A comparison of faecal sample collection times for estimating faecal output and total tract digestibility using inert markers in sheep offered three ryegrass cultivars at two allowances

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Abstract

The objective of this study was to compare different faecal sampling regimes (collected in the morning (am) or the afternoon (pm), or the weighted mean of both times) for determining the concentrations of faecal markers (the natural odd-chain *n*-alkanes (C27 – C35) contained in feed, the dosed (once daily in the morning) synthetic *n*-alkanes (C32 and C36) and titanium dioxide (TiO_2)) and their accuracy for estimating dry matter digestibility (DMD) and faecal dry matter (DM) output from wethers (n = 30) fed cut ryegrasses. The concordance correlation coefficient (CCC) with measured faecal DM output was strong (CCC = 0.8) for faecal DM output predicted from the mean concentration of C32 in morning and afternoon faeces (am+pm), moderate (CCC = 0.6-0.7) when predicted from TiO_2 am and am+pm, and weak when predicted from C36. The DMD predicted by C27 and C33 at either collection time, and by C29 and C31 for samples collected in the pm, were similar to measured DMD. The estimated DMD based on faecal C35 had a negligible CCC with measured DMD (CCC<0.1), while other natural *n*-alkanes had a low to moderate CCC (0.3-0.6) with measured DMD, especially for the samples collected pm and am+pm. For applications with grazing animals, where marker dosing and faecal sampling is often restricted to once daily in the morning for practical reasons, TiO_2 was the most accurate marker to estimate faecal DM output and C31 or C33 were the most accurate and precise markers to estimate DMD.

Keywords: indigestible marker; predicted dry matter intake; grazing; total faecal collection

Introduction

The feeding value of perennial ryegrass (*Lolium perenne* L.) is determined mainly by dry matter intake (DMI) and dry matter digestibility (DMD), both of which are difficult to estimate under grazing conditions, the dominant livestock farming system in New Zealand (Waghorn & Clark 2004). Feeding value of forage is an important parameter not only for animal performance, but also for determining environmental impact of grazing ruminants. Dry matter intake is the main driver of CH_4 emissions, nitrogen (N) intake is the main driver of N excretion and consequently nitrous oxide (N_2O) emissions and DMD is the main determinant of faecal DM excretion which also contributes to CH_4 emissions (Fick 2016).

The DMI of grazing animals can be estimated in the field from faecal DM output and DMD of the diet eaten, which both can be estimated using external (i.e. dosed) or internal (i.e. a component in the diet) inert markers (Lippke 2002). Faecal DM output is often estimated using an external marker such as titanium dioxide (TiO_2) or synthetic even-chain *n*-alkanes. Dry matter digestibility can be estimated using internal makers such as natural odd-chain *n*-alkanes (C27 to C35) (Dove and Mayes 1991; Glindemann et al. 2009; Lippke 2002). External markers require oral dosing whereas internal markers require the collection representative feed samples (to know the dietary concentration of marker). Both markers require the collection of faecal samples. It is generally recommended to include multiple dosing's of the marker and faecal sample collection per day, over several days (Dove and Mayes 1991; Glindemann et al., 2009). This is, however, not very practical and disruptive to the animal, which likely impairs animal performance during the dosing and sampling period.

Ideally, estimates using faecal markers should be calibrated against measured values. This can only be done within indoor experiments because direct measurement requires known DMI and total collection of faeces. The objective of this study was to compare the concentrations of *n*-alkanes and titanium dioxide (TiO_2) in faeces collected in the morning (am) or the afternoon (pm) from sheep fed a diploid high-sugar ryegrass (HSG), a conventional diploid ryegrass (CRG) and a tetraploid ryegrass (TRG) cultivar, each offered at 1.1 and 1.8 multiples of maintenance energy requirements (MEm). The faecal concentrations of marker for the different sample collection times were subsequently compared for their accuracy and precision in estimating DMD and faecal dry matter (DM) output.

