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PROJECT TITLE:	Diagnostic Tests For Greenhouse Gas Production
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Final Report (30th June 2008)

1. Introduction

1.1 General approach

The starting point for this project was the difficulty of existing techniques for measuring emissions of methane and urinary nitrogen (nitrous oxide precursor) from animals. The methodological difficulties are particular problems when working with free-grazing animals, which are the norm in New Zealand.

There are a number of broad approaches that are being used to address greenhouse gas issues, including plant breeding, animal breeding and animal nutrition. However, in each case, the rate of progress is restricted by our inability to make accurate measurements using individual animals.

This project explored the potential for diagnostic measurements using readily accessible samples to provide indices of both methane production and N-use efficiency (NUE) for individual cows and herds. Our objective was to complete feasibility studies by June 2008 using existing samples.

1.2 Difficulties of current techniques for measuring methane output

The 'gold standard' method for measuring methane output from animals is the respiration chamber. These have been used for over 100 years, often directed more at understanding energy metabolism. They are being reintroduced because of the recent focus on methane production, and newer versions are more animal friendly and lower cost (Pinares-Patino et al., 2008). Despite their strengths and importance for work to understand mechanisms, they are not useful for studies with grazing animals.

The interaction between grazing animals and pasture will be important for progress in addressing methane emissions in New Zealand and so alternative techniques, notably the sulphur hexafluoride (SF_6) approach have been developed. Pinares-Patino and Clark (2008) reviewed the use of this technique to measure methane production from individual animals. They concluded that there is a much higher level of between-animal variation with this technique in comparison with chamber studies. It seems likely that this reflects problems with the technique and issues such as alternative routes for emission of both marker gas and methane (Grainger et al., 2007) and effects of the release rate of marker gas (Vlaming et al., 2007). The variability means that the technique is appropriate for large treatment groups, but not for

characterising individual animals – this is a major limitation to genetic approaches and a significant cost factor in designing other experiments.

1.3 Difficulties with current techniques for measuring urinary N output

The traditional approach to measuring the efficiency of utilisation of nitrogen is to conduct a full balance study – measuring N intake and excretion in faeces, urine and milk. This type of study is also receiving new attention because of the importance of urinary N as a precursor of a nitrous oxide.

The technique was reviewed by Spanghero and Kowlaski (1997) and there are a number of problems in addition to the fact the technique is both laborious and not suited for in situ grazing. Spanghero and Kowlaski (1997) found that the technique provided unrealistically high estimates of the amount of N retained within dairy cows – 39 g/day which equates to over 1 kg of lean tissue. This overestimation may result from a number of problems – notably incomplete collection of faeces and urine or volatilisation of N during the preparation of faeces and urine for analysis.

1.4 The diagnostics approach

The problems of techniques to measure outputs of methane and urinary N are similar to problems encountered in assessing rumen function and we may learn some lessons from the application of a diagnostics approach to that area.

The use of diagnostic tests to describe rumen function was described by Dewhurst et al. (2000). The literature concerning rumen microbial protein synthesis and microbial efficiency has been reviewed on many occasions, but yields few reliable predictors (Agricultural Research Council, 1984; Firkins, 1996). This is both because of the complexity of the rumen system and the technical difficulties of estimating microbial protein synthesis, particularly *in vivo*. There are two distinct problems, namely estimating how much material leaves the rumen and then assessing what proportion of that material is microbial protein. These estimates have involved the use of fistulated cows which has tended to add to these problems, through low replication. Titgemeyer *et al.* (1997) reviewed the literature and showed that on average treatment groups of 12 animals are required in order to identify treatment differences of 10% in rumen microbial efficiency. This combination of a laborious technique and low precision is similar to the current situation with estimating methane production.

New momentum in rumen studies was provided by the development of less-invasive approaches to estimating microbial protein synthesis. This latter approach has the attraction of offering in-built technology transfer through the development of diagnostic tests, based on samples of milk or urine. Some of these techniques offer a description of rumen function that is less rigorously quantitative (in terms of microbial protein synthesis), but more usefully qualitative.

