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 (^{15}N-urea 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 endo-genous or exogenous markers such as ^{35}S, ^{15}N, ^{3}H-Leucine, ^{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 (^{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 ^{15}N exogenous marker, Abel *et al*. (1990) tried to measure microbial protein synthesis in rumen simulation fermentor (Rusitec) by using small quantities of ^{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 ^{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 ^{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 ^{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 $(^{15}NH_2)_2CO$ was weighed and dissolved in 100 ml distilled water.

Table 1. Nutritional composition of the feed components

Ingredient (g)	DM	Ash	CP	XL	CF	NDF	ADF
	%	% DM					
Hay	93.4	7.92	15.8	1.32	30.2	57.3	33.9
Barley	87.6	2.48	12.7	2.48	7.26	30.8	8.46
Soy bean meal	89.2	7.12	52.2	1.82	4.66	10.6	5.86
Urea treated cocoa pod	90.5	8.58	15.9	0.74	51.9	76.9	59.4

Table 2. Composition of the feed rations

	Ration					
	R1	R2	R3	R4	R5	R6
Ingredients						
Hay (g)	10	10	10	10	10	10
Barley (g)	0	2.4	1.8	1.2	0.6	0
Soy bean meal extr. (g)	0	1.6	1.2	0.8	0.4	0
Cocoa pod (g)	0	0	1.0	2.0	3.0	4.0
Mineral mix (g)	0.15	0.15	0.15	0.15	0.15	0.15
Nutrient contents						
DM (%)	93.5	91.9	92.1	92.3	92.5	92.7
ASH (% DM)	9.73	7.64	8.02	8.40	8.78	9.15
CP (% DM)	15.6	15.9	15.8	15.8	15.7	15.7
NPN (% DM)	2.3	2.4	4.3	6.3	8.3	10.3
XL (% DM)	1.30	1.60	1.49	1.37	1.26	1.14
CF (% DM)	29.7	23.6	26.7	29.8	32.8	35.9
NDF (% DM)	56.4	48.9	52.2	55.5	58.8	62.1
ADF (% DM)	33.3	26.5	30.0	33.6	37.0	40.5

Five ml of the solution was added to 5 l of fresh buffer. The amount of ^{15}N injected daily was calculated as follows:

^{15}N (mg/d) = (0.2259 g/100 ml) (1000 mg/1g) (5ml/5000 ml) (28/60) (95/100) (ml overflow/d)
= 0.00100149 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, Simadzu) equipped with a packed column (10% Carbowax 20 MTPA SP 1000 with 1% H_3PO_4 on Cromosorb WAW 80/100) and flame ionisation detector connected to a chromato-integrator (D-2000 Merck-Hitachi). Hydrogen at 120 kPa pressure served as carrier gas. The injection oven temperature was set at 170 ^oC while the column and detector temperatures were set at 120 ^oC and 220 ^oC respectively.

Concentration of NH_3-N was measured by means of an electrode. Methane production was measured by gas chromatography (SimadzuGC-8A) in collected gas samples on a packed silica gel column, at 80^0C injection port and detector temperature and 40^0C column temperature (isothermal), TC-detector (Simadzu C-R1B) 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 analysing 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 thymolphthalein (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 into100 ml effluent. The solution was distilled and the distillate was collected in a beaker glass containing 0.1 N H_2SO_4. Basically, 0.1 N H_2SO_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_4-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 ^oC to give a volume of 3 – 4 ml. The temperature was then increased to 100 ^oC to further reduce the volume to750 µl. The remaining solution was transferred into a tin cup using a pipette and further dried at 105 ^oC. The cup was then formed to a small pellet and placed into a pallet. 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:

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

Where, r_i = infusion rate (mg/d), s_i = ^{15}N-excess in infusion, s_p = ^{15}N-excess in NH_4-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:

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

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 (TB). The sample was kept in an ice bath during the preparation to prevent microbial activity.

