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Using ^{15}N Dilution Method to Correct for Microbial Contamination When Assessing *in situ* Protein Degradability of Fresh Ryegrass

Research Article

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ABSTRACT

This experiment was done to investigate microbial contamination and *in situ* disappearance rates of dry matter (DM), N and ^{15}N of fresh labeled ryegrass. Perennial ryegrass (*Lolium perenne*) were labeled with ^{15}N during growth in a glasshouse, harvested at 4th leaves stage and were incubated up to 34 h *in situ* in the rumen of 3 individually housed sheep. The animals were fed 800 g/d chopped alfalfa and had free access to drinking water. Six bags were placed in the rumen of each sheep simultaneously and removed after 0, 3, 7, 12, 21 and 33 h after incubation. The results were fitted to a model describing the degradation of DM and total N with time. It was found that residues from the washed zero time bags had lower ^{15}N enrichments (7.7% *vs.* 8.3% enriched) than the original fresh samples. Under-estimation of effective degradability (ED) of protein in fresh forages by about 4% would have potential consequences for predictions of ruminally fermentable and escape protein and thus for dietary protein feeding management. However, because the correction assumes contaminating microbial N is unlabeled, but microbes attached to labeled ryegrass would become labeled to some extent, the true error and effective degradability may still be underestimated. Studies with two markers would help us to better understand the errors associated with the *in situ* technique.

KEY WORDS degradation, *in situ*, labeled nitrogen, microbial contamination, ryegrass.

INTRODUCTION

Current systems of protein evaluation in ruminants involve accurate estimates of both ruminal protein degradability and intestinal digestibility of the undegraded protein fraction of feedstuffs (Arroyo *et al.* 2009). *In situ* techniques mimic *in vivo* conditions and are widely adopted in most modern protein feeding systems (Jarrige, 1987). Estimation of ruminal degradability *in situ* may be affected by several factors (Colombini and Broderick, 2008). The effects of the method of preparation of fresh clover were compared by Cohen and Doyle (2001). They concluded that no sample preparation method will be ideal and that the preferred method for *in situ* studies will vary for fresh forages compared with conserved forages and concentrates. The isotope of nitrogen, ^{15}N has been employed many times in plant physiology studies (Broadbent and Carlton 1980; Danso *et al.* 1988; Harris and Hesterman, 1990), but it has only infrequently been used to study ruminal metabolism of forages (Varvikko and Lindberg, 1985; Van Bruchem *et al.* 1997; Kamoun *et al.* 2014). The principle of the *in situ* method is that microbes should enter the bag and degrade the feed in a similar way to that if the feed was consumed directly by the animal. Mathers and Aitchison (1981) observed that residues remaining in the bags after incubation in the rumen were contaminated with microbial matter. Lower N content in forages and longer incubation period causes higher microbial contamination (González *et al.*

2007). A correction for this contamination should be considered when assessing N degradability values. The use of ^{15}N appears to be a precise and consistent method of identifying microbial N (Kamoun *et al*. 2007). Natural labelling of forage N with ^{15}N is an appropriate technique for studying metabolism of plant N (Hristov *et al*. 2001). Labelling the plant material with ^{15}N will determine the extent of microbial ^{14}N contamination *in situ* and from this, the errors in predicting effective degradability (ED) can be corrected. It is difficult to make field measurements of the ED of fresh forage ingested by grazing ruminants. The *in situ* technique is often used for estimating ruminal protein digestibility of forage samples, but microbial attachment to feed residues in the bags causes true degradability coefficients to be underestimated (Noceck and Grant, 1987). In addition, the forage samples are often dried or frozen before being placed in the bags and this may affect microbial attachment. Internal and external microbial markers have been used to correct for microbial contamination (Noceck and Grant, 1987). In this study, an alternative approach, i.e. labelling the plant material perennial ryegrass, (*Lolium perenne*) with ^{15}N was used to determine the extent of microbial ^{14}N contamination *in situ* and from this, the errors in predicting ED.