Materials and methods

The marker excretion measurements presented here were conducted as part of research to determine N and methane emissions from sheep fed different ryegrass cultivars (Jonker et al. 2014; Jonker et al. 2017). The marker release rate data (presented herein) was collected along with actual DMI and DMD during the indoor trial and this information subsequently used in grazing trials described by Cosgrove et al. (2015) during SF_6 tracer technique measurements (A. Jonker and G.P. Cosgrove, AgResearch, Palmerston North, unpublished). The animal experiments reported were reviewed and approved by the Grasslands Animal Ethics Committee (Palmerston North, NZ; approval #13004) in accordance with the AgResearch Code of Ethical Conduct.

Animals, feeding, experimental procedures and sample collections

A total faecal collection trial was performed at

Grasslands Research Centre (AgResearch Ltd., Palmerston North, NZ) in spring 2013 (23 September to 1 October) as described by Jonker et al. (2014; 2017). Thirty romney wether sheep (mean live-weight 33.5±1.7 kg), born around October 2012, were fed one of three different perennial ryegrass cultivars; Alto (CRG), AberMagic (HSG) or Base (TRG) perennial ryegrass (each containing AR1 endophyte) at either 1.1 or 1.8 × MEm according to Australian Feed Standards (CSIRO 2007) (resulting in six dietary treatments; n=5 sheep per treatment) at 15:30 h and 08:30 h the next morning. Treatment allocation, feeding and acclimatization to the pastures were detailed by Jonker et al. (2017) and pasture management by Cosgrove et al. (2015). Oral dosing of a gelatine capsule once daily around 08:15 h started five days before the start of faecal collection to reach an equilibrium concentration of the markers in the faecal excretion, as suggested by Glindeman et al. (2009). The capsules contained 2.5 g TiO_2, 43 mg C32 alkane and 58 mg C36 alkane. Only sheep fed at 1.8 × MEm received capsules with C32 due to a shortage of the C32 alkane. Dosing of the markers was continued till the end of the total faecal collections described below.

Total faecal collection was performed in individual metabolism crates as detailed by Jonker et al. (2014) with sheep being moved into metabolism crates three days before the five-day collection period. Harnesses were fitted for attachment of faecal collection bags which were replaced before every feeding (pm and am) as described above. A 10% aliquot by weight of the faeces collected twice daily was pooled per sheep separately for am and pm, and stored in the freezer at -20°C. Forage subsamples were collected daily during the five-day total collection period. Triplicate subsamples were dried at 105°C for 48 h to determine the dry matter (DM) content (samples discarded after drying) and a fourth subsample was stored in the freezer at -20°C. Refusals of feed offered during the preceding 24 h were collected in the afternoon, before feeding, and dried at 65°C for 48 h to determine DM. Grass was harvested each at about midday, and weighed into separate aliquots for feeding that afternoon (35% of the daily total allowance) or placed in a chiller overnight for feeding following morning (65% of the daily total allowance). Feed offered and faeces were freeze-dried after the trial and ground through a 1 mm screen.

Feed and faecal analysis

Feed and faecal samples were analysed for *n*-alkanes according to the method of Dove & Mayes (1991), and for TiO_2 concentration using a colorimetric technique as described by Short et al. (1996). Recovery of all markers in faeces was calculated as: [marker output in faeces (mg/d) / marker intake or dose rate (mg/d)] × 100. Faecal DM output estimated by dosed markers (TiO_2, C32 and C36) was calculated as: marker dose rate (mg/d) / marker concentration in faeces (mg/kg DM). Dry matter digestibility estimated by natural *n*-alkanes (C27 – C35) was calculated as: 1 – [*n*-alkane concentration in faeces (mg/kg DM) / *n*-alkane concentration in feed (mg/kg DM)]. Dry matter intake (kg/d) was then calculated from the combination of a faecal DM output marker and a DMD marker as: faecal DM output (kg/d) / [100 – DMD (%)].

