Comparison of Indirect and Direct Determination of Microbial Growth in The Rumen Simulation Technique (RUSITEC)

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ABSTRACT

A study to compare the indirect and direct measurements of microbial protein synthesis in Rusitec using stable isotope nitrogen (\textsuperscript{15}N-ammonia with 95% enrichment) as a tracer have been conducted. Six types of ration containing 16% CP, 24 – 36% CF, 49 – 62% NDF and 27 – 41% ADF with gradual content of non protein nitrogen (NPN) have been put into nylon bag and fed daily to Rusitec fermentor for 15 days observation. Indirect microbial protein synthesis (MPS) estimated MPS from N-turnover of the ammonia pool, while direct method estimated MPS from microbial isolated. The direct measurement of microbial-N in bacterial isolates resulted in higher microbial protein synthesis compared to the indirect method based. The results obtained are compared and discussed in relation to theoretical stoichiometric data of rumen fermentation.

Key words: direct measurement, indirect measurement, microbial protein synthesis, Rusitec

INTRODUCTION

Low protein content of tropical grasses (8 – 12%) forces the farmer to use high protein concentrate feed. This condition limits ruminant production in the tropical area. To avoid utilisation of expensive high protein feed, sometimes, non protein nitrogen (NPN) is added into the ruminant ration. In this condition, microbial protein (MP) has become the major crude protein contributor and provides high quality protein for the ruminants. To have a figure of protein supply for ruminant, it is therefore important to quantify microbial protein synthesis in the rumen.

Several methods have been used to assess microbial protein synthesis. For instance, (1) measurement of microbial protein on protein free diets and assuming that all protein flowing to the duodenum is of microbial origin, (2) distinguishing feed and microbial protein on the basis of amino acid profiles, (3) use of endogenous or exogenous markers such as \textsuperscript{35}S, \textsuperscript{14}N, \textsuperscript{2}H-Leucine, \textsuperscript{32}P, diaminopimelic acid (DAPA), RNA, and purine and pyrimidine bases to label microbial protein material, and (4) near infrared reflectance spectroscopy methods (Obispo and Dehority, 1999; Dewhurst et al., 2000).

So far there is no sophisticated method available. Dewhurst et al. (2000) reviewed that each of the methods has its own problems including safety (radioisotopes), cost (\textsuperscript{15}N and amino acid profiles), difficulties in analysis (RNA and DNA) and contamination of feeds, thereby lacking specificity to the microbial fraction (DAPA, nucleic acid and their bases).

To overcome the cost problem in using \textsuperscript{15}N exogenous marker, Abel et al. (1990) tried to measure microbial protein synthesis in rumen simulation fermentor (Rusitec) by using small quantities of \textsuperscript{15}N and applying the continuous tracer infusion method. The method is based on the rate of incorporation of nitrogen into microbes through ammonia pools (indirect method). It is assumed that ammonia was the only nitrogen source for microbial protein synthesis.

Van Nevel et al. (1975) had expressed their objection to the \textsuperscript{15}N based methods which do not take into consideration the direct incorporation of amino acid or peptide nitrogen into microbial cells instead of ammonia. The direct incorporation of amino acid or peptide nitrogen could amount to 20% of the total nitrogen incorporated. With the indirect \textsuperscript{15}N-based methods the microbial synthesis could, therefore, be under-estimated.

The objective of this study is to compare the indirect (ammonia pool) and direct (microbial isolate) measurements of microbial protein synthesis using tracer stable isotope nitrogen \textsuperscript{15}N in rumen simulation fermentor (Rusitec).
MATERIALS AND METHODS

Materials

Six different rations containing (% DM) CP 16, NFE 41 – 52, CF 24 – 36, NDF 49 – 62 and ADF 27 – 41 were used in this experiment. The nutritional composition of the ration ingredients is shown in Table 1. The daily amounts of different feed components supplied to six fermenters in Rusitec and the chemical composition are shown in Table 2.

