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# Effect of feed $^{15}N$ incorporation into solid-associated bacteria on the *in situ* nitrogen degradability of $^{15}N$ labelled Italian ryegrass

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## Abstract

The objective of this study was to examine the effect of microbial contamination of the incubation residue on the *in situ* nitrogen degradability of Italian ryegrass. Microbial nitrogen in the bag residues was estimated by the feed $^{15}N$ dilution procedure, and the incorporation of feed $^{15}N$ into the adherent bacteria was assessed after isolating a bacterial pellet and determining its $^{15}N$ enrichment. Isotopically labelled Italian ryegrass (fertilized with $^{15}NH_4{}^{15}NO_3$) was harvested either in spring (early cut) or in summer (late cut). Forages were incubated in the rumen of two steers for 6, 24 and 48 h. The proportion of microbial N per unit of total N in the bag residue increased with incubation time, ranging from 184 to 853 mg microbial N/g total N at 6 and 48h, respectively. $^{15}N$ enrichment in the bacterial pellet was highest after 6 h of incubation (3.6 mg $^{15}N$/g N) and then declined steadily (0.8mg$^{15}N$/gN in 48-h residues). Microbial $^{15}N$ represented up to 0.422 g/g total $^{15}N$ in the bag residue, but the incorporation of feed $^{15}N$ into bacterial N did not account for more than 57 mg/g $^{15}N$ incubated, this incorporation rate decreasing progressively with incubation time. Correction of apparent N disappearance for microbial N resulted in higher values of N degradability, especially for the late cut grass. A further correction considering the amount of microbial $^{15}N$ had little effect on the estimation of corrected N degradability values. Therefore, some of the feed $^{15}N$ is incorporated into bacterial N, but this fraction has a minor effect on the estimation of total microbial N in the bag residue by the feed $^{15}N$ dilution approach, and hence on the estimation of corrected degradability values.

**Abbreviations:**

ADF, acid detergent fibre; $D_c$, N disappearance corrected for microbial contamination; $D_{c*}$, N disappearance corrected for the microbial contamination and for the amount of $^{15}N$ of microbial origin; EC, early cut grass; LC, late cut grass; NDF, neutral detergent fibre; $N_i$, nitrogen incubated in the bag; $N_r$, nitrogen remaining in the bag residue after incubation; $N_m$, microbial nitrogen in the bag; $N_{SAB,}$ nitrogen in solid-associated bacteria; SAB, solid-associated bacteria

**Keywords***:* Microbial contamination; $^{15}N$ excess; $^{15}N$ incorporation; N degradability

## 1. Introduction

The *in situ* polyester bag technique is a method adopted by most protein evaluation systems to assess the protein degradability of feedstuffs in the rumen. Several methodological factors affect considerably this technique, and contribute to variability across studies (Beever and Cottrill, 1994). Microbial contamination of residues remaining in the bags after incubation in the rumen and subsequent rinsing and washing may be one of the most influential factors, especially when the technique is used to estimate protein degradability of forages (Varvikko and Lindberg, 1985; Wanderley et al., 1999; Dixon and Chanchai, 2000). Protein degradability may be underestimated because part of the considered undegraded nitrogen is of microbial origin. The extent of microbial contamination may be variable depending on a number of factors, such as incubation time, type of feedstuff incubated and marker used for the identification of microbial and feed nitrogen fractions (Varvikko and Lindberg, 1985; Beckers et al., 1995). $^{15}N$ can be used as a suitable and reliable marker either to label feed protein (Varvikko and Lindberg, 1985; Wanderley et al., 1999) or, more frequently, amino acids synthesized de novo by ruminai microorganisms and incorporated into the microbial protein (Beckers et al., 1995; Dixon and Chanchai, 2000). The technique based on feed $^{15}N$ dilution following incubation in the rumen of labelled feed takes into account all the microorganisms attached to the feed particles remaining in the nylon bag, and may

result in accurate and reliable estimations of microbial N in the incubation residues. In this technique it is assumed that all the $^{15}N$ measured in the bag residue is only of feed origin, whereas, if present, any microbial $^{15}N$ would be negligible (Varvikko and Lindberg, 1985; Wanderley et al., 1999). However, it seems reasonable to think that attached microorganisms may incorporate some of the feed $^{15}N$ into their own amino acids. The aim of this study was to assess the $^{15}N$ incorporation into microbial protein of solid-associated bacteria (SAB), examining how this fraction may affect the estimation of the extent of microbial contamination in the incubation residues.