Statistical analysis

Animal data were analysed with cultivar, intake level and their interaction as fixed effects and for the overall data set with cultivar, intake level, season and all interactions as fixed effects. Individual sheep were the experimental unit (*n*=5 per treatment).

All statistical analysis was performed using GenStat (18th edition; VSN international, Hemel Hempstead, UK) (Payne et al. 2009). Faecal marker concentrations were analysed with cultivar, feeding level, faecal sampling time and their two and three way interactions as fixed effects in ANOVA (unbalanced due to one sheep being excluded). Comparison of marker method estimates with measured values was performed using ANOVA with method (marker and sampling time combination) as fixed effect and using Lin`s concordance correlation coefficient (CCC), which consists of Pearson correlation coefficient (R; precision) × a bias correction factor called Cb (accuracy) (Payne et al. 2009). The correlations were judged as negligible (0.0 – 0.3), low (0.3 – 0.5), moderate (0.5 – 0.7) and strong (0.7 – 1.0). Multi-treatment comparison was performed using the Fisher`s unprotected LSD at $P<0.05$. Significance was declared at $P<0.05$ and trends at $P<0.10$.

Results

Concentration of natural *n*-alkanes in faeces differed among cultivars ($P<0.001$; Table 1). C27 and C29 were lower in CRG than HSG and TRG; C31, C33 and C35 were highest in HSG; C31 was lowest in CRG; and C33 and C35 were lowest in TRG. Faecal concentration of C27 and C29 were similar for sheep fed either of two feed offer levels, while faecal concentration of C33 was greater ($P=0.02$) and C33 and C35 tended to be greater ($P<0.10$) in sheep at the low feed offer. Faecal natural *n*-alkane concentrations were similar in samples collected in the morning or the afternoon. For dosed markers, faecal C32 concentration was similar among sheep fed either of three cultivars, but was greater ($P<0.001$) in faecal samples collected in the morning than in the afternoon. Faecal C36 and TiO_2 concentrations were greater ($P<0.001$) in sheep fed TRG than sheep fed CRG or HSG, greater ($P<0.001$) in sheep fed at the low compared with the high feeding level and greater [tendency for C36; $P=0.06$)] in faecal samples collected in the morning than in samples collected in the afternoon.

Recovery of dosed markers in faeces was not influenced by cultivar or feeding level, averaging (±CV) 91±9, 106±6 and 64±9 % for TiO_2, C32 and C36, respectively. Recovery of natural *n*-alkanes C31, C33 and C35 in faeces were lower ($P<0.02$; except for C27, $P=0.10$) for sheep fed TRG than sheep fed CRG and HSG (C29 was similar for TRG and HSG). For C27, recovery did not differ among cultivars ($P=0.10$) and for C29 recovery was similar for TRG and

Table 1 Measured faecal DM output (FO DM), forage and faecal *n*-alkane (C27-C36) and titanium oxide (TiO2) concentrations from sheep fed three different cultivars of perennial ryegrass (a high-sugar (HSG), a conventional (CRG) and a tetraploid (TRG) ryegrass cultivar), each offered at 1.1 and 1.8 multiples of maintenance energy (MEm) and with faeces collected around 8:30 h (am) and 15:30 h (pm).