Methods

The Rusitec system consisted of 6 simultaneously operating 1060 ml fermenters. At the beginning of each run, each fermenter was filled with 690 ml of rumen fluid and 80 g of solid fraction of rumen content taken from rumen fistulated Jersey steers. The solid fraction was placed into a nylon bag. Simultaneously, two other nylon bags, one of 100 µm pore size containing 10 g hay and another of 60 µm pore size containing 4 g concentrate (barley, extracted soybean meal, urea treated cocoa pods and mineral mixtures in different proportions as shown in Table 2) were introduced. Urea was used to treat cocoa pod with a level of 20 g/kg fresh substances to constitute the proportion of NPN. The fermenters were then filled to volume with pre-warmed incubation buffer and operated during the following days according to the established procedure of Rusitec (Czerkawski and Breckenridge, 1977). The bag containing the solid ruminal fraction was removed after 24h and replaced by bags containing the feed ration. The ration containing bags were removed after 48h of incubation.

The Rusitec was run three times. Each run consisted of 9 days of preliminary phase and 5 days of collection phase. Each fermenter was infused with about 620 ml buffer (McDougal, 1948) daily using an automatic pump. The overflows were collected in 1000 ml Erlenmeyer flasks connected to each fermenter, while the gas was collected in gas bags.

$^{15}$N-urea with 95% enrichment was used as a tracer for microbial growth measurements. The amount of 0.2259 g ($^{15}$NH$_2$)CO was weighed and dissolved in 100 ml distilled water.

<table>
<thead>
<tr>
<th>Ingredient (g)</th>
<th>DM %</th>
<th>Ash %</th>
<th>CP</th>
<th>XL</th>
<th>CF</th>
<th>NDF</th>
<th>ADF</th>
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<tr>
<td>Hay</td>
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<td>7.92</td>
<td>15.8</td>
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<td>30.2</td>
<td>57.3</td>
<td>33.9</td>
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<td>2.45</td>
<td>12.7</td>
<td>2.48</td>
<td>7.26</td>
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<td>8.46</td>
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<td>Soy bean meal</td>
<td>89.2</td>
<td>7.12</td>
<td>52.2</td>
<td>1.82</td>
<td>4.66</td>
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<td>5.86</td>
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<td>Urea treated cocoa pod</td>
<td>90.5</td>
<td>8.58</td>
<td>15.9</td>
<td>0.74</td>
<td>51.9</td>
<td>76.9</td>
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<table>
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<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
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<td>2.4</td>
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<td>0.6</td>
<td>0</td>
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<tr>
<td>Soy bean meal extr. (g)</td>
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<td>1.2</td>
<td>0.8</td>
<td>0.4</td>
<td>0</td>
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<tr>
<td>Cocoa pod (g)</td>
<td>0</td>
<td>0</td>
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<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
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<tr>
<td>Mineral mix (g)</td>
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<td>0.15</td>
<td>0.15</td>
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<table>
<thead>
<tr>
<th>Nutrient contents</th>
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<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
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<tr>
<td>DM (%)</td>
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<td>92.5</td>
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<td>7.64</td>
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<td>8.78</td>
<td>9.15</td>
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<tr>
<td>CP (% DM)</td>
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<td>15.9</td>
<td>15.8</td>
<td>15.8</td>
<td>15.7</td>
<td>15.7</td>
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<td>2.4</td>
<td>4.3</td>
<td>6.3</td>
<td>8.3</td>
<td>10.3</td>
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<td>1.30</td>
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<td>1.49</td>
<td>1.37</td>
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<td>23.6</td>
<td>26.7</td>
<td>29.8</td>
<td>32.8</td>
<td>35.9</td>
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<tr>
<td>NDF (% DM)</td>
<td>56.4</td>
<td>48.9</td>
<td>52.2</td>
<td>55.5</td>
<td>58.8</td>
<td>62.1</td>
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<tr>
<td>ADF (% DM)</td>
<td>33.3</td>
<td>26.5</td>
<td>30.0</td>
<td>33.6</td>
<td>37.0</td>
<td>40.5</td>
</tr>
</tbody>
</table>
Five ml of the solution was added to 5 l of fresh buffer. The amount of $^{15}$N injected daily was calculated as follows:

