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The Effects of Seasonal Variation on the Microbial-N Flow to the Small Intestine and Prediction of Feed Intake in Grazing Karayaka Sheep

Mustafa Salman, Nurcan Cetinkaya, Zehra Selcuk, Bugra Genc

***Abstract*—**The objectives of the present study were to estimate the microbial-N flow to the small intestine and to predict the digestible organic matter intake (DOMI) in grazing Karayaka sheep based on urinary excretion of purine derivatives (xanthine, hypoxanthine, uric acid, and allantoin) by the use of spot urine sampling under field conditions. In the trial, 10 Karayaka sheep from 2 to 3 years of age were used. The animals were grazed in a pasture for ten months and fed with concentrate and vetch plus oat hay for the other two months (January and February) indoors. Highly significant linear and cubic relationships ($P<0.001$) were found among months for purine derivatives index, purine derivatives excretion, purine derivatives absorption, microbial-N and DOMI. Through urine sampling and the determination of levels of excreted urinary PD and Purine Derivatives / Creatinine ratio (PDC index), microbial-N values were estimated and they indicated that the protein nutrition of the sheep was insufficient.

In conclusion, the prediction of protein nutrition of sheep under the field conditions may be possible with the use of spot urine sampling, urinary excreted PD and PDC index. The mean purine derivative levels in spot urine samples from sheep were highest in June, July and October. Protein nutrition of pastured sheep may be affected by weather changes, including rainfall. Spot urine sampling may useful in modeling the feed consumption of pasturing sheep. However, further studies are required under different field conditions with different breeds of sheep to develop spot urine sampling as a model.

***Keywords*—**Karayaka sheep, spot sampling, urinary purine derivatives, PDC index, microbial-N, feed intake.

I. Introduction

THE Karayaka sheep is one of the indigenous breeds reared on the coastline of the Black Sea region of Turkey. The breed is mainly kept for its high meat quality because its milk production is lower than other native breeds. Karayaka sheep are well adapted to the wet climate of the region and there is a total population of approximately one million [1]. The quality of grazing in the areas of its distribution is much better than in most of the other regions and the grazing season is longer.

The use of spot urine sampling has been proposed to predict protein nutrition and digestible organic matter intake in grazing sheep [2] and goats [3]. Microbial protein flow to the small intestine has been estimated total of purine derivatives excreted in the urine of ruminants [4], [5]. Rumen microbes constitute the major source of protein supply to the ruminants. The purines from the rumen microbes are metabolized and excreted in the urine as their derivatives, hypoxanthine, xanthine, uric acid and allantoin. Nucleic acids leaving the rumen are essentially of microbial origin. That is because ruminant feeds usually have a low purine content, most of which undergoes extensive degradation in the rumen as the result of microbial fermentation. In sheep, hypoxanthine and xanthine are converted to uric acid by xanthine oxidase, and uric acid is further converted to allantoin by uricase. All four compounds are excreted in the urine of sheep. The synthesis of microbial protein is dependent on ruminal ammonia nitrogen supply [6] and digestible organic matter intake [7], [8].

Reports that estimate microbial-N flow to the small intestine are mostly from European sheep breeds [2], [8]-[11].

The objectives of the present study were to examine the microbial-N flow to the small intestine and to predict DOMI in grazing Karayaka sheep on the basis of urinary excretion of PD by the use of spot urine sampling under field conditions and to investigate the effects of seasonal variation on ruminal microbial synthesis.

II. Material and Methods

A. Animal and Feed Materials

A total of 10 Karayaka sheep aged between 2 and 3 years, and with live weight ranging from 42.4 to 47.6kg, were used in the this study that was undertaken in Akyazı village of Bafra town, Samsun province, Turkey. The animals were grazed in a pasture for ten months and fed a concentrate and vetch plus oat hay for the other two months (January and February) indoors.