The rumen diagnostics approach is based on the measurement of compounds that derive from rumen microbes and which appear in urine or milk. Early efforts focussed on urinary allantoin, which is derived from the purine bases within nucleic acids, and so builds on experience of using RNA or purine bases as a marker for microbial content in duodenal digesta. More recently, we have tried to identify markers in milk – since it is easier to collect milk in a quantitative way. Vlaeminck et al. (2006) reviewed progress in using odd- and branched-chain fatty acids in milk as rumen microbial markers. Again, the principle is that these compounds are derived from rumen micro-organisms but present in samples which are easily accessible.

1.5 Diagnostic test for N-use efficiency (urinary N production)

This potential diagnostic test uses analysis of the ^{15}N content of milk, urine and faeces to predict N partitioning without the need for quantitative total collections. The approach relies on the phenomenon of N isotopic discrimination. This involves differential use of the stable isotopes of N (^{14}N and ^{15}N) by the different pathways of N metabolism that result in either product (milk protein) or waste (urinary N). This discrimination results from the different masses of the isotopes and has been demonstrated in a range of other biological systems (e.g. Sick et al., 1997; Hobson et al., 1993). It is anticipated that it could be used to identify differences in N-use efficiency in cows. We have some limited, but encouraging, data from earlier studies conducted in Portugal (Cabrita et al., 2003).

1.6 Diagnostic test for Archaea (methane production)

For methane production we investigated possible markers for rumen methanogenic archaea. Archaeal lipids are comprised of isoprenoid chains linked together through highly resistant ether linkages: mainly glycerol diethers (archaeols) and glycerol tetraethers (caldarchaeols). These are highly resistant to degradation and have been used as markers of archaea in archaeological samples (van Dongen et al., 2006). These resistant fatty acids might remain undigested and appear in faeces, providing a marker for archaea.

1.7 Cost and ease of operation of diagnostic tests

In both cases, the ultimate objective is a test that can be based on spot samples, or a series of spot samples, taken from a commercial farm – for example alongside traditional herd testing protocols. Analytical costs are modest- around $10-20 for isotope ratio mass spectrometry for ^{15}N and $50-100 for the HPLC-MS analysis of ether lipids. Labour costs will be greatly reduced in comparison with costs of conducting full nutrition/balance studies. The precision of these approaches is not yet know and so it is not possible to comment on what will be the trade of in terms of cost and precision associated with the approaches. The precision of the SF_6 technique is already low, so the target for a new diagnostic approach is not particularly high.

2. Objective 1: N-use efficiency test

2.1 Description of the study used to provide samples

In order to test the concept of using ^{15}N measurements on samples of milk or urine from dairy cows to assess N-use efficiency (and urinary N output), we utilised samples from a full N balance study that had been conducted previously. The study was conducted in the United Kingdom during 2005 and samples had been stored frozen in the interim. Freeze-dried samples of feed, urine, milk and urine were transferred to Lincoln University for ^{15}N analysis using a PDZ Europa GSL 20/20 Isotope Ratio Mass Spectrometer. Urine was reconstituted in water prior to ^{15}N analysis, whilst all other samples were weighed as dried material. All samples were analysed in duplicate and results for mixed forage diets were checked against values for individual forage components. The objective was to weigh up enough material to provide 2 to 4 mg of N. One set of milk samples (period 1) was unavailable.

The basal study was chosen because of the availability of results and stored samples from a very wide range of N intakes, resulting from the use of diverse forages of widely differing N contents. The experimental details were as follows.

Dietary treatments

Cows were given a flat-rate of concentrates (4 kg/day of standard dairy concentrates (21% crude protein), unless stated otherwise (one treatment was give a low-protein concentrate mix based on 2 kg of rolled barley, 2 kg of molassed sugar beet pulp and milking cow mineral/vitamin mix).

