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Comparison of direct and indirect methods for estimating microbial protein synthesis in sheep

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Abstract. The aim of this work was to compare the values of rumen microbial N duodenal flow (MNDF) obtained from duodenal flow measurements using ^{15}N as microbial marker with those estimated from the urinary excretion of purine derivatives (PD) and allantoin. Six ruminally and duodenally cannulated sheep were used in a partially replicated Latin Square design. The four experimental diets had forage:concentrate ratios of 70:30 (HF) or 30:70 (HC) and either alfalfa hay (A) or grass hay (G) as forage, and were designated as HFA, HCA, HFG and HCG. Duodenal digesta flows were determined by the dual-phase marker technique using Cr-mordanted fibre and Co-EDTA as solid and fluid digesta marker, respectively. The MNDF was estimated from duodenal flow of ^{15}N using as reference liquid- (LAB) and solid-associated (SAB) bacteria isolated from the rumen, and indirectly from the urinary excretion of PD and allantoin using response models from the literature. Estimates of MNDF with ^{15}N-SAB as reference were 8.3, 7.1, 11.1 and 9.8% greater for HFA, HCA, HFG and HCG diets, respectively, than those obtained with ^{15}N-LAB, but both bacterial isolates detected the same significant differences among diets. Compared with ^{15}N, the urinary excretion of both total PD and allantoin produced lower estimates of MNDF for HFA, HCA and HCG, but values for the HFG diet were similar. Averaged across diets, MNDF values obtained from the urinary excretion of PD and allantoin were 14.9 and 7.9 % lower than those obtained with ^{15}N and LAB as reference, and 22.3 and 15.9% lower than those obtained with ^{15}N and SAB as reference. All methods detected the same significant differences among diets, indicating that urinary excretion of PD constitutes a reliable method for estimating MNDF.

Keywords. Sheep – Microbial protein – ^{15}N – Purine derivatives – Allantoin – Forage:concentrate.

Comparaison des méthodes directes et indirectes d'estimation de la synthèse des protéines microbiennes chez les ovins

Résumé. *L'objectif de ce travail était de comparer les valeurs de flux duodénal d'azote microbien (MNDF) obtenues à partir de mesures de flux duodénal en utilisant ^{15}N comme marqueur microbien avec celles estimées à partir de l'excrétion urinaire des dérivés puriques (PD) et de l´allantoïne. Six moutons munis de canules ruminal et duodénal ont été utilisés selon un dispositif en carré latin double. Les quatre régimes expérimentaux désignés HFA, HCA, HFG et HCG ont des rapports fourrage : concentré de 70 : 30 (HF) ou 30 : 70 (HC) et le foin de luzerne (a) ou le foin (G) d'herbe comme fourrage. Les flux de digesta duodénal ont été déterminés par la technique de marqueur de double phase en utilisant les fibres mordancées au chrome et le Co-EDTA comme marqueurs des phases, respectivement, solide et liquide. La MNDF a été estimée à partir du flux duodénal de ^{15}N en utilisant comme référence les bactéries associées à la phase liquide (LAB) et à la phase solide (SAB) du rumen, et indirectement à partir de l'excrétion urinaire de PD et d'allantoïne utilisant des modèles de réponse rapportés dans la littérature. Les estimations de MNDF avec ^{15}N-SAB comme référence ont été 8,3; 7,1; 11,1 and 9,8% plus élevés pour les régimes HFA, HCA, HFG et HCG, respectivement, que ceux obtenus avec ^{15}N-LAB, mais les deux isolatss bactériens ont détecté les mêmes différences significatives parmi des régimes. Comparée avec le ^{15}N, l'excrétion urinaire totale des PB et de l´allantoïne ont produit de faibles estimations de MNDF pour les régimes HFA, HCA and HCG, mais les valeurs pour le régime HFG ont été semblables. Les valeurs de MNDF obtenues à partir de l'excrétion urinaire du PD et l'allantoïne on été 14,9 et 7,9 % inférieurs à celles obtenues avec ^{15}N et LAB comme référence, et 22,3 et 15,9% inférieurs à celles obtenues avec ^{15}N et SAB comme référence. Toutes les méthodes ont détecté les mêmes différences significatives parmi des régimes, indiquant que l'excrétion urinaire du PD est une méthode fiable pour estimer MNDF.*