$$\text{^{15}N (mg/d)} = \frac{0.2259 \text{ g/100 ml}}{1000 \text{ mg/lg}} \times \frac{5 \text{ ml/5000 ml}}{(28/60)} \times \frac{(95/100) \text{ ml overflow/d}}{28/60 \times 95/100} = 0.00100149 \text{ mg/ml (ml overflow/d)}.$$  

The quasi-steady state condition in the fermenters with a nearly constant $^{15}$N-concentration of the ammonia pool was achieved after 5 days. After continuous infusion of $^{15}$N-urea, the microbial protein synthesis was measured either indirectly by measuring the $^{15}$N-enrichment of the ammonia pool or directly by analyzing the $^{15}$N-enrichment in microbial isolate.

The proximate nutritional compositions of feed rations and solid residues after fermentation in Rusitec were analyzed according to conventional Weende method (Naumann and Bassler, 1997). A differential fibre analysis according to Van Soest et al. (1991) was also performed. Short chain of fatty acid (SCFA) were detected using a gas chromatograph (GC-14B, Shimadzu) equipped with a packed column (10% Carbowax 20 MTPA SP 1000 with 1% H$_3$PO$_4$ on Chromosorb WAW 80/100) and flame ionisation detector connected to a chromat integrator (D-2000 Merck-Hitachi). Hydrogen at 120 kPa pressure served as carrier gas. The injection oven temperature was set at 170 °C while the column and detector temperatures were set at 120 °C and 220 °C respectively.

Concentration of NH$_3$-N was measured by means of an electrode. Methane production was measured by gas chromatography (ShimadzuGC-8A) in collected gas samples on a packed silica gel column, at 80°C injection port and detector temperature and 40°C column temperature (isothermal), TC-detector (Shimadzu C-RIB) and argon as carrier gas (Da Costa Gomez, 1999).

After continuous infusion of $^{15}$N-urea, the microbial protein synthesis was measured either indirectly by measuring the $^{15}$N-enrichment of the ammonia pool or directly by analyzing the $^{15}$N-enrichment in microbial isolate. Details of the two methods are described below.

**Indirect method of MPS measurement**

The estimation of MPS from N-turnover of the ammonia pool was done using the same method described by Abel et al. (1990). Three drops of silicon oil (anti-foam agent), 8 drops of thyminophthaldehyde (2% in ethanol solution) or methyl red, 10 ml boric acid buffer (in 1 N KCl solution adjusted with 1 N KOH to pH 9.5) and 4.5 ml 1N KOH were added into 100 ml effluent. The solution was distilled and the distillate was collected in a beaker glass containing 0.1 N H$_2$SO$_4$. Basically, 0.1 N H$_2$SO$_4$ will be sufficient to bind the NH$_3$ released. The beaker volume was filled up to 40 ml with distilled water. The distillation process was terminated when 200 – 250 ml of distillate has been collected; this process took approximately 12 minutes.

Part of the distillate volume containing approximately 0.5 mg nitrogen was evaporated. The amount of distilled volume to be evaporated was calculated as the volume of distillate in the beaker divided by NH$_3$-N/100 ml rumen fluid and divided by 2. This amount was transferred into a 50 ml beaker glass and evaporated at a temperature of 60°C to give a volume of 3 – 4 ml. The temperature was then increased to 100°C to further reduce the volume to 750 μl. The remaining solution was transferred into a tin cup using a pipette and further dried at 105°C. The cup was then formed to a small pellet and placed into a pellet. The pellets were analysed for $^{15}$N enrichment by means of a mass spectrometer (Finnigan MAT Delta C, Bremen, connected to an elemental analyser, Fisons 1108 Rodano, Milano).