## 2. Material and methods

### *2.1. Forages and $^{15}N$ labelling*

A sward of Italian ryegrass (*Lolium multiflorum* Lam.) was established in October on a 20 $m^2$ plot. In December, the plot was fertilized at a rate 80 N units (kg N)/ha with a mixture (50:50; w/w) of $^{15}NH_4{}^{15}NO_3$ (atoms 5% excess) and $NH_4NO_3$ (spreading a mixture containing 240 g of each fertilizer over the 20 $m^2$ plot). The application of $^{15}N$ labelled fertilizer allowed for the incorporation of this stable isotope into the herbage proteins. The plot was divided in two equal subplots, and one of them was harvested in March at an early stage of maturity (early cut, EC), whereas the herbage from the other subplot was collected in June (late cut, LC). The sward was cut by hand using scissors at a height of approximately 5 cm. Herbage samples were freeze-dried and ground through a 1-mm screen.

### *2.2. In situ procedures and isolation of bacterial pellet from incubation residues*

To determine N degradability of forage in the rumen, herbage samples were incubated in the rumen of two steers (fitted with ruminai cannula of 150 mm i.d. and weighing approximately 1000 kg) according to the method described by Ørskov and McDonald (1979). Each animal was fed a mixed diet containing (per kg) 666 g of meadow hay and 333 g of a concentrate mixture consisting of 400 g beet pulp, 300 g barley, 150 g gluten feed, 120 g soybean meal and 30 g minerals per kg concentrate. Average daily dry matter intake was 70 g/kg $BW^{0.75}$, and the diet was supplied daily in two equal meals at 08:00 and 15:00.

The bags (100 mm × 150 mm) were made of precision nylon cloth with a pore size of 42.5 μm. Bags contained 5 g of freeze-dried matter and were attached to a PVC tube connected to the stopper of each cannula by fish line. The bags were incubated in the rumen for 6,24 and 48 h. Sixteen bags were incubated for each feed (EC and LC herbage), animal and incubation time (for a total of 192 bags =16 bags × 2 feeds × 2 animals × 3 incubation times = 192). After incubation, all the bags were immediately rinsed under cold tap water and then in a washing machine (3 rinses of 5 min in cold water). Residual matter of two bags (weighed after freeze-drying) was used to determine dry matter disappearance. Then, residues of both bags were pooled (one incubation residue sample per forage, animal and incubation time), milled through a 1 mm screen and analyzed for N and $^{15}N$ ($N_r$ and $^{15}N_r$, respectively). On the other hand, after rinsing and washing, the contents of the other 14 bags were mixed thoroughly and used to obtain a microbial pellet of SAB. The residual matter thus obtained was homogenised by crushing and stirring in a Stomacher blender for 7 min to detach bacteria from digesta particles. After filtration and differential centrifugation, a pellet of bacteria detached from solid particles was obtained according to the method described by Legay-Carmier and Bauchart (1989). This pellet was freeze-dried and analyzed to determine its total N and $^{15}N$ ($^{15}N_{SAB}$) contents.

### *2.3. Chemical analyses*

Chemical composition was determined in forage samples. Dry matter (method ID 934.01), ash (method ID 942.05), crude protein (method ID 984.13) and acid detergent fibre (ADF, method ID 973.18) contents were determined following the methods of AOAC (1999). Neutral detergent fibre (NDF) was determined using the procedure of Van Soest et al. (1991), using sodium sulphite, but not α-amylase, in the neutral detergent solution. Both NDF and ADF were expressed inclusive of residual ash. Chemical composition of both forages is shown in Table 1.