The objective of this study was to investigate *in situ* disappearance rates of DM, N and ^{15}N of fresh labeled ryegrass. It was assumed that the amount of ^{15}N placed in the rumen in all of the bags was approximately similar to the amount when labeled forage ingested *in vivo*. Thus, the rumen microbes in the bags could be expected to be labeled to about the same extent and that there is microbial contamination in nylon bags incubation residues during estimating protein degradability values.

MATERIALS AND METHODS

Planting and labelling fresh ryegrass

The ryegrass was grown in 4 L pots in a climate-controlled glass house (temperature and humidity were controlled). The soil used for planting was almost free of N and therefore, at the time of planting, 8 g of Multigro fertilizer (N=10.1%, P=3.5%, K=5.5%, S=16.3%, Ca=7.8%) was added to the pots. Ryegrass plants were cut for the first time at 30 d after planting when they were at the four-leaf stage. After the first harvest, pots were allocated at random to two groups; the first group was assigned to grow un-labeled ryegrass (control) and watered with tap water. The second group was assigned to grow labeled ryegrass. These pots were fertilized with a mixture of fertilizers including P, K, S, Ca (8.8, 50, 11 and 20%, respectively) and N was applied as $^{15}NH_4{}^{15}NO_3$. These pots were initially watered with $^{15}NH_4{}^{15}NO_3$ solution providing 30 mg ^{15}N and a further 12 mg ^{15}N was given to each pot weekly.

The same quantity of un-labeled N was provided as fertilizer to the control plants.

In situ procedure

About 30 min after harvesting, 10 g of fresh ryegrass samples (13% dry matter (DM), 18% crude protein (CP), ^{15}N enrichment=8.3%) were chopped by scissors to approximately 5 mm lengths and weighed into nylon bags (size 150 mm×80 mm, pore size 44 µ) together with a marble (about 5 g). The marble was added to ensure that the bags remained in the rumen digesta during incubation and to facilitate the removal of the bag from the rumen when the desired incubation periods had been ended. The bags were then tied firmly with a monofilament fishing line before being suspended in the rumen of the three male merino lambs (BW=27.7±SD 2.3 kg). All animal care, handling techniques, and surgical procedures were approved by the Animal Ethics Committee of The University of New England before the initiation of research and followed the guidelines issued by the committee on care, handling and sampling of the animals. Animals had free access to drinking water and received a basal diet of chopped alfalfa hay (800 g) daily at about 0900 h 00. Six bags were placed in the rumen of each sheep (each animal was considered as replicate) simultaneously and removed after 0, 3, 7, 12, 21 and 33 h after incubation. The bags were then washed thoroughly with running cold tap water while being squeezed gently until no more visible color came from the bags and their contents. Bags designated 'time zero' were never placed in the rumen, but were otherwise treated in exactly the same way as bags placed in the rumen. After drying (65 ˚C, 48 h), bags were cooled in a desiccator and weighed and the residues were used for N and ^{15}N analysis.

In situ measurement

Chemical analysis

N concentrations in bag residues were determined on the dry samples after they had been ground to pass a 1 mm sieve (Foss Tecator, 1093 cyclotec sample mill). Total-N content of feed samples was determined on finely ground sample DM (1 mm sieve) either using an N analyzer (Leco FP 2000) or, when N was required for ^{15}N analyses, by micro-Kjeldhal digestion followed by steam-distillation and titration of the resulting ammonia. Forage samples, *in situ* bag residues were analyzed to measure isotopic ratio (^{15}N enrichment) by mass spectrometry (Tracermass; Europe Scientific Ltd). The ^{15}N excess was calculated assuming a natural abundance of 0.3663 atoms percent.

Calculations

Rumen degradability parameters were calculated as the percentage of disappearance of DM, N and ^{15}N from the

bags at different times during incubation and fitted by the following equation (Orskov and McDonald, 1979):
$P_t = a + b(1-exp^{-ct})$ Equation 1

Where:
P_t: indicates the percentage of the material disappearance from the bag at time t.
a: soluble potentially degradable fraction.
b: insoluble potentially degradable fraction.
c: degradation rate constant for fraction b.

The percentage disappearance with time values were fitted by using the 'Neway' program for Microsoft Excel (Chen, 1997).