Isolation processes. Samples of reconstituted digest were strained using two layers of cheese cloth. The strained fluid was centrifuged at 500 ***g*** (4 ^{o}C) for 10 min. The supernatant was centrifuged again at 18 000 ***g*** (4 ^{o}C) for 25 minutes to retain the bacterial pellet. The sediment was washed by re-suspension in NaCl solution (9 g/l) which was then followed by centrifugation at 18 000 ***g*** (4^{o}C) for 25 minutes. This sediment was washed again by re-suspension in distilled water and then again centrifuged at 18 000 ***g*** (4 ^{o}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 ^{15}N 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; Karnati *et al*., 2007).

The protozoal 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)

Variables	Dimension	Average	Maximum	Minimum	STD
pH		6.56	6.65	6.49	0.06
Protozoal number	cts/ml	6240	9691	3465	1795
CH_4	mmol/d	6.70	8.47	5.21	0.96
NH_4^+	mmol/d	6.80	8.63	5.11	0.97
SCFA	mmol/d	31.0	37.6	22.5	4.16
Acetate	mmol/d	17.4	20.0	13.5	1.87
Propionate	mmol/d	7.62	9.75	5.50	1.24
i-Butyrate	mmol/d	0.18	0.30	0.08	0.06
n-Butyrate	mmol/d	4.09	5.98	2.44	0.99
i-Valerate	mmol/d	0.84	1.16	0.52	0.19
n-Valerate	mmol/d	0.78	1.49	0.42	0.28
OMAD	g/d	5.10	5.79	4.13	0.56
OMAD	%	45.47	48.99	41.63	2.39

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

Variables	Direct			Indirect		
	average	maximum	minimum	average	maximum	minimum
^{15}N enrichment (%)	0.61	0.68	0.56	0.88	1.00	0.81
Microbial-N (mg/d)	90.3	100.1	80.2	63.1	70.2	54.3
Microbial-N/OMAD (mg/g)	17.8	21.8	15.5	12.4	14.9	10.9

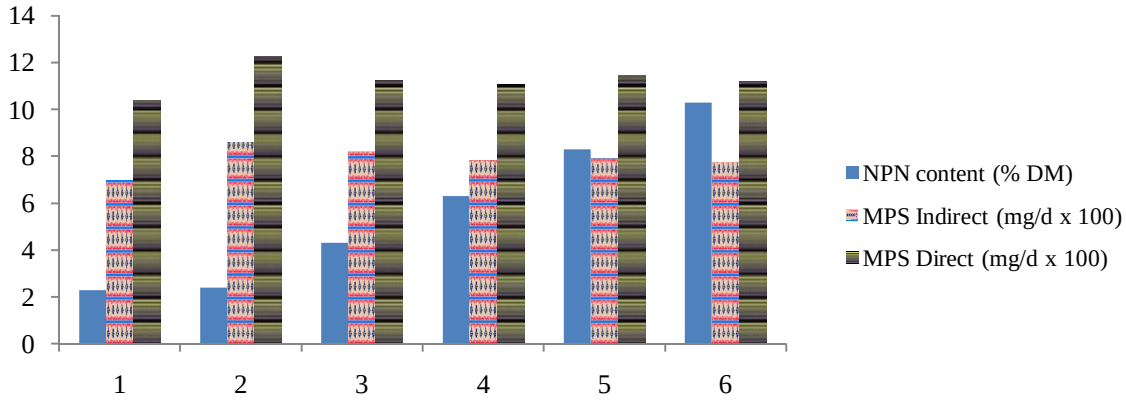

Figure 1. Relationship between differences amount of NPN in ration and MPS measured from different methods

Microbial Protein Synthesis

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

The ^{15}N 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 ^{15}N-enrichment was taken from the bacterial isolate instead of taking it from the ammonia pool.

Microbial-N measured by means of ^{15}N-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

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 = hexose fermented (mol/d) = (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 x 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.

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