	FO DM*	C27	C29	C31	C33	C35	C32	C36*†	TiO_2*†
	(g)	(mg/kg DM)							
Feed									
CRG	-	16.3	70.5	144.8	128.5	15.0	7.4	0	-
HSG	-	18.8	81.7	161.3	153.9	19.8	9.5	0	-
TRG	-	19.3	87.5	159.1	123.3	12.5	8.0	0.3	-
Faeces									
CRG	85	85[a]	382[a]	752[a]	617[b]	66[b]	226	215[a]	13.3[a]
HSG	86	105[b]	459[b]	926[c]	799[c]	91[c]	222	220[a]	12.8[a]
TRG	75	97[b]	458[b]	832[b]	571[a]	48[a]	233	266[b]	15.71[b]
SED	3.3	4.5	12.6	15.9	11.5	1.5	16.3	10.7	0.75
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.849	<0.001	<0.001
1.1 × MEm	66	96	434	846	669	69	-[1]	284	17.2
1.8 × MEm	96	94	431	822	647	66	23	188	11.0
SED	2.7	3.7	10.3	13.0	9.4	1.2	-	8.7	0.62
P value	<0.001	0.650	0.780	0.070	0.024	0.061	-	<0.001	<0.001
am	107	98	438	838	656	67	257	243	15.9
pm	57	93	427	828	659	68	193	226	12.1
SED	2.7	3.7	10.3	12.9	9.4	1.2	13.3	8.7	0.61
P value	<0.001	0.189	0.301	0.435	0.796	0.186	<0.001	0.063	<0.001

[a-c]Means within a column with different superscripts are significantly different (P<0.05). SED, standard error of the difference. *Level × time P<0.001; †Cultivar × level P<0.01

[1]C32 alkanes were dosed only to sheep fed at 1.8 × MEm.

Table 2 Dry matter intake, faecal DM output and apparent total tract DM digestibility in sheep fed three different cultivars of perennial ryegrass (high-sugar (HSG), a conventional (CRG) and a tetraploid (TRG) ryegrass cultivar), each offered at 1.1 and 1.8 multiples of maintenance energy (MEm).

	Cultivar					Feeding level			
	CRG	HSG	TRG	SED	P value	1.1 × MEm	1.8 × MEm	SED	P value
Dry matter intake (kg/d)	0.81[a]	0.91[b]	0.76[a]	0.03	<0.001	0.69	0.95	0.024	<0.001
Faecal DM output (g/d)	169.6[b]	171.5[b]	149.6[a]	5.95	<0.001	132.5	192	4.857	<0.001
DM digestibility (% of DM)	79.1[a]	81.1[b]	80.5[b]	0.62	0.011	80.6	79.8	0.5	0.102

[a-b]Means within a column with different superscripts are significantly different (P<0.05). SED, standard error of the difference.

HSG. Recovery was not affected by feeding level, except for C29 which had a greater (P=0.05) recovery at the high compared to that at the low feeding level (data not shown).

Daily DMI and measured faecal DM output were greater (P<0.001) in sheep fed HSG and CRC compared with sheep fed TRG, and were lower (P<0.001) at the low compared to those at the high feeding level (Table 2). Faecal DM output estimated by C32 and TiO_2 in faecal samples collected am and am+pm were similar to measured faecal DM output, while DM output was overestimated (P<0.05) by C36 (Table 3). Faecal DM output predicted from C32 am+pm had a strong CCC (0.8) and predicted from TiO_2 am and am+pm moderate CCC (0.6-0.7) with measured faecal DM output, while the CCC with C36 was low.

Total tract apparent DMD measured by total collection was greater (P=0.02) in sheep fed HSG and TRG than in sheep fed CRG (81.1, 80.5 and 79.1 %, respectively) and numerically greater (P=0.11) at low vs high feed offer (80.6 vs. 79.8 %; Table 2). The DMD predicted by C27 and C33 at any collection time and by C29 and C31 for samples collected in the pm were similar compared to measured DMD. In comparison, C35 under-predicted DMD and C29 and C31 from the am sample and average of am+pm over predicted DMD (Table 4). The estimated DMD based on faecal C35 had a negligible CCC with measured DMD (CCC<0.1), while other natural *n*-alkanes had a low to moderate CCC (0.3-0.6) with measured DMD, especially for the samples collected pm and am+pm. Dry matter digestibility predicted by faecal C31 had numerically the strongest CCC with measured DMD within any of the faecal collection times.