Mots-clés. *Moutons – Protéine microbienne – ^{15}N – Dérivés puriques – Allantoïne – Fourrage : concentré.*

I – Introduction

The urinary excretion of purine derivatives (PD) has been proposed as a non-invasive method of estimating microbial N duodenal flow (MNDF) to the duodenum in ruminants, and response models (RM) have been developed in sheep (Balcells *et al*., 1991; Chen *et al*., 1990, 1992) and other species. These RM assume that nucleic acids flowing to the duodenum are mostly of microbial origin, and after intestinal digestion and absorption, purine base catabolites are proportionally recovered in the urine as allantoin, hypoxanthine, xanthine and uric acid (Pérez *et al*., 1996). However, the usefulness of the RM needs to be validated by comparison of estimates with values obtained using cannulated animals and microbial markers. Previous studies have compared both methods of estimating MNDF in sheep fed diets with different straw:barley grains ratio (Pérez *et al*., 1996) and different levels of intake (Chen *et al*., 1992), but no comparative studies have been conducted in sheep fed diets representative of those used in practical feeding. Because ^{15}N has been shown to be more accurate and precise than other microbial markers (Perez *et al*., 1996; Carro and Miller, 2002), we decided to use ^{15}N for measuring MNDF in cannulated sheep fed four different diets representative of those most frequently used under practical feeding conditions in Spain. The aim of this study was to compare the MNDF values with those obtained using RM developed for sheep, in order to analyse the strength of the urinary excretion of PD as a method to estimate MNDF under practical feeding conditions.

II – Materials and methods

1. Animals and diets

Six ruminally and duodenally cannulated Merino sheep [59.0 ± 4.46 kg body weight (BW)] were used in a partially replicated 4 x 4 Latin square. Sheep were housed in individual pens, had continuous access to fresh water and vitamin/mineral block over the experimental period and were cared and handled in accordance with the Spanish Animal Care Regulations. Four total mixed diets were formulated according to a 2 x 2 factorial arrangement of treatments. The diets had forage:concentrate (F:C) ratios (dry matter (DM) basis) of 70:30 (HF) or 30:70 (HC) with either alfalfa hay (A) or grass hay (G) as forage, and were designated as HFA, HCA, HFG and HCG. The concentrate was based on barley, gluten feed, wheat middlings, soybean meal, palmkern meal, wheat, corn and mineral-vitamin premix in the proportions of 215, 204, 200, 135, 115, 50, 50 and 31 g/kg, respectively (fresh matter basis). Crude protein content was 186, 177, 121 and 160 g/kg DM for HFA, HCA, HFG and HCG, respectively, and neutral-detergent fibre content was 426, 374, 499 and 401 g/kg DM. Diets were offered to the animals twice daily (08:00 and 20:00 h) at a daily rate of 56 g DM/kg $BW^{0.75}$ to minimise feed selection. Samples of diets and refusals were collected daily over the trial and composited weekly. Samples were dried at 55°C in an oven for 48 h and ground to pass through a 1-mm screen before chemical analyses.

2. Experimental procedure and analytical methods

Each 26-day experimental period consisted of 15 days of dietary adaptation and 11 days for sample and data collection. On day 13, sheep were moved to metabolism cages equipped for quantitative collection of faeces and urine. After 2 days of adaptation, urine voided by each sheep in 12 h was collected for 6 days. Urine was collected in a solution of 3.6 M H_2SO_4 to keep the pH below 3. The volume of urine at each sampling was determined, and a subsample (20%) was taken for each sheep and frozen until analyzed for total N and PD. Cr-mordanted fibre and Co-EDTA were used as solid and fluid phase markers, respectively, to assess the duodenal flow of digesta, and ^{15}N was used as a microbial marker. From days 18 to 26, 15 g of Cr-mordanted fibre were administered daily via the ruminal cannula into four equal portions at 08:00, 14:00, 20:00