Forage samples (early- and late-cut ryegrass), *in situ* bag residues and SAB pellets were analyzed to measure isotopic ratio ($^{15}N$ enrichment) by mass spectrometry (VG SIRA 12, UK) following the determination of nitrogen as described by Bremner (1965). The $^{15}N$ excess was calculated assuming a natural abundance of 0.3663 at.% (3.663 mg $^{15}N$/gN). To verify the $^{15}N$ uniformity in the various nitrogenous fractions of the forage, $^{15}N$ excess was determined not only in feed N, but also in the soluble and insoluble nitrogenous fractions. N solubility was determined after soaking the sample (for lh and under continuous agitation) in a buffer solution

(containing $Na_2HPO_4 \cdot 12H_2O$, $NaHCO_3$, NaCl and KCl), according to the procedure described by Vérité and Demarquilly (1978).

***Table 1.*** *Chemical composition and N enrichment of different nitrogenous fractions of early and late cut labelled Italian ryegrass*

| **Forages** | **Italian ryegrass, early cut** | **Italian ryegrass, late cut** |
|---|---|---|
| Chemical composition (g/kg dry matter) | | |
| Organic matter | 912 | 934 |
| Crude protein | 138 | 88 |
| Neutral detergent fibre | 447 | 467 |
| Acid detergent fibre | 244 | 262 |
| $^{15}$N excess in forage samples (mg$^{15}$N/g N) | | |
| Total N | 10.7 | 9.7 |
| Soluble N | 10.6 | 9.7 |
| Insoluble N | 10.7 | 9.7 |

*2.4. Calculations*

The proportion of microbial N in the total N determined in the nylon bag residues ($N_m$; mg microbial N/g total residual N) was estimated according to the forage $^{15}$N dilution procedure (Varvikko and Lindberg, 1985) as $N_m = (1 - (^{15}N_r/^{15}N_i)) \times 1000$, where $^{15}N_r$ and $^{15}N_i$ are the $^{15}$N enrichments (mg $^{15}$N/g total N) in the bag residues and in the incubated feed, respectively. In this procedure it is assumed that contribution of $^{15}$N from feed to adhering microbes is probably insignificant.

The amount of $^{15}$N of microbial origin ($^{15}N_m$, mg microbial $^{15}$N/g total residual N) present in the bag residues was estimated from $N_m$ and $^{15}$N enrichment of the SAB pellet ($^{15}N_{SAB}$, mg $^{15}$N/g total N) as $^{15}N_m = {}^{15}N_{SAB} \times (N_m/(1000 - {}^{15}N_{SAB}))$. The proportion of $^{15}$N of microbial origin (mg microbial $^{15}$N/g total residual $^{15}$N) was calculated as $^{15}N_m/^{15}N_r$.

The incorporation rate of dietary $^{15}$N into SAB (Inc, mg microbial $^{15}$N/g incubated feed $^{15}$N) was calculated as $Inc = (N_r \times {}^{15}N_m)/(N_i \times {}^{15}N_i) \times 1000$, where $N_r$ and $N_i$ are the amounts (g) of nitrogen remaining in the bags after incubation and initially incubated, respectively.

The apparent N disappearance from the bags ($D_{app}$, mg N degraded/g N incubated) at each incubation time was calculated from $N_r$ and $N_i$ as $D_{app} = ((N_i - N_r)/N_i) \times 1000$. N disappearance corrected for microbial contamination ($D_c$) was calculated subtracting the amount of microbial N from the residual N: $D_c = ([N_i - N_r(1 - N_m/1000)]/N_i) \times 1000$. In a further correction for microbial contamination ($D_{c*}$) the amount of $^{15}$N of microbial origin was taken into account: $D_c* = ([N_i - N_r(1 - (N_m + {}^{15}N_m)/1000)]/N_i) \times 1000$.