Effective rumen degradabilities of DM and protein were calculated using the fractional outflow rate of solid from the rumen (kp) according to the following equation.

$P= a + ((bc)/(c+k_p))(1-e^{-ct})$ Equation 2

Where:
P: effective extent of degradation at time t(h).
k_p: particle flow rate constant assumed to be 0.05/h (Hristov and Broderick 1996).
a: soluble potentially degradable fraction.
b: insoluble potentially degradable fraction.
c: degradation rate constant for fraction b.

Values for fractional loss of N and ^{15}N from the bags during incubation were fitted by the equation 3 (Orskov and McDonald, 1979).

Fractional loss= $a + b(1-exp^{-ct})$ Equation 3

The rumen microbial N (RMN^{15}_N) in the feed residues derived from ^{15}N values, was calculated using the equation given by Varvikko and Lindberg (1985):

(1-(^{15}N % of total residual N/^{15} N % of total original N) × 100) Equation 4

The percentage of error in the feed N loss estimates resulting from the microbial contamination of the feed sample residues was calculated from the following equation.

(1-(% ^{15}N disappearance/% total N disappearance) × 100) Equation 5

Statistical model and methods

Data were analyzed using the general linear models (GLM) procedure of (SAS, 2003) with the following statistical model:

$Y_{ijk} = \mu + treat_i + sheep_{ij} + period_{ijk} + e_{ijkh}$

Where:
Y_{ijk}: performance during the k^{th} period of the i^{th} treat and j^{th} sheep, (i=1, 2; j=1, 2, .., ni; k=1, 2).
μ: overall mean effect.
$Treat_i$: effect of the i^{th} treat (i=1, 2).
$Sheep_{ij}$: effect of the j^{th} sheep on the i^{th} treat, (j=1,…4).
$Period_{ijk}$: effect of the kth period (k=1, 2).
e_{ijk}: random error.

RESULTS AND DISCUSSION

In situ disappearance (%) of DM (●), N (○) and ^{15}N is shown in Figure 1. There were no statistically significant differences between the 3 sheep (as replicates) in the values for DM, N and ^{15}N disappearance during the *in situ* incubation of the fresh ryegrass. Therefore, the respective mean degradabilities of fresh ryegrass are presented in Figure 1.

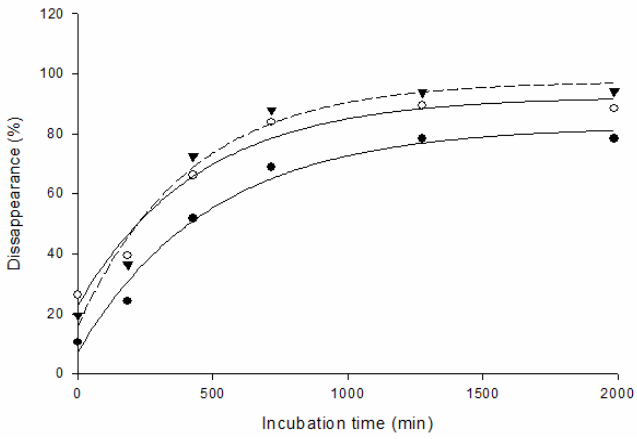

Figure 1 *In situ* disappearance (%) of DM (●), N (○) and ^{15}N (▼) for sheep

The ^{15}N removal from the bag represents the loss of ^{15}N ryegrass, but there would still be some ^{15}N present in rumen bacteria. True values for ^{15}N ryegrass N removal will be slightly higher than the ^{15}N values shown here, i.e. close to 100%. However, *in vivo*, some soluble protein and peptides and AA will leave the rumen before being degraded. Apparent and corrected proportional N disappearance of *in situ* incubated fresh ryegrass is presented in Figure 2. **Error! Reference source not found.**2 shows both *in situ* apparent and corrected proportional N disappearance when the fresh ryegrass was incubated in sheep.

Error! Reference source not found.3 shows the predicted ED of forage N (crude protein) before and after correction for microbial contamination, for various periods of digestion in the rumen. Values of fractional degradation rate (parameter kd) of potential and ED were significantly

increased when a correction for microbial contamination of the residues was applied (Kamoun *et al*. 2014).