Meadow and pasture samples were collected by using the visual estimation method. Plant samples were taken by hand clipping to ground level at the beginning of the grazing experiment and repeated every month. They were collected from one square meter area in six different locations in a 0.5-1 ha area [12]. The important part of meadow and pasture was formed by *Agropyro ncristatum*, *Lotus corniculatus* L., *Agropyron elongatum*, *Bromus inermis*, *Convolvulus* sp., *Trifolium pretense, Trifolium repense*, and *Dactylis glomerata*. All plant samples were dried to a constant weight in a forced-air oven at 65°C for 48h. Dry matter (DM), nitrogen (N), neutral detergent fibre (NDF), and acid detergent fibre (ADF) were determined after the grinding through a 1mm screen. Ash

Mustafa Salman is with the Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139, Samsun, Turkey (e-mail: msalman@omu.edu.tr).

Nurcan Cetinkaya, Zehra Selcuk, and Bugra Genc are with the Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139, Samsun, Turkey.

content was determined by heating in a muffle furnace at 550°C [13]. Organic matter (OM) was calculated as DM–ash. N content was analyzed with the Kjeldahl method, with the use of a semi-automated N analyzer. NDF and ADF were determined according to the methodology of van Soest et al. [14].

The levels of purine derivatives were determined according to the methodology of Chen et al. [15] using a spectrophotometer (Shimadzu UV-1700). Allantoin is first hydrolyzed under weak alkaline conditions and at 100°C to allantoic acid which is further hydrolysed to urea and glyoxylic acid in a weak acid solution. The glyoxylic acid is reacted with phenylhydrazine hydrochloride to produce a phenylhydrazone derivative of the acid. The product forms an unstable chromophore with potassium ferricyanide. The colour was read at 522nm. Xanthine and hypoxanthine are converted to uric acid by treatment with xanthine oxidase and are thus determined as uric acid, the amount of which is determined by its absorbance at 293nm, although other compounds may also absorb at this wavelength. When samples are treated with uricase, uric acid is converted to allantoin and other compounds that do not absorb UV at 293 nm. Therefore, the reduction in absorbance reading after treatment with uricase is correlated with the concentration of uric acid in the sample. After treatment, the absorbance of the standards should be zero, if the conversion is complete. Creatinine reacts with picrate ion formed in alkaline medium and a red-orange colour develops. The colour produced from the sample is then compared in a colorimeter at 505nm with that produced by a known amount of creatinine under the same conditions [5].

B. Urinary Sample Collection

Spot urinary samples were collected from each sheep between 10:00 am and 12:00 pm (2h after grazing) on two consecutive days per month for a year. Urine samples were collected by closing the mouth and nose of the sheep by hand. Samples were usually collected over a 10 to 35second period. After collection, a 10ml sub-sample was taken, acidified with 1ml of 10% H_2SO_4 and diluted to 1:4 with distilled water. The samples were stored at -20C^{o} until analysis for purine derivatives.

C. Estimation of Microbial-N Supply

The intestinal flow of microbial-N supply was estimated using the following equation for sheep 5.

$$Y = 0.84X + (0.150\ W^{0.75\ e\text{-}0.25X})$$

where X (mmol/d) is absorption of purines (X mmol/d) and Y (mmol/d) is PD excretion in the urine.

The supply of microbial-N was estimated below from the relationship derived by CHEN and GOMES 11. The assumptions used in the above equations are: digestibility of microbial purines is 0.83. The N content of purines is 70 mg N/mmol and the ratio of purine-N: total-N in mixed rumen microbes is 11.6:100 [5]. Therefore, the equation to determine microbial-N is:

Microbial-N estimated from purine derivatives excretion corresponds to the quantity of microbial biomass reaching the duodenum, rather than that synthesized within the rumen. It is expressed as grams of microbial-N per kilogram of digestible organic matter apparently digested in the rumen. The model assumes that the digestible organic matter in the rumen is 65% of digestible organic matter intake [16].

D. Estimation of PDC Index

PDC index is determined by calculating the creatinine concentrations and total purine derivatives in the urine. The following equation is used to index the PDC:

$$\text{PDC index} = (PD/C)xW^{0.75}$$

where W is the body weight (kg), and PD and C are purine derivatives and creatinine concentrations, respectively in mmol/L.

$$\text{PD excretion (mmol/d)} = \text{PDC index} \times C$$

where C is the daily creatinine excretion (mmol/kg W0.75) for a specific breed of animals, which should have been previously measured from the complete urine collection. Average daily creatinine excretion was taken to be 503μmol/kg CA0.75 for sheep [5].