Discussion

Faecal DM output and DMD can be estimated using external and/or internal indigestible markers. An ideal marker should be indigestible and be completely recovered

Table 3 The overall mean daily faecal DM output from sheep fed three cultivars of perennial ryegrass and concordance correlation coefficient (CCC), Pearson correlation (R) and bias correction factor (Cb) with faecal DM output predicted using concentrations of C32, C36 and TiO_2 in faeces collected around 8:30 h (am) and 15:30 h (pm), and the mean of the am and pm concentrations (am+pm).

Marker	Sample time	Mean	CCC	R	Cb
Actual faecal DM output (1.1 and 1.8 × MEm)[1]		163.2[a]			
Predicted faecal DM output					
alkane C36	am+pm	257.3[bc]	0.29	0.93	0.31
	am	247.9[b]	0.28	0.89	0.31
	pm	291.2[c]	0.23	0.89	0.26
TiO_2	am+pm	180.1[a]	0.82	0.92	0.90
	am	163.2[a]	0.79	0.79	0.99
	pm	249.8[b]	0.30	0.83	0.36
P-value		<0.001			
SED		18.63			
Actual faecal DM output (1.8 × MEm)[1]					
Predicted faecal DM output		192.1[b]			
alkane C32[1]	am+pm	189.3[b]	0.80	0.85	0.94
	am	166.6[a]	0.28	0.68	0.41
	pm	231.5[c]	0.27	0.77	0.35
P-value		<0.001			
SED		9.36			

[a-d]Means within a column with different superscripts are significantly different (P<0.05). SED, standard error of the difference for the method comparison

[1]C36 and TiO_2 were dosed to sheep fed at both 1.1 and 1.8 × MEm, C32 alkanes were dosed only to sheep fed at 1.8 × MEm.

Table 4 The overall mean daily dry matter digestibility (%; DMD) from sheep fed three perennial ryegrass cultivars and concordance correlation coefficient (CCC), Pearson correlation (R) and bias correction factor (Cb) with DMD predicted from natural *n*-alkane concentrations (C27 – C35) in faeces collected at 8:30 h (am) and 15:30 h (pm), and the mean of am and pm (am+pm).

	Sample time	Mean	CCC	R	Cb
In vivo DMD		80.2[bcd]			
Predicted DMD					
alkane C27	am+pm	80.8[cde]	0.33	0.38	0.87
	am	81.0[cde]	0.17	0.23	0.74
	pm	80.1[bc]	0.47	0.52	0.91
alkane C29	am+pm	81.5[e]	0.34	0.47	0.73
	am	81.6[e]	0.24	0.33	0.73
	pm	81.2[de]	0.49	0.58	0.84
alkane C31	am+pm	81.3[e]	0.50	0.69	0.72
	am	81.4[e]	0.44	0.63	0.69
	pm	81.1[de]	0.58	0.70	0.83
alkane C33	am+pm	79.3[b]	0.47	0.55	0.84
	am	79.4[b]	0.40	0.47	0.85
	pm	79.3[b]	0.51	0.58	0.87
alkane C35	am+pm	76.3[a]	0.05	0.18	0.30
	am	76.0[a]	0.03	0.11	0.29
	pm	76.6[a]	0.09	0.24	0.35
P-value		<0.001			
SED		0.51			

[a-e]Means in a column with different superscripts are significantly different (P<0.05). SED, standard error of the difference for the method comparison.

in faeces, and should be maintained at a steady-state concentration in faeces throughout the faecal collection period. Furthermore, when used to predict faecal DM output or DMD it should result in a precise and accurate relationship with measured faecal DM output or DMD (Lippke 2002). For grazing trials, it is not possible to measure faecal DM output or DMD and concentrations of inert markers in faecal spot-samples are required to estimate these values. To obtain these samples usually requires that animals are temporarily removed from pasture to holding yards. This is labour-demanding and can be disruptive to animals. For these reasons it is generally good practice to conduct faecal spot-sampling at the same time as other trial-specific or routine tasks. For example, in grazing trials where methane emissions are measured using the SF_6 technique, this would usually be at the time of day when the sample collection canisters are changed, which is normally in the morning (Pinares-Patiño et al. 2016). In dairy trials, faecal samples would most easily be collected when animals are routinely yarded for milking (Cosgrove et al., 2017).