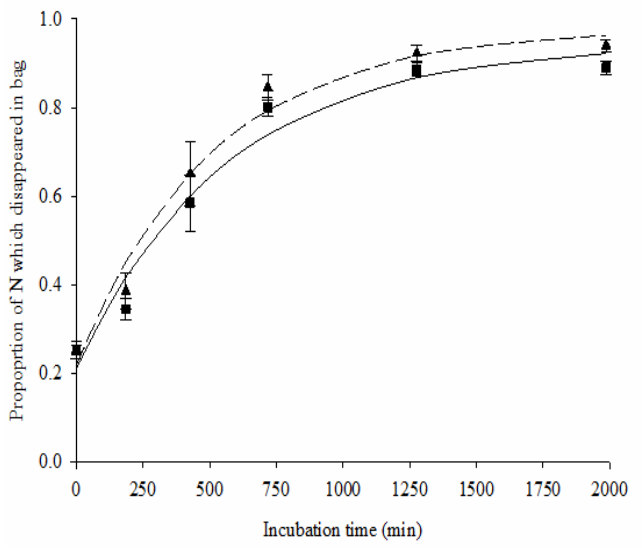

Figure 2 Mean (±SE) fresh ryegrass N disappearance (apparent ■, ___ fitted; corrected ▲, ---- fitted) from nylon bags in sheep

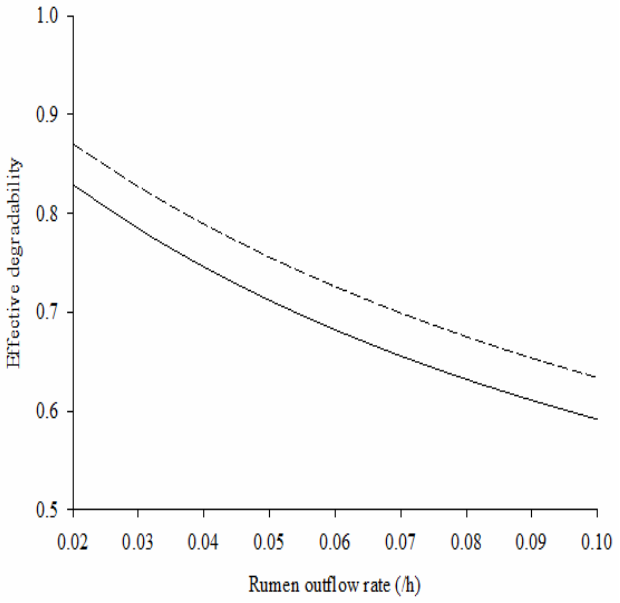

Figure 3 Effective degradability estimates at increasing ruminal outflow rates (true, ___ fitted; corrected, ---- fitted)

As it shown in Figure 3, N disappearance and ED of protein in fresh ryegrass were underestimated. Underestimation of ED of protein in fresh ryegrass by about 4% would have potential consequences for predictions of ruminally fermentable and escape protein and thus for dietary protein feeding management. However, because the correction assumes contaminating microbial N is unlabeled, but microbes attached to labeled ryegrass would become labeled to some extent, the true error (and ED) may still be underestimated. Studies with two markers would help us to better understand the errors associated with the *in situ* technique. Microbial contamination of feed residues is shown in Figure 4. Plant cell soluble protein had slightly higher ^{15}N:^{14}N than cell walls. After washing the zero time bags, it was found that plant residues from the washed zero time bags had slightly lower ^{15}N concentration (7.7% enriched) than the original fresh samples (8.3% enriched). Results for incubated bag residues were corrected accordingly. After a lag period, microbial contamination in the bags increased over time (Figure 4).

