elemental requirements for methanogenesis or other rumen microbes involved, such as fibre-degrading microbes.

When *in vitro* methane data were correlated with *in vivo* methane production (g/kg DMI) by sheep fed *L. pedunculatus* (11.5), Lu (20.6), Hc (17.5) (Waghorn *et al.* 2002), Ku (20.7) (Ulyatt *et al.* 2002) and adjusted emissions from cattle fed *L. corniculatus* (17.9) (Woodward *et al.* 2004), Hc (17.5) (Woodward *et al.* 2002) and Ku (21.0) (Ulyatt *et al.* 2002) there was a positive correlation between 24 h *in vitro* and *in vivo*

emissions (adjusted $R^2 = 0.62$; $P = 0.035$). The animal methane emission (Y) was $0.600X + 8.33$ where 'X' is the *in vitro* methane production at 24 h and all values were given the units g CH_4/kg DMI. The adjustments made to cow data were a 10% reduction in CH_4/kg DMI, based on inventory estimates (NZ Climate Change Office 2003).

In vitro incubations (Ungerfeld *et al.* 2005) can amplify effects of methanogenic inhibitors (e.g. oils) relative to *in vivo* measurements (Machmüller *et al.* 2000), presumably because *in vivo* fermentation has potential to ameliorate effects of toxic substances and the closed incubations used here may have affected a slower digestion of forages with high concentrations of CT. However the 13% reduction in methane emissions per DMI attributed to CT from cows fed *L. corniculatus* with 2.6% CT in the DM and an 11 % increase in milk solids production (Woodward *et al.* 2004) could not have been associated with reduced forage digestion. Any reduction in rate or extent of digestion, associated with a methane inhibitor will be counterproductive, irrespective of methane emissions.

In summary, the *in vitro* technique is able to give values that are comparable to *in vivo* measurements in NZ. The observed reduction in *in vitro* methane emissions from forages containing CT can be attributed to effects of CT, or possibly ash content of the forage. The future focus of this research is to evaluate a broad selection of forages grazed by ruminants in NZ pastures for methane emissions using the *in vitro* technique described here.

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