From the dosed markers, faecal DM output estimated by C32 am+pm and TiO_2 am+pm and TiO_2 am had a moderate to strong CCC, R and Cb and statistically similar absolute values compared with measured faecal DM output, and therefore seem the most suitable markers to use for grazing sheep. Overall, recovery of dosed TiO_2 was marginally incomplete, averaging 91%, which is within the range reported previously (Glindemann et al. 2009; Titgemeyer et al. 2001). The TiO_2 concentration was, however, higher

in the am than in the pm faecal sample, as was also found by Glindeman et al. (2009). The TiO_2 was dosed once daily in the am, and faecal DM output estimated with TiO_2 am was similar to measured DM output, suggesting that TiO_2 am could be used to estimate DM output accurately and precisely.

From the natural *n*-alkanes, DMD estimated with C31 and C33 from any faecal spot-samplings (am, pm and am+pm), and also from C27 pm and C29 pm, had a medium CCC, R and Cb with actual DMD. This differs from Dove and Mayes (1991) who indicated in their review that C35 might be the most useful internal marker to estimate DMD because of its high recovery. The CCC, R and Cb of DMD estimated by natural *n*-alkanes were in general greatest in the pm samples, lowest in the am sample and intermediate in the am+pm sample, which was opposite to findings for TiO_2 described above. Interestingly, dosed markers had in general a higher R than Cb, while this was the reverse for the natural *n*-alkanes suggesting that dosed markers were relatively more precise and natural *n*-alkanes relatively more accurate. In contrast to dosed markers, the concentrations of natural *n*-alkanes were similar in faecal samples collected am and pm. This would be expected since dosed markers were pulse-dosed once daily while natural *n*-alkanes in herbage were ingested at a comparatively steady rate, with every bite of pasture.

Faecal DM output estimated by dosed marker in combination with *in vitro* DMD of feed can be used to estimate DMI (Lippke 2002; Pinares-Patiño et al. 2016). It is, however, well documented that actual DMD decreases with increasing intake level (CSIRO 2007) and this was also observed in the current study. This interaction between DMI and DMD cannot be identified by an *in vitro* assay. The concentrations of C31, C33 and C35 were lower (or tended to be) in faeces from sheep fed at 1.8 compared with 1.1 × MEm, consistent with the expected effect of intake level. Furthermore, there was no interaction between predicted DMD determined by the different markers and actual DMD with feeding level, suggesting that these three *n*-alkanes could distinguish the effects of the different feeding levels on DMD.

Predicted DMD was greater for sheep fed HSG compared with sheep fed CRG when estimated using C31 and C33 in faecal samples collected at either sampling time. This ranking of cultivars was similar for predicted and actual DMD. For TRG, however, DMD predicted by C31 and C33 was lower than for HSG and similar to CRG (data not shown), a ranking which was different to actual DMD.

For faecal spot-samples collected once daily in the morning as a practical method for field trials, TiO_2 and C31 or C33 were the most promising markers to estimate faecal DM output and DMD, respectively, to use for calculating DMI. Indeed, for DMI the strongest CCC (>0.73 with R >0.75 and Cb >0.94; data not shown) was found when DMI was predicted from faecal DM output estimated using TiO_2 am and DMD estimated using either C31 or C33. The number observations and feeding conditions in the current study are, however, narrow and results presented here need further validation over a wider range of DMI levels and pasture qualities. Furthermore, other markers, and other faecal sampling schedules may give different results to those presented here. The ability to accurately and precisely estimate DMI by grazing ruminants is important for many reasons, for example efforts improve the efficiency of production or reduce the intensity of greenhouse gas emissions, yet remains challenging. Research to improve the reliability of techniques in this area has stalled.

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