There were 9 dietary treatments:

1. Perennial ryegrass silage (cv. AberDart) ad libitum. (PRG).
2. Timothy silage (cv. Promesse) ad libitum. (TIM).
3. Tall Fescue silage (cv. Excella) ad libitum. (TF).
4. Red clover silage ad libitum. (RC).
5. Red clover silage/whole-crop oat silage mixture (40% red clover on a DM basis) ad libitum. (40RCWCO).
6. Red clover silage/whole-crop oat silage mixture (40% red clover on a DM basis)- feed intake restricted to the same level as treatment (1). (40RCWCOr).
7. Red clover/whole-crop oat silage mixture (40% red clover on a DM basis) ad libitum- with 4 kg/day of the low protein concentrate mixture instead of the 21% crude protein concentrate. (40RCWCOlp).
8. Red clover silage/whole-crop oat silage mixture (25% red clover on a DM basis) ad libitum. (25RCWCO).

9. Red clover/maize silage mixture (40% red clover on a DM basis) ad libitum. (40RCMZ).

Cows and design

Nine multiparous Holstein-Friesian cows, which were around peak lactation (6-8 weeks) at the start were used in a 3-period changeover design experiment (with 3 week periods):

Measurements

Nitrogen balance measurements were conducted according to standard procedures in the final week of each period. This involved measurement and sampling over 6 consecutive days for: feed intake, faecal output, milk production and urine output. Samples were preserved and analysed for N content so that a full N balance could be calculated.

The Nitrogen balance results had already been analysed using an analysis of variance (Table 1) and showed a wide range in both Nitrogen intake and the partitioning of Nitrogen between faeces, urine and milk. It was also evident, however, that the technique is difficult to accomplish with potential for quite large errors (in agreement with Spanghero and Kowalski, 1997). This reinforces the need for a simpler and more robust technique. It also points to the need for care in interpreting relationships with ^{15}N results because the Nitrogen balance technique is not the 'gold standard' technique that might have been envisaged.

TABLE 1 Nitrogen partitioning results from the basal study used to provide samples for the ^{15}N study conducted in this project

	PRG	TIM	TF	RC	40RCMZ	40RCWCO	40RCWCOlp	40RCWCOr	25RCWCO	s.e.d.	Sig
N intake (g/d)	604.5	547.1	494.4	626.0	511.7	472.6	378.6	463.9	358.8	36.35	***
Milk N (g/d)	114.5	103.0	90.5	121.4	127.8	125.2	100.2	114.6	115.9	6.10	***
Urine N (g/d)	302.4	259.3	256.4	275.1	180.8	148.3	116.9	161.5	122.0	18.81	***
Faecal N (g/d)	157.1	157.5	142.5	177.2	160.9	158.2	113.9	116.2	119.6	13.41	**
Milk N/Feed N (g/g)	0.188	0.185	0.186	0.196	0.254	0.267	0.271	0.248	0.328	0.0210	***

PRG, Perennial ryegrass; TIM, Timothy; TF, Tall fescue; RC. red clover; red clover/maize 40:60%; red clover/whole crop oats 40:60%; red clover/whole crop oats 40:60%, restricted intake; red clover/whole crop oats 40:60% plus low protein concentrate; red clover/ whole crop oats 25:75%.

2.2 Nitrogen-15 results and their relationship with N partitioning

Preliminary analysis of the nitrogen-15 results showed that there had been isotopic discrimination with faeces and milk enriched in ^{15}N relative to the original diet, whilst urine was depleted (Figures 1 and 2).