*2.5. Statistical analyses*

Data were examined by analysis of variance using the general linear model (GLM) procedure of SAS ( 1999), with herbage maturity stage (early- or late-cut ryegrass) and incubation time (6, 24 or 48 h) as the main sources of variation (Steel and Torrie, 1980). Effects of microbial contamination and dietary $^{15}$N incorporation on the nitrogen degradability were assessed from the differences (error) between apparent and corrected disappearance values. Significance of the differences between apparent and corrected values for each forage and incubation time was established from a Student *t*-test (Steel and Torrie, 1980).

## 3. Results and discussion

### *3.1. Incorporation of dietary $^{15}N$ into microbial proteins*

$^{15}N$ was uniformly distributed into soluble and insoluble nitrogenous fractions of each forage (Table 1). Therefore, it can be assumed that incorporation of dietary $^{15}N$ into SAB proteins would not be affected if the N used by the bacteria is taken from either the soluble or the insoluble nitrogenous fractions.

In pellets isolated from bag residuals of both forages, $^{15}N$ enrichment of SAB increased very quickly during the first hours of ruminai incubation to reach a peak value and then decreased steadily (Table 2). In the ruminai-digesta, feed particles are progressively colonized by bacteria that degrade feed proteins and immediately incorporate ammonia and amino acids, hence becoming increasingly enriched with $^{15}N$. Average disappearance of $^{15}N$ is faster than that of total N (Varvikko and Lindberg, 1985). Inside the bags, ruminai bacteria would use in first instance the N of the feed contained in the bag, but afterwards bacteria need to use ammonia originated from the degradation of dietary proteins. This ammonia is unlabelled, explaining the progressive decline (dilution) in $^{15}N$ enrichment observed in SAB pellets as incubation time is increased. This decline was faster with the EC ryegrass, probably due to its higher N degradability at early incubation times (6h).

The proportion of $^{15}N$ of microbial origin (from SAB) in the total residual N was always lower than 1.2 g/kg, with no differences between both forages (Table 2). This proportion was lower after 48 h of incubation than at earlier incubation times. However, when expressed as proportion of the total $^{15}N$ enrichment of the bag residue, microbial $^{15}N$ represented between 70 and 420 g/kg, and this proportion was increased with incubation time in both forages. As a result, estimates of feed $^{15}N$ incorporation into SAB showed values of up to 57 g/kg, with higher values for LC than for EC ryegrass (P=0.020), and with a decreasing trend in the rate of feed $^{15}N$ incorporation as incubation time was extended (P=0.002) (Table 2 and Fig. 1), in agreement with the suggestion that at early incubation times uptake of N by microorganisms is mostly from the degradation of feed proteins contained inside the bag. However, after this initial stage ruminai microorganisms use ammonia of the ruminai environment as a major source of nitrogen to satisfy their needs.

The proportion of the ammonia nitrogen incorporated into bacterial cells may be highly variable, ranging from 300 to 800 g ammonia N/kg total bacterial nitrogen (Salter et al., 1979; Wallace et al., 1999). Our results indicate even a higher rate of incorporation of ammonia-N into microbial protein, assuming the incorporation rate of $^{15}N$ represents the direct incorporation of feed N. However, the incorporation of feed $^{15}N$ represents just the rate of direct incorporation of N from feed N contained in the bag, whereas SAB attached to the residue particles may also incorporate N compounds derived from degradation of proteins supplied by the diet ingested by the animals.

***Table 2.*** *$^{15}N$ enrichment of solid-associated bacteria isolated from incubation residues, and estimation of the extent of microbial contamination in the residues and of $^{15}N$ incorporation from labelled forage to microbial nitrogen*