Assuming steady state conditions, microbial nitrogen was derived according to the following equation:

$$\text{Microbial N (mg/d)} = \frac{\ell \times (s_i/s_p)}{1}$$

Where, $\ell$ = infusion rate (mg/d), $s_i$ = $^{15}$N-excess in infusion, $s_p$ = $^{15}$N-excess in NH$_3$-N pool.

Microbial protein synthesis (MPS) in mg/d can be calculated as microbial-N divided by (8/100), assuming 8% N in microbial cells (Czerkawski, 1986).

The efficiency of microbial protein synthesis (EMPS) is calculated according to the following formula:

$$\text{EMPS (mg/g OMAD) = MPS/OMAD}$$

Where OMAD is organic matter apparently degraded.

**Direct isolation method of MPS measurement**

Samples reconstitute. The isolation method to estimate microbial protein synthesis in this study used the same principle as described by Carro & Miller (1999). The samples taken from
the overflow and the solid residuals contained in
the nylon bag on day 10 and 12 of Rusitec were
well mixed using a low speed blender to
reconstitute the total digesta. The sample was
used to isolate bacterial pellets (1B). The sample
was kept in an ice bath during the preparation to
prevent microbial activity.

Isolation processes. Samples of recon­
stituted digest were strained using two layers of
cheese cloth. The strained fluid was centrifuged
at 500 g (4 DC) for 10 min. The supernatant was
centrifuged again at 18 000 g (4 °C) for 25
minutes to retain the bacterial pellet. The sedi­
ment was washed by re-suspension in NaCl
solution (9 g/l) which was then followed by
centrifugation at 18 000 g (4 °C) for 25 minutes.
This sediment was washed again by re­
suspension in distilled water and then again
centrifuged at 18 000 g (4 °C) for 25 minutes.
The resultant pellet was freeze dried.

The freeze dried isolate was put into a tin
cup and the cups were formed to pellets. The
enrichment of ~ excess in the isolate was detected
using a mass spectrometer (Finnigan MAT Delta
C, Bremen, connected to an elemental analyser,
Fisons 1108 Rodano, Milano).

Assuming steady state conditions, microbial
protein synthesis (MPS) and efficiency of
microbial protein synthesis (EMPS) were
estimated as described above, however, by
inserting 15 N-excess of the microbial isolate
instead of the 15 N-excess of the ammonia pool
into the formula.

RESULTS AND DISCUSSION

Fermentation characteristic and
organic matter degraded

Fermentation characteristics inform the
activities of microbial in a media. Fermentation
characteristics and organic matter apparently
degraded (OMAD) in the fermenters used in this
experiment are shown in Table 3. The values
represent mean of pooled samples from different
ration with different proportion of NPN and
collection phase (n = 18).

The pH values of the fermenters (6.49 -
6.65) in this experiments shows that fermentation
conditions were suitable for rumen microbial
growth (pH > 6). A higher pH than those
obtained in this experiment would create an
environment that is more conducive to the growth
of rumen cellulolytic bacteria (Harrison et al.,
1988; Beharka and Nagaraja, 1991; Yoon and
Stern, 1996), ultimately increasing fibre
digestion. The fermenter pH values in this
experiment were the same as those found by
Miller et al. (2009) and Carro et al. (2009).
Decreasing pH below 6, even in a short duration
of a cyclic nature, will cause a moderate
depression in fibre digestion (Hoover, 1986).