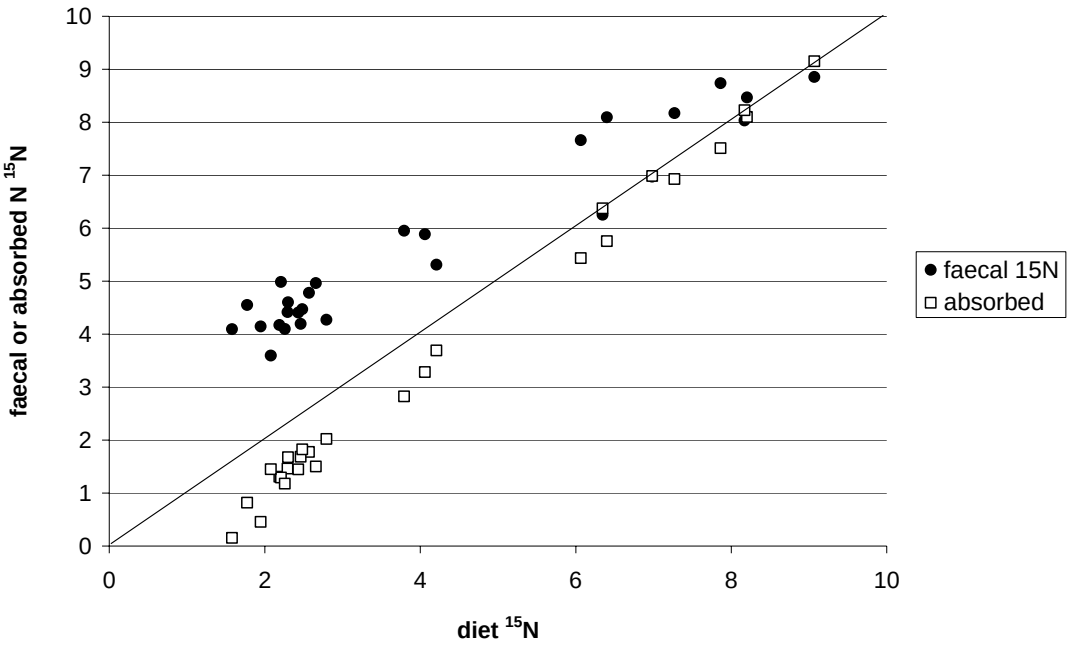

Figure 1 ^{15}N enrichment of faecal N (and calculated enrichment of absorbed N) in relation to dietary ^{15}N enrichment.

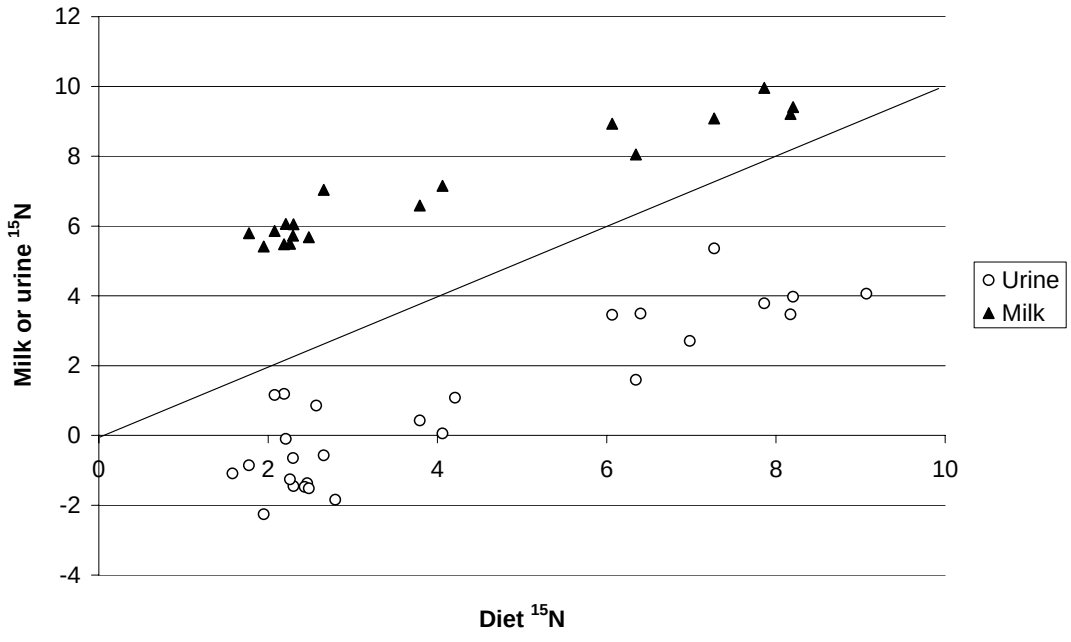

Figure 2 ^{15}N enrichment of milk and urine in relation to dietary ^{15}N enrichment.

The primary purpose of this work was to investigate whether there was any relationship between the N partitioning results (Table 1) and ^{15}N measurements made on the samples of faeces, urine and milk. Initial inspection of relationships suggested that there was a high level of between-cow variation and so further analysis used treatment means taken from analysis of variance (REML) that used a model including effects of cow and period.