and 02:00 h. Co-EDTA and $^{15}NH_4Cl$ (10% atom excess; Tracer SA, Madrid, Spain) were dissolved in distilled water and infused into the rumen (250 ml/d) at constant daily rate of 60 mg of Co and 30 mg of ^{15}N by means of a peristaltic pump. On days 23, 24 and 25 duodenal digesta samples were collected at 6 h intervals. The sampling time was adjusted ahead 2 h daily in order to obtain a sample representative of daily duodenal flow. Samples were pooled by sheep and stored at -20ºC. Duodenal samples were thawed at 4ºC, homogenized, and half of each sample was centrifuged (1000 *x g*, 5 min) to obtain particulate matter (Faichney, 1975). Both samples of whole duodenal digesta and particulate matter were freeze dried and analyzed for ash, non-ammonia N (NAN) and ^{15}N. On day 26, about 200 g of rumen contents was withdrawn from each sheep at 0, 4 and 8 h after the morning feeding. Rumen contents were squeezed through four layers of cheesecloth and the solid digesta was combined with an equal volume of saline solution (0.9% NaCl) at 38ºC, mixed gently, and squeezed again to remove residual liquid-associated bacteria (LAB). The filtrate obtained at each sampling time was kept at 4ºC, pooled by sheep, and used to isolate LAB by differential centrifugation (Ranilla and Carro, 2003). The solid digesta was treated with saline solution containing 0.1% methylcellulose as described by Ranilla and Carro (2003) before isolation of solid-associated bacteria (SAB). Bacterial pellets were lyophilized, ground to a fine powder with a mortar and pestle, and analyzed for N and ^{15}N enrichment. Procedures for determination of chemical composition of feeds and ^{15}N analysis in feeds, digesta and bacterial isolates have been reported by Carro and Miller (1999). Concentration of PD in urine was analyzed by HPLC as described by Balcells *et al.* (1992).

3. Calculations and statistical analyses

Duodenal flow was calculated from the concentrations of Cr and Co in duodenal digesta phases using the dual-phase maker method of Faichney (1975). Samples were mathematically reconstituted to create a representative sample from the concentrations of each analyzed nutrient in each of the duodenal phases (Faichney, 1975). The MNDF was calculated using ^{15}N as a microbial marker and LAB or SAB as reference bacteria as follows: microbial NAN flow (g/d) = DM digesta flow (g/d) x NAN in digesta (g/g DM) x (^{15}N atom% excess in digesta / ^{15}N atom% excess in bacterial reference). The MNDF was also indirectly estimated from urinary excretion of total PD according Chen *et al.* (1992) and of allantoin according to Balcells *et al.* (1991). Data were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC). The effects of F:C ratio, type of forage (FOR), period, and the interaction F:C x FOR were considered fixed, and sheep effect was considered random. When a significant effect of treatment ($P<0.05$) was detected, differences among means were tested using the Tukey's multiple comparison test. Correlations between different estimates of MNDF were determined by Pearson correlation analysis using the PROC CORR of SAS.

III – Results and discussion

In agreement with previous results (Ranilla and Carro, 2003), N and ^{15}N enrichment in LAB were lower ($P=0.07$ and <0.001, respectively) for HC than for HF diets (Table 1). Solid-associated bacteria isolated from sheep fed HC diets also presented lower ^{15}N enrichments ($P<0.001$) compared with those from sheep fed HF diets.