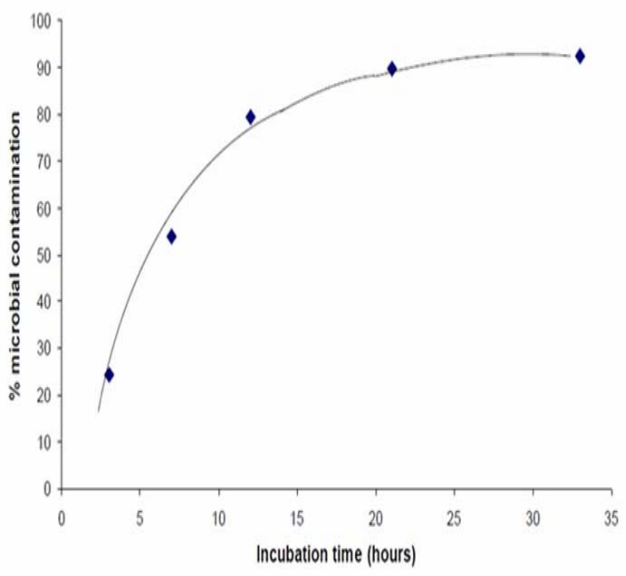

Figure 4 Microbial contamination (microbial ^{14}N: plant^{15}N)

During ingestion of fresh forage by ruminants, there is considerable particle size reduction by mastication (Ulyatt *et al.* 1986) and about 60% of the cell contents of fresh herbages are released (Waghorn *et al.* 1989). Any type of sample preparation is likely to inadequately represent the processes of ingestion, formation of a bolus and swallowing.

AFRC (1992) recommended that, for *in situ* estimation of CP degradation in grass and silage, samples should be chopped to lengths of about 1 cm. However, in this experiment the forages were chopped to about 5 mm to better simulate the chewing activity of sheep.

Barrell *et al*. (2000) reported that ranking of forages for digestibility was affected by method of preparation and they considered that mincing is preferable to either chopping or freeze drying, as particle sizes are closer to those present in real rumen contents. Deinum and Maassen (1994) investigated the effects of different methods of sample preparation.

They proposed for example, mincing causes more damage to the plant cells structure or grinding would have increased the surface area of plant cells exposed for microbial attack compared with chopping. Currently, none of the methods of sample preparation has been nominated as the preferred method. It is likely that the most appropriate method for *in situ* studies will vary depending on whether fresh herbages or conserved forages and concentrates are being incubated (AFRC, 1992).

Microbial nitrogen yield (MN) is of great importance to protein metabolism in ruminants (Broderick *et al*. 2010). The quantification of its flow to the small intestine is therefore important in calculating the amount of digestible MN available to the animal. Various microbial markers (MM) can be used to estimate MN flow (Ipharraguerre *et al*. 2007). Among the techniques used, ^{15}N, purine bases (PB), and urinary excretion of purine derivatives are the most common (Ma *et al*. 2014).

The microbial contamination and adhesion sites are directly related to the nature of the plant tissues. In the adjusted models to predict the values of corrected fractions, certain variables were used to represent the cell wall and its qualities, including neutral detergent fiber (NDF), neutral detergent insoluble protein (NDIP) and indigestible neutral detergent fiber (iNDF) (Machado *et al*. 2013). The method using labeled forage sometimes gave lower rates of microbial contamination. Varvikko and Lindberg (1985) found that calculation of microbial colonization of feed residues in nylon bags using ^{15}N dilution might lead to an underestimate due to the re-use of the feed ^{15}N degraded inside the bags. However, Kamoun *et al*. (2007), using ^{15}N-labeled ryegrass, reported a slight effect on estimation of microbial contamination of the residues on feed N degradability when microbes incorporate feed ^{15}N. They also mentioned that, at early incubation times, the maximum value of incorporation was 5.7% of the ^{15}N incubated in the bag and decreased progressively with incubation time. In their experiment Machado *et al*. (2013) concluded that A and B fractions and kd of CP could be highly biased by microbial CP contamination and therefore these corrected values could be obtained mathematically, replacing the use of microbial markers. In our experiment, during the first hour after incubation, the net disappearance of ^{15}N was greater than of total N. This result could be explained by the entry of rumen micro-organisms which have unlabeled N into the bag. Therefore, the presence of unlabeled N would lead to an underestimation of true forage N degradability. The net disappearance rates ranked as follow: $^{15}N > N > DM$. Because total N and DM fractions left in the bags were contaminated with micro-organisms, their rate of apparent or net loss can be expected to be slower than for ^{15}N, which is present only in plant N. The results of present study agree with the results of Varvikko and Lindberg (1985). *In situ* incubation has been widely used for estimation of feed DM and protein degradation in the rumen but microbial colonization of feed particles in the bag leads to an underestimate of protein degradability by adding non-plant N to the residual N inside the bags (Mathers and Aitchinson, 1981).