There were highly significant effects of diet on the ^{15}N content of urine, milk and faeces (Table 2). These differences followed the same pattern as differences in dietary ^{15}N and so the real interest is in whether there were diet effects on the partitioning of ^{15}N between faeces, urine and milk. The effects of diets on these differences are also shown in Table 2.

Table 2 Effects of diets on ^{15}N levels in diet, faeces, urine and milk, as well as the differences in ^{15}N levels between these fractions.

	PRG	TIM	TF	RC	40RC MZ	40RC WCO	40RCWC Olp	40RCW COr	25RC WCO	s.e.d.	Sig
Diet ^{15}N	6.593	8.381	7.148	2.274	4.029	2.366	2.586	2.290	1.815	0.348	***
Faecal ^{15}N	7.946	8.761	7.042	4.271	5.746	4.196	4.531	4.615	4.271	0.369	***
Absorbed ^{15}N	6.047	8.248	7.193	1.530	3.264	1.410	1.807	1.485	0.453	0.419	***
Urine ^{15}N	4.104	3.872	2.650	1.000	0.498	-1.171	-1.260	-0.864	-1.427	0.532	***
Milk ^{15}N	9.090	9.759	8.765	5.323	6.796	5.535	6.569	6.053	5.592	0.378	**
Diet – Urine ^{15}N	2.485	4.422	4.588	1.191	3.494	3.419	3.948	3.355	3.178	0.488	***
Faecal – Diet ^{15}N	1.507	0.151	-0.030	1.692	1.653	2.058	1.851	2.402	2.613	0.211	***
Milk – Faeces ^{15}N	1.144	0.584	1.535	1.906	1.271	1.165	1.473	1.437	1.388	0.0368	***
Milk – Urine ^{15}N	4.392	6.144	6.102	4.542	6.316	6.973	7.357	6.803	6.956	0.4887	*

Using these treatment means and corresponding treatment means from the N balance study identified significant relationships between the extent of isotopic discrimination (the difference between milk ^{15}N and urine ^{15}N) and both N-use efficiency (milk N as a percentage of N intake; Figure 3) and with N intake (Figure 4). The fact that the strongest relationship was with N intake leads us to question the amount of experimental error associated with the components of the N balance measurements. As stated earlier, the N balance technique is susceptible to a number of problems in obtaining precise estimates (Spanghero and Kowalski, 1997).