The urinary excretion of PD was greater ($P=0.006$) in sheep fed HC diets than in those fed HF diets, and tended ($P=0.05$) to be greater for diets containing A than for G diets (Table 2). Similar differences among diets were observed in the urinary excretion of allantoin, but excretion of uric acid, xanthine and hypoxanthine was not affected by the type of forage ($P=0.26$, 0.29 and 0.83, respectively). In agreement with previous results in sheep (Pérez *et al.*, 1996,1997; Carro *et al.*, 2000,2006), allantoin represented between 72.4 and 77.9% of total urinary PD and this proportion was not affected by either F:C ratio or type of forage ($P=0.57$ and 0.27, respectively). Urinary

excretion of creatinine is considered as an index of lean body mass, and the lack of differences among diets in our study (see Table 2) is consistent with the lack of effects of F:C ratio (P=0.93) and type of forage (P=0.62) on sheep BW (mean values were 59.3, 59.3, 59.0 and 59.1 kg for HFA, HCA, HFG and HCG diets, respectively).

Table 1. Effect of forage:concentrate ratio (F:C) and type of forage (FOR) on N content (mg N/g dry matter) and ^{15}N enrichment (atoms % excess) of liquid-associated (LAB) and solid-associated (SAB) bacteria isolated from the rumen of sheep fed diets with F:C ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage

Item	Diet					Significance of effects (P =)		
	HFA	HCA	HFG	HCG	SEM	F:C	FOR	F:C x FOR
N-LAB	66.4^{b}	64.4^{ab}	62.6^{a}	61.7^{a}	1.22	0.07	0.02	0.67
N-SAB	68.7	64.8	66.6	67.9	0.95	0.22	0.64	0.02
^{15}N-LAB	0.0712^{a}	0.0601^{a}	0.1332^{c}	0.0844^{b}	0.00428	<0.001	<0.001	0.001
^{15}N-SAB	0.0646^{ab}	0.0549^{a}	0.1170^{c}	0.0757^{b}	0.00447	<0.001	<0.001	0.004

$^{a, b}$ Within a row, means with unlike superscripts differ (P<0.05).

As a consequence of the differences in ^{15}N enrichment between LAB and SAB, estimates of MNDF with SAB were 8.3, 7.1, 11.1 and 9.8% greater for HFA, HCA, HFG and HCG diets, respectively, than those estimated with LAB as reference (Table 3). These differences are in the range of those reported by others (Carro *et al.*, 2002; Ipharraguerre *et al.*, 2007), and suggest that the overestimation of MNDF by using SAB as reference was not affected (P = 0.76) by the diet fed to sheep in the present study. The use of LAB or SAB as reference detected the same differences among diets, showing that although values were affected by the bacterial isolate, the interpretation of results was unchanged. The MNDF was greatest (P<0.02) for HCA and lowest (P<0.02) for HFG, with HFA and HCG having similar intermediate values (P=0.45). The lower microbial N production found in HFG compared with the other diets could be partially explained by the lower N content of this diet.

Table 2. Effect of forage:concentrate ratio (F:C) and type of forage (FOR) on urinary excretion (mmol/d) of purine derivatives (PD) and creatinine in sheep fed diets with F:C ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage

Item	Diet					Significance of effects (P =)		
	HFA	HCA	HFG	HCG	SEM	F:C	FOR	F:C x FOR
Total PD	13.2^{ab}	15.1^{b}	11.0^{a}	14.1^{b}	0.75	0.006	0.05	0.45
Allantoin	10.3^{b}	11.5^{b}	7.92^{a}	10.9^{b}	0.708	0.01	0.05	0.22
Uric acid	0.56	0.70	0.60	0.76	0.059	0.08	0.26	0.89
Xanthine	0.82^{a}	1.62^{b}	0.92^{a}	1.09^{ab}	0.191	0.08	0.29	0.13
Hypoxanthine	1.51	1.36	1.57	1.40	0.225	0.48	0.83	0.94
Creatinine	9.89	10.3	9.50	9.88	0.45	0.43	0.54	0.83

$^{a, b}$ Within a row, means with unlike superscripts differ (P<0.05).