| Forages | Incubation time, *h* | $N_r$[a] | $^{15}N_{SAB}$[b] | $N_m$[c] | $^{15}N_m$[d] | $^{15}N_m/^{15}N_r$[e] | Inc[f] |
|---|---|---|---|---|---|---|---|
| Early cut | 6 | 67.0 | 3.88 | 184 | 0.65 | 72 | 25.3 |
| | 24 | 78.6 | 1.59 | 706 | 1.10 | 350 | 17.8 |
| | 48 | 74.4 | 0.76 | 853 | 0.65 | 422 | 5.7 |
| Late cut | 6 | 76.4 | 3.21 | 367 | 1.15 | 183 | 57.0 |
| | 24 | 80.3 | 1.66 | 674 | 1.10 | 343 | 21.7 |
| | 48 | 78.2 | 0.86 | 795 | 0.70 | 333 | 13.1 |
| RSD[g] | | 3.75 | 0.741 | 86.5 | 0.176 | 86.9 | 8.62 |
| Forage effects | P value | 0.052 | 0.704 | 0.550 | 0.108 | 0.925 | 0.020 |
| Time effects | P value | 0.054 | 0.002 | <0.001 | 0.027 | 0.007 | 0.002 |

[a] $N_r$= N content in microbial pellet (mg microbial N/g dry matter of pellet).
[b] $^{15}N_{SAB}$= $^{15}N$ excess in solid-associated bacteria (mg$^{15}N$/g N).
[c] $N_m$ = proportion of microbial N in incubation residue (mg microbial N/g total residual N).
[d] $^{15}N_m$ = proportion of $^{15}N$ of microbial origin (mg microbial $^{15}N$/g total residual N).
[e] $^{15}N_m/^{15}N_r$ = mg microbial $^{15}N$/g total $^{15}N$ in residue.
[f] Inc = incorporation rate of dietary $^{15}N$ into SAB (mg microbial $^{15}N$/g incubated feed $^{15}N$).
[g] RSD = residual standard deviation (residual degrees of freedom = 8).

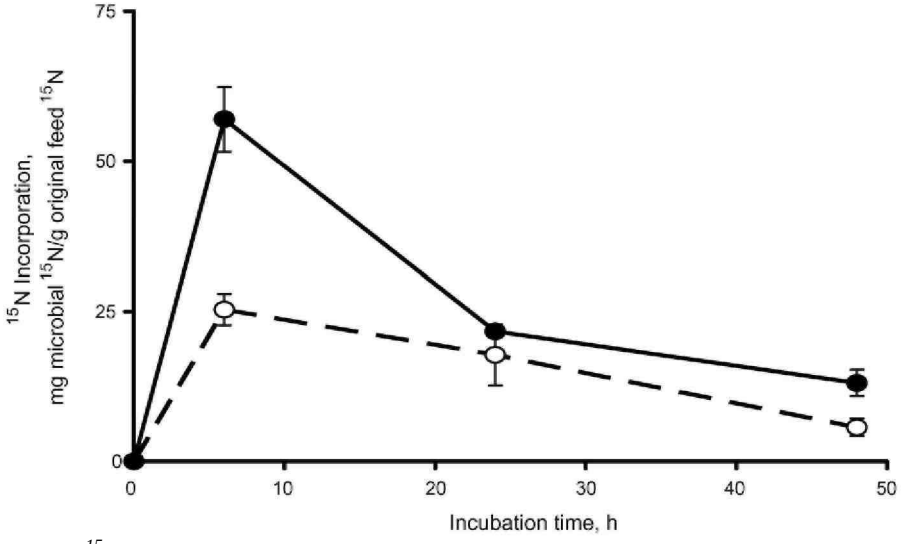


***Fig. 1***. *$^{15}N$ incorporation rate into solid-associated bacteria in the residual particles from the orig inal$^{15}N$ labelled Italian ryegrass (early cut (○) and late cut (●)).*

For the synthesis of microbial protein, not only ammonia N is used, amino acids and small peptides may be directly incorporated into bacterial proteins (Wallace et al., 1999), especially at initial stages of fermentation. SAB are mainly cellulolytic bacteria that grow at a slow rate and thus incorporate almost exclusively ammonia nitrogen (Wallace et al., 1999), although some non-ammonia N may be directly incorporated, albeit to a small extent, at short incubation times. Microorganisms digesting low N forages are much more dependent on rumen ammonia as N substrate than those digesting high N forages (Dixon and Chanchai, 2000). As degradation rate of feed proteins is faster (as with EC ryegrass), direct incorporation of feed N into microbial protein is restricted to a shorter time after feeding, explaining why at 6 h of incubation incorporation of feed $^{15}N$ into microbial N was double with LC compared with EC ryegrass.