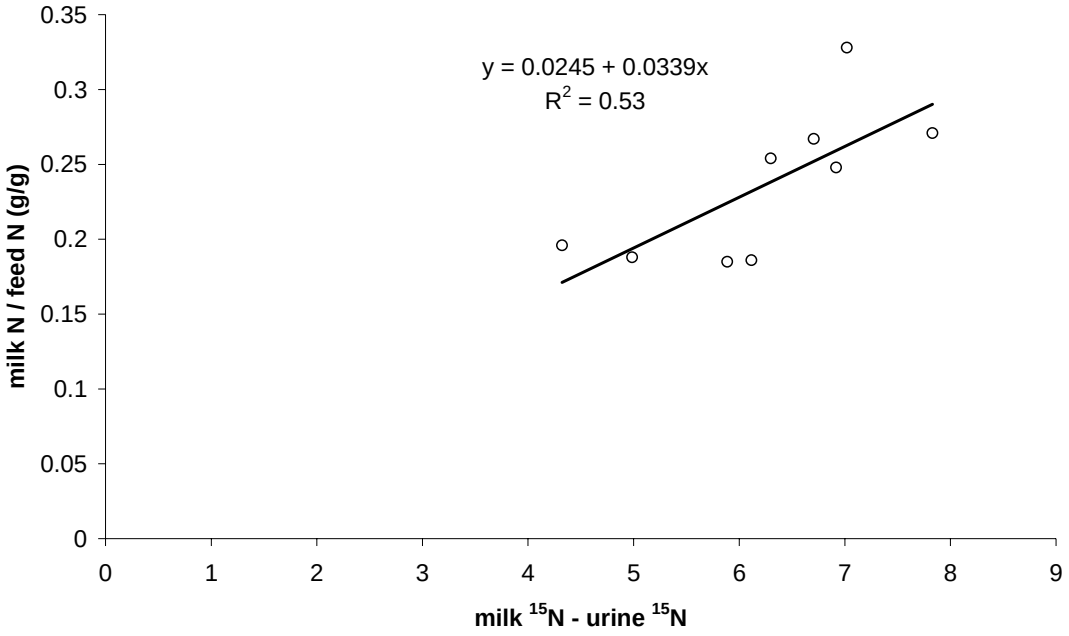

Figure 3 Relationship between the extent of isotopic disproportionation (milk ^{15}N – urine ^{15}N) and N-use efficiency (efficiency of conversion of feed N into milk N). Treatment means.

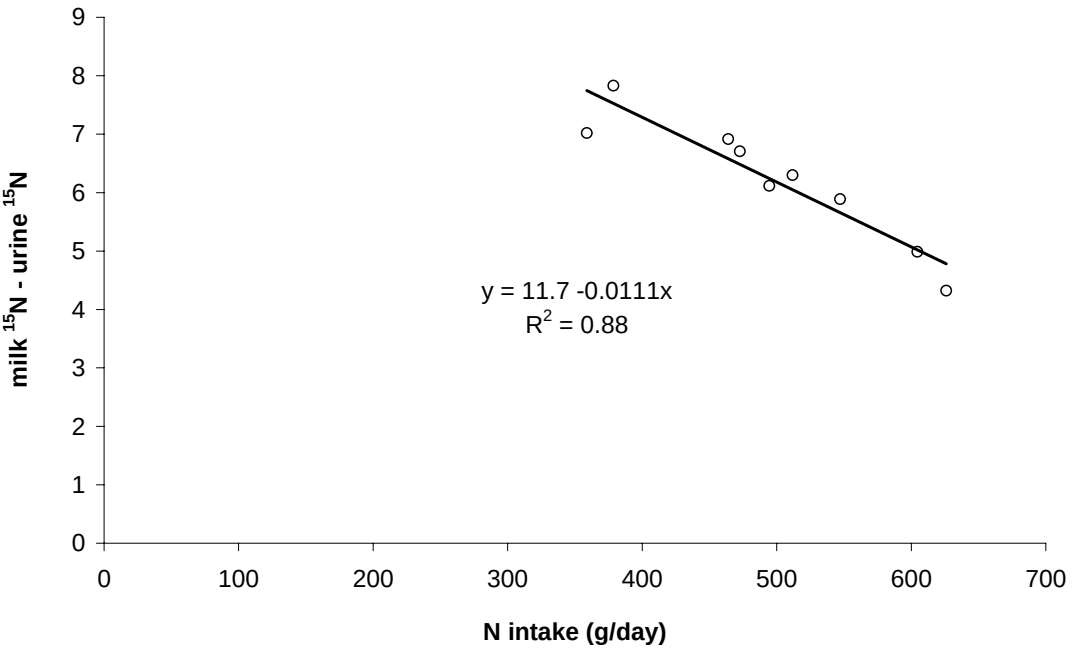

Figure 4 Relationship between N intake and the extent of isotopic disproportionation (milk ^{15}N – urine ^{15}N). Treatment means.

2.3 Conclusions

There were very strong effects of diet on levels of ^{15}N in faeces, urine and milk; these are mainly related to diet differences in ^{15}N.

There were also highly significant effects of diet on the degree of isotopic disproportionation – that is the relative proportions of ^{15}N and ^{14}N going into milk and urine. It seems likely that these differences are related to differences in N-use efficiency within the animal. However, the analysis has cast some doubt on the accuracy of the underlying N balance measurements. Whilst it seems likely that the ^{15}N approach offers a more precise approach than the traditional N balance approach, this conclusion awaits further investigation.

Interpretation of these results was made more complicated by the wide range of diet types included and further modelling analysis may be helpful in unravelling some of the effects. Studies with varying levels of the same diet (e.g. pasture) or graded levels of the same protein supplement will also help develop the interpretation.