When all values were analysed together, MNDF values estimated from the urinary excretion of both total PD and allantoin were lower than those obtained with ^{15}N in LAB (P=0.002 and 0.07, respectively) and SAB (P<0.001). There were, however, differences among diets. For diet HFG, there was a good agreement between the MNDF values estimated by all methods (P=0.17 to

0.87). In contrast, total PD underestimated MNDF values compared to ^{15}N in LAB by 13.8, 23.8 and 15.7% for HFA, HCA and HCG diets, respectively, and by 16.6, 23.9 and 13.8% when using SAB as reference. Compared with ^{15}N in LAB, the use of allantoin resulted in MNDF values which were 7.8, 18.0 and 6.8% lower for HFA, HCA and HCG diets, respectively, and 16.6, 23.9 and 13.8% lower when SAB were used as reference. Our results are in accordance with those from Pérez *et al.* (1996), who found that allantoin-based estimations of MNDF were between 24 and 35% lower than those obtained with ^{15}N in sheep fed four diets varying in straw:barley grains ratio. Similarly, Pérez *et al.* (1997) observed that values of MNDF estimated from urinary PD and allantoin were on average 27.7 and 37.8% lower than those measured by using ^{15}N in sheep fed diets with different protein sources. The average values of efficiency of microbial synthesis were 24.8, 27.4, 21.1 and 22.8 g of microbial N/kg of organic matter apparently digested in the rumen when estimated from ^{15}N-LAB, ^{15}N-SAB, total PD and allantoin, respectively. Values differed significantly between methods of estimation (P<0.05), but were in the range of those previously reported for sheep (Pérez *et al.*, 1996,1997).

Table 3. Effect of forage:concentrate ratio (F:C) and type of forage (FOR) on microbial N duodenal flow (MNDF; g N/d) and efficiency of microbial synthesis (EMS; g N/kg organic matter apparently fermented in the rumen) in sheep fed diets with F:C ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage

Item	Diet					Significance of effects (P =)		
	HFA	HCA	HFG	HCG	SEM	F:C	FOR	F:C x FOR
MNDF†								
^{15}N-LAB	13.2^{b}	16.9^{c}	9.45^{a}	14.4^{bc}	0.88	0.001	0.005	0.59
^{15}N-SAB	14.6^{b}	18.2^{c}	10.8^{a}	15.5^{bc}	0.95	0.001	0.008	0.55
PD	11.4^{b}	13.1^{b}	9.35^{a}	12.1^{b}	0.62	0.001	0.003	0.39
Allantoin	12.6^{b}	13.9^{b}	10.0^{a}	13.4^{bc}	0.74	0.01	0.06	0.18
EMG								
^{15}N-LAB	21.8^{a}	31.1^{b}	17.9^{a}	29.5^{b}	1.63	<0.001	0.10	0.61
^{15}N-SAB	24.1^{a}	33.5^{b}	20.4^{a}	32.2^{b}	1.69	<0.001	0.18	0.48
PD	18.9^{a}	23.3^{b}	17.8^{a}	24.7^{b}	1.13	0.001	0.83	0.26
Allantoin	20.8^{a}	24.7^{b}	18.9^{a}	27.7^{b}	1.12	0.002	0.73	0.15

$^{a, b}$ Within a row, means with unlike superscripts differ (P<0.05).

† Estimated from measurements of duodenal flow and ^{15}N with liquid- (LAB) and solid-associated bacteria (SAB) as reference, from urinary excretion of total purine derivatives (PD; Chen *et al.*, 1992) and from urinary excretion of allantoin (Balcells *et al.*, 1991).

Although there were differences in the values of MNDF between the methods investigated in our study, all of them detected similar differences (P<0.05) among diets, and therefore did not influence the interpretation of results. In addition, there was a positive relationship between the values of MNDF obtained using ^{15}N (mean values for LAB and SAB) and those estimated from urinary excretion of either PD (r=0.75; P<0.001; n=16) or allantoin (r=0.73; P<0.001; n=16).

IV – Conclusions

Under the conditions of the present study, the urinary excretion of PD and allantoin underestimated the MNDF compared with ^{15}N for high-quality diets, but produced similar values for a medium-quality diet. All methods detected the same significant differences among diets, and

there were positive relationships between the MNDF values obtained with ^{15}N and those estimated from urinary excretion of PD and allantoin. These results strength the use of urinary excretion of PD as a simple method to estimate MNDF in sheep.

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