### 3.2. Extent of microbial contamination and effect on protein degradability

The extent of microbial contamination in nylon bags residues estimated by feed $^{15}N$ dilution was high even at early incubation times (Table 2). Microbial N as proportion of total residual N ranged between 180 and 850g/kg, depending upon incubation time and forage incubated. This content was increased at longer incubation times. On the other hand, at short incubation times (6 h) microbial N in the bag residue was higher in LC than in EC ryegrass (367 *versus* 184 g/kg, respectively), whereas after 48 h of incubation, residues from EC ryegrass showed a higher contamination with microbial N (853 *versus* 795 g/kg for EC and LC ryegrass, respectively).

***Table 3.*** *In situ nitrogen disappearance from nylon bags: apparent ($D_{app}$) and corrected values for the microbial contamination without ($D_c$) or with ($D_c$*) considering the microbial $^{15}N$, and errors associated (relative differences between apparent and corrected values)*

| **Forages** | **Incubation time, *h*** | $D_{app}$ | $D_c$ | $D_{c*}$ | **Error$_1$**[a] | **Error$_2$**[b] |
|---|---|---|---|---|---|---|
| Early cut | 6 | 565 | 647 | 648 | 15.1 | 15.2 |
| | 24 | 830 | 949 | 950 | 14.5 | 14.6 |
| | 48 | 905 | 986 | 986 | 9.0 | 9.0 |
| Late cut | 6 | 497 | 684 | 685 | 38.0 | 38.2 |
| | 24 | 805 | 937 | 937 | 16.4 | 16.5 |
| | 48 | 808 | 961 | 961 | 19.0 | 19.0 |
| RSD[C] | | 36.6 | 23.0 | 23.0 | 9.00 | 9.03 |
| Forage effects | P value | 0.017 | 0.976 | 0.986 | 0.055 | 0.056 |
| Time effects | P value | <0.001 | <0.001 | <0.001 | 0.159 | 0.157 |

[a] $Error_1 = 100[1 - (D_{app}/D_c)]$.
[b] $Error_2 = 100[1-(D_{app}/D_{c*})]$.
[c] RSD = residual standard deviation (residual degrees of freedom = 8).

The extent of microbial contamination of the bag residues and its effects on forage nitrogen degradability were similar to those observed by other authors (Olubobokum et al., 1990; Dixon and Chanchai, 2000). Given the high extent of microbial contamination of the bag residues, correction of apparent N disappearance for microbial N estimated by feed $^{15}N$ dilution resulted in higher values of N degradability. Differences between apparent and corrected values were larger with LC than with EC ryegrass, confirming previous observations indicating that microbial contamination of the bag residues is greater as the nitrogen content of the feed incubated is lower (Varvikko and Lindberg, 1985; Beckers et al., 1995; Dixon and Chanchai, 2000). In spite of the apparent important proportion of microbial $^{15}N$ in total $^{15}N$ determined in the bag residue, the quantity of feed $^{15}N$ incorporated into bacterial cells did not affect the assessment of the contamination values based on the forage $^{15}N$ dilution (Table 3). This result confirms the assumption suggested by some authors concerning the dietary $^{15}N$ incorporation phenomenon, in that microbial $^{15}N$ represents a small proportion of the total microbial N estimated by feed $^{15}N$ dilution (Varvikko and Lindberg, 1985; Wanderley et al., 1999).

## 4. Conclusion

*In situ* N disappearances require a correction for microbial contamination for a more accurate estimation of forage protein degradability in the rumen. When microbial N is estimated using the feed $^{15}N$ dilution technique there is some incorporation of feed $^{15}N$ into microbial cells, although this had been assumed non-existent by the authors designing the method. Nevertheless, this incorporation has a negligible effect on the estimation of total microbial N, and it may be not necessary for the correction to improve the accuracy of the estimation of forage N degradability.

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