3. Objective 2: Archaea diagnostic test.

3.1 Method development

A number of methods had already been published for ether lipids, mainly in archaeological samples. This provided a starting point and also an indication of the stability of these compounds over long time periods. Two approaches were identified. In each case the first steps are extraction of lipids from the sample and fractionation of the lipids to isolate the phospholipid fraction which contains the archaeal ether lipids. These could then either be measured as intact ether lipids using high-peformance liquid chromatography mass spectrometry (HPLC-MS) or broken down to the constituent hydrocarbon chains that could be measured by gas chromatography. We opted to use the former approach to avoid the safety issues associated with the use of strong acid digestions for the latter. Our early work was hampered by the detector that we had available, but later in the project we borrowed an APCI detector that proved much better. The method eventually adopted was based on that of Fang et al. (1996, 2006) and is outlined in the next section.

3.2 Method for extraction and separation of archaeal ether lipids

Extraction:

1. Weigh 0.8 g ground faeces (DM) into a 15-ml test tube.
2. Add 2.5ml dichloromethane (DCM), 5ml methanol and 2.0ml of potassium phosphate buffer pH=7.4. (1:2:0.8)
3. Vortex for 1min, wrapped with foil, stand in the dark for 24 hours at 4ºC.

4. Add 2.5ml DCM and 2.5ml buffer (Potassium phosphate, 0.05 M; pH=7.4) to the tubes, vortex for 1 min, make the final ratio of extract to 1:1:0.9 (DCM:MeOH:buffer).
5. Stand overnight at 4ºC.
6. Discard the upper aqueous phase.
7. The lower organic phase is decanted through a No.4 cellulose filter paper (Labserve, LBS0004) with vacuum.
8. The solid of residue is washed three times with 1ml DCM.
9. The total extract is dried under a nitrogen stream.
10. The dry residue is re-dissolved in 1 ml hexane:DCM mixture (70:30).

Fractionation:

1. SPE cartridge (STRATA 8B-S009-FBJ, NH2, Phenomenex) is conditioned by flushing with 3ml methanol, 3 ml chloroform, and 1ml hexane twice.
2. Load 1ml extract onto the column.
3. Elute 4ml DCM slowly; discard.
4. Elute 4ml acetone; collect.
5. Elute 4ml methanol; collect.
6. Combine acetone and methanol fractions.
7. Analyze by LCMS.

Note: Vacuum is applied at every step while using the SPE cartridge.

LCMS:

Interface: APCI
Interface temperature: 400ºC
CDL temperature: 250ºC
Heat Block temperature: 200ºC
Nebulising gas: 2.5L/min

Event1: Positive Scan
Scan Speed: 2000
Detector Voltage: 1.7kv
Event time: 0.50 second
Interface Voltage: 4.5kv
CDL Voltage: -50V
Q-array Voltage: Use scan tuning
Start M/Z: 500
End M/Z: 1350

Event2: Positive SIM (Selected Ion Mode)
Event time: 0.2 second
Selected positive ions (m/z): 165,181,359,598,609,625,636,787,807,908,949,1245,12921293,1296,1298, 1300,1302

Solvent A: Chloroform

Solvent B: Methanol
Flow rate: 0.2ml/min

LC program:

Time (mins)	B%
0.00	0
1.00	0
10.00	50
15.00	50
16.00	0
25.00	0

This method was applied to samples from the experiment described under Section 2.1 in order to obtain a preliminary indication about variability in levels of these marker lipids found in faeces. Samples were bulked according to dietary treatment.