Measures of contamination of feed residues in this current experiment suggest that, as the period of incubation became longer, disappearance of ^{15}N from the bags decreased but the fraction present as microbial N increased. Microbial contamination correction is necessary for forage based diets but it has less importance when animal fed concentrate based diet (Krawielitzki *et al*. 2006). The level of microbial contamination increases with increasing incubation time and has been shown to be greater in substrates with high fiber and low N concentrations and the cellulose content is the main factor determining the accumulation of micro colonies in feed particles, which is also responsible for the accumulation of microbial N (Beckers *et al*. 1995; Rodríguez and González, 2006). Conversely, Mitchell *et al.* (1997) found that with increasing plant maturity the protein residue was contaminated to a lower extent by rumen microbes, and they suggested that higher lignification may be associated with lower bacterial colonization.

In contrast, Hoffman *et al.* (1993) found no relationship between extent of bacterial CP and maturity stage of forage species and correction for bacterial CP decreased the estimated undegraded CP fraction.

The ED of protein was consistently increased by microbial contamination correction, with values similar to those reported by González *et al.* (2007) and Kamoun *et al.* (2007). After 24 h incubation, forages residues were highly contaminated (up to 90%), which was in the same range as reported by Olubobokun *et al.* (1990). The correction of the microbial contamination taking place during rumen incubation has negative effects ($P<0.05$) not only on the rumen undegraded fraction but also on its intestinal effective degradability for all controlled fractions of wheat grain (Arroyo *et al.* 2009).

According to the results it seems clear that, in the nylon bag technique microbial attachment to the feed residues may be an important source of error in quantitative feed protein degradation. The degradation results also show that the extent of error (%) in the N loss measurement depends on the N content of the feeds and on the rumen degradation rate. The very high contamination with microbial amino acids (AA) indicates unacceptable errors for the estimates of the rumen undegraded fraction, which furthermore were very variable between AA in both green and ensiled Italian ryegrass (González *et al.* 2009). Therefore, they concluded the need to correct the microbial contamination to obtain accurate estimates of the AA supply.

In their experiment Arroyo *et al.* (2009) revealed that corrected values of intestinal digested CP, as an indicator for the protein value of wheat protein, is closely related to the microbial protein synthesis derived from its organic matter (OM) rumen fermentation. This synthesis and the content of intestinal digested undegraded protein may be respectively higher and lower than is usually assumed in feed tables. Therefore, accurate estimating of protein degradability of feedstuffs in the rumen need that incubation residues to be corrected for microbial contamination. Labeling methods using ^{15}N as a marker are appropriate substitutes for determining microbial N in the residues. However, they are excessively expensive and time-consuming to be applied as routine techniques (Kamoun *et al*. 2014).

CONCLUSION

In conclusion, ^{15}N can be used as a consistent marker to estimate the microbial N fraction of undigested residues in the nylon bags. True ED for fresh ryegrass was about 82% (about 4% higher than uncorrected ED) and may still be underestimated, however, because the ^{15}N-based correction depends on the assumption that contaminating microbial N is unlabeled. It is likely that bacteria attached to digesting labeled ryegrass would become labeled to some extent. Experiments with alternative markers (eg. microbial purines) or more than one marker may help to provide further insights into the errors associated with the *in situ* technique. Because of the high quantity (about 95%) of microbial contamination achieved in this experiment on estimated N degradability, especially with fresh forages, it is necessary to consider microbial contamination in estimation of degradability parameters. Accuracy in estimating the extent of degradation of protein in the rumen from *in situ* disappearance curves is significantly improved when values are corrected for microbial contamination of the bag residue. However, considering the cost and labor required, simpler and cheaper methods would be desirable so that prediction of such microbial contamination is feasible under practical conditions.

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