The ammonium concentration in the liquid
phase (5.11 - 8.63 mmol/d) during the
experiments shows that the conditions in the
fermenters were within the physiological range
for rumen microbes (Satter and Slyter, 1974;
McDonald et al., 1995). However, the numbers
of protozoa in the rumen fluid (3.4 - 9.7 x 10^3
counts/ml) were less than those in the normal
rumen physiology which were up to 10 x 10^6/ml
(McDonald et al., 1995; Kamati et al., 2007).
The protozoa numbers were even decreased
in Rusitec to about 5000 counts/ml during the
collection phase. Apart from methodological
reasons associated with the discrepancy between
protozoal generation interval and substrate
exchange, the survival of only one tenth of the
initial protozoal numbers introduced during the
collection phase might also have been caused by
the restriction in substrate available for protozoa
when high fiber diets were used (Weller and
Pilgrim, 1974; Coleman et al., 1980). The low
numbers of protozoa (10^3) in Rusitec fermenters
were also found by Hillman et al. (1991).

Total of SCFA (22.5 - 37.6 mmol/d) were
lower than the values found by Carro et al.
(2009) which were range from 84 - 130 mmol/L.
If the average of effluent per day was about 600
ml, then total SCFA found in the fermenters were
only 37.5 - 62.7 mmol/L. The low SCFA values
found in this experiment may be due to SCFA
measurement procedure using liquid gas
chromatography equipment. The low value of
SCFA using liquid gas chromatography was also
found by Hutabarat et al. (2009) in comparison to
steam distillation methods by Asti et al. (2009).
In average, proportion of acetic, propionic, and
butyric acids were 56.13, 24.58 and 13.77. The
ratio of acetic to propionic acids (2.2 - 2.5 to 1)
found in this experiment was also lower than that
found by Carro et al. (2009).

OMAD from all fermenters expressed low
microbial activities. Maximum ration degradation
coefficient was <50%. The OMAD values were
in line with SCFA fermentation products.
Table 3. OMAD and fermentation characteristics in Rusitec (n = 18)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Dimension</th>
<th>Average</th>
<th>Maximum</th>
<th>Minimum</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>cts/ml</td>
<td>6.56</td>
<td>6.65</td>
<td>6.49</td>
<td>0.06</td>
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<tr>
<td>Protozoal number</td>
<td>cts/ml</td>
<td>62.49</td>
<td>96.91</td>
<td>34.65</td>
<td>1795</td>
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<tr>
<td>CH₄</td>
<td>mmol/d</td>
<td>6.70</td>
<td>8.47</td>
<td>5.21</td>
<td>0.96</td>
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<tr>
<td>NH₃</td>
<td>mmol/d</td>
<td>6.80</td>
<td>8.63</td>
<td>5.11</td>
<td>0.97</td>
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<tr>
<td>SCFA</td>
<td>mmol/d</td>
<td>31.0</td>
<td>37.6</td>
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<td>Acetate</td>
<td>mmol/d</td>
<td>17.4</td>
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<tr>
<td>Propionate</td>
<td>mmol/d</td>
<td>7.62</td>
<td>9.75</td>
<td>5.50</td>
<td>1.24</td>
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<tr>
<td>i-Butyrate</td>
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<td>0.18</td>
<td>0.30</td>
<td>0.08</td>
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<tr>
<td>n-Butyrate</td>
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<td>5.98</td>
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<td>i-Valerate</td>
<td>mmol/d</td>
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<td>OMAD g/d</td>
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<td>5.79</td>
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<td>45.47</td>
<td>48.99</td>
<td>41.63</td>
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</table>

Table 4. Average values of ¹⁵N enrichment (% atom excess), microbial protein synthesis and efficiency using the direct and indirect method

<table>
<thead>
<tr>
<th>Variables</th>
<th>Direct average</th>
<th>Direct maximum</th>
<th>Direct minimum</th>
<th>Indirect average</th>
<th>Indirect maximum</th>
<th>Indirect minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹⁵N enrichment (%)</td>
<td>0.61</td>
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<td>0.56</td>
<td>0.88</td>
<td>1.00</td>
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<td>Microbial-N (mg/d)</td>
<td>90.3</td>
<td>100.1</td>
<td>80.2</td>
<td>63.1</td>
<td>70.2</td>
<td>54.3</td>
</tr>
<tr>
<td>Microbial-N/OMAD (mg/g)</td>
<td>17.8</td>
<td>21.8</td>
<td>15.5</td>
<td>12.4</td>
<td>14.9</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Microbial Protein Synthesis

Incorporation rate of ¹⁵N into microbes (% atom excess) which was measured directly in microbial isolates and indirectly through the ammonia pool is given in Table 4.