3.3 Results and Discussion

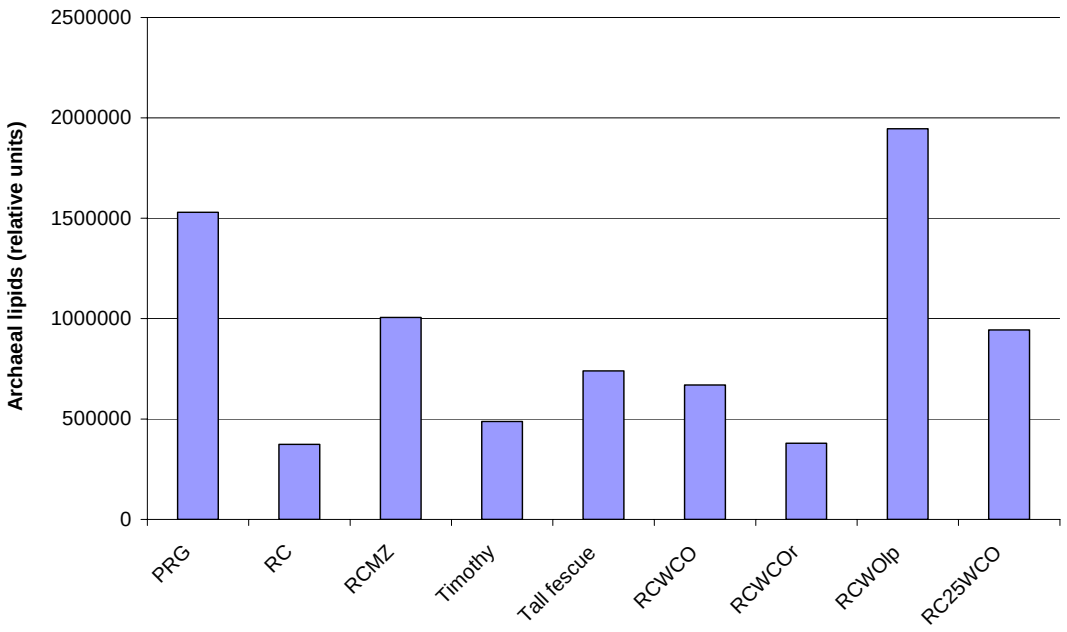

Figure 5 Concentration of archaeal lipid (relative units) in a range of faecal samples

These results are for single bulked samples and so cannot be subject to statistical analysis. However, they do suggest a high degree of variation in the concentrations of archaeal lipids in faeces from cows consuming a wide range of diets. Values were also considerably lower than in a faecal sample taken from a cow consuming high-quality pasture at Lincoln University Dairy Farm (values not directly comparable owing to method changes, but approximately 3.5 million on the same relative scale). It is not clear why the ryegrass silage, grazed pasture and red clover/whole-crop oat silage with low protein concentrate should all lead to higher levels of archaeal lipids – a tentative

suggestion is that it may relate to levels and types of carbohydrate available in the rumen (the low protein concentrate involved molassed sugar beet pulp).

We have not yet been able to source a standard for the method – we have tracked down one potential standard in the US and are trying to get hold of some at this time.

4. Conclusions and Proposals for Further Work

4.1 Conclusions

In both of these research areas, we have demonstrated variation between samples and potential for application of the analytical methods as diagnostic tests of N utilisation and methane output respectively. The ^{15}N approach in particular showed a high level of ability to discriminate between dietary treatments that had been imposed. However, both areas need further work – firstly to refine methods and further model results, and secondly to apply the approaches to new sets of samples that are more relevant to New Zealand feeding situations.

At the moment, it is not clear whether variability in the relationship between ^{15}N measurements and N-use efficiency from N balance measurements is the result of problems with one or other method, or both. Although we used treatment means from a REML model including period and cow effects to obtain better means, it is entirely possible that the experimental errors are mainly in the N balance technique. In other words, it is still possible that the ^{15}N approach could be applied to assessing N-use efficiency in individual animals.

4.2 Proposals for further work

Further validation of the ^{15}N approach needs to utilise a test that avoids the technical difficulties associated with the N balance procedure – for example using measurements of urea kinetics or controlled feeding studies with graded levels of urea addition. Once proven, the next step would be to investigate potential of the approach to identify between-cow variation in N-use efficiency with cows of varying potential for milk protein synthesis given a single feed (pasture).

The next steps in the archaeal lipid work are: firstly, to use the standard to refine the method and provide quantitation, and secondly to apply the method to faeces from experiments in which significant differences in methane output have been recorded.

Towards the end of the project (June 2008) we came across a reference for a gas chromatography method for the intact ether lipids (Fritze et al., 1999). This is a good option to investigate as this lower cost equipment would be available in more laboratories than HPLC-APCI-MS.

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