The ¹⁵N enrichment in bacterial isolate (direct method) was lower than in the ammonia pool (indirect method). This resulted in higher bacterial-N fixation calculated for the direct microbial isolate method compared to the indirect approach. Correspondingly, microbial-N per gram of organic matter apparently degraded (OMAD) was higher when ¹⁵N-enrichment was taken from the bacterial isolate instead of taking it from the ammonia pool.

Microbial-N measured by means of ¹⁵N-enrichment in the microbial isolate was higher than that determined from the ammonia pool. It may support the argument of Van Nevel et al. (1975) that the method based on the rate of incorporation of nitrogen through the ammonia pool (indirect method) did not take into consideration the direct incorporation of amino acid or peptide nitrogen into microbial cells. The direct incorporation of amino acids or peptide nitrogen may amount to 20% of the total nitrogen incorporated. In this experiment, however, unaccounted nitrogen incorporation as proportion of total nitrogen incorporated reached 33% (different value between direct and indirect methods). Feed particle contamination in
Feed and Nutrition

bacterial isolates may have contributed to the lower $^{15}$N-enrichment thus leading to higher calculated microbial N-incorporation with the direct method.

A comparison of the measured MPS values with the MPS to be expected theoretically from the stoichiometry of rumen fermentation may be helpful. The amount of synthesised microbial cells can be calculated from SCFA production. According to Demeyer et al. (1995) the amount of hexose fermented (HF) can be calculated as follows:

$$HF = \text{hexose fermented (mol/d)} = \frac{(A + P)}{2} + B + V$$

Where $A$ is acetate, $P$ is propionate, $B$ is butyrate and $V$ is valerate expressed in mol/d respectively.

The amount of hexose fermented (HF; g/d) is calculated as $162 \times HF$ (mol/d). The fermentation of 100 g hexose is assumed to result in the synthesis of 30 g microbial cells (Bergner and Hofmann, 1996). If the average SCFA values from Table 3 are taken for the formula above and assuming 8% nitrogen in microbial cells (Czerkawski, 1986), 68 mg microbial-N are theoretically expected per day. This is lower than the average microbial-N measured with the direct method, but higher than that calculated with the indirect method. Relationship between MPS measured using indirect and direct methods and the amount of NPN in the ration were shown in Figure 1. The figure showed that the differences MPS measured from indirect and direct methods were constant and did not depend on the amount of NPN in the ration. The fact shows that the microbe synthesized the protein was mainly from ammonia-N and only small proportion of the microbe were synthesized directly from amino acid-N and other peptide-N (< 30%). Therefore, it is necessary to protect extensive degradation of high quality feed protein and replaced with cheaper NPN sources.

CONCLUSIONS

Fermentation characteristics show an optimal condition for rumen microbial growth. However, low degradation activities have lead to low fermentation product and microbial protein synthesis. For the range of rations used, it can be concluded that microbial-N synthesis in Rusitec measured by the direct method (based on $^{15}$N-incorporation in microbial isolates) is higher than that determined with the indirect method (based on the rate of incorporation of nitrogen into microbes through the ammonia pool). The direct method also results in higher microbial-N synthesis compared to the theoretically expected microbial-N synthesis. To maximize microbial protein synthesis in the rumen, the amount of about 30% nitrogen in form of amino acid and other peptide are needed. Since the rumen microbe synthesize its protein mainly from ammonia-N sources, therefore, extensive degradation of expensive feed protein, should be protected and replaced with cheaper NPN sources.

REFERENCES