E. Estimation of Digestible Organic Matter Intake (DOMI)

The spot measurement of the PDC index can provide an estimate of feed intake. Digestible organic matter intake was calculated with the following equation [5]:

$$\text{DOMI (g /d)} = 59.7xPDC\text{-}678$$

F. Statistical Analysis

Data were summarized with descriptive statistics for means, and the standard errors of the means were analyzed with analysis of variance (ANOVA), using the Least Square Method of the GLM procedure of the SAS [17]. The differences between the groups were analyzed via 3rd order orthogonal polynomials. All results were summarized as mean ± standard error of mean (SEM). Ordinary linear regression and Pearson correlation analyses were performed with the use of variables, namely allantoin, microbial-N, DOMI and purine derivatives excretion, and digestible organic matter digested in the rumen.

III. Results

The dry matter (DM), organic matter (OM), crude protein (CD), neutral detergent fiber (NDF) and acid detergent fiber (ADF) values of meadow and pasture samples (g/kg DM) collected monthly are presented in Table I. Levels of creatinine and allantoin, uric acid, hypoxhanthine and xanthine in spot urine samples are shown in Table II. The purine derivatives index (PDC index), purine derivatives excretion, purine derivatives absorption, microbial-N and DOMI for spot urine samples are shown in Table III. Monthly variations in both mean values of the PDC index and microbial-N (Fig. 1) and mean values of DOMI are presented in Fig. 2.

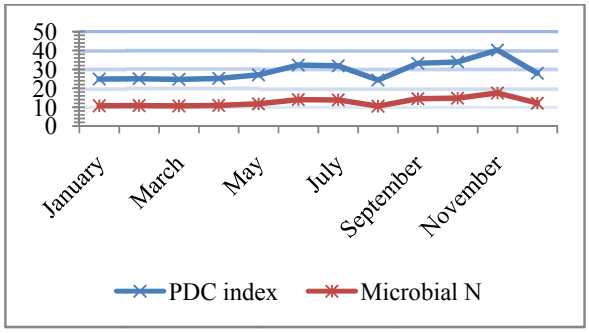

Fig. 1 Monthly changes of mean values of PDC index and Microbial-N (g N/d) for grazing Karayaka sheep

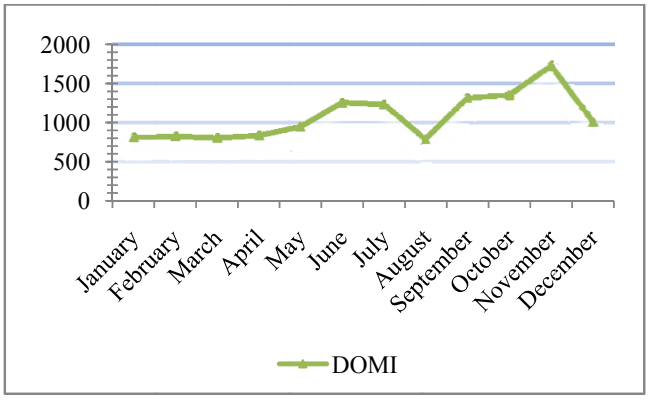

Fig. 2 Monthly changes of mean values of DOMI (g/d) for grazing Karayaka sheep

TABLE I
THE NUTRIENT COMPOSITION OF MEADOW AND PASTURE SAMPLES COLLECTED MONTHLY (G/KG DM).

Years	Period Months		DM	OM	CP	NDF	ADF
	June		913.0	838.4	134.0	437.2	351.7
	July		931.2	866.5	148.0	476.8	337.7
	August		892.5	801.2	106.5	500.2	392.1
2010	September		909.3	830.9	143.7	479.4	334.9
	October		905.6	824.5	131.1	442.1	313.5
	November		913.0	837.6	107.5	468.4	345.5
	December		904.3	827.3	101.2	543.7	415.5
	January	1	902.1	829.4	118.0	613.0	469.6
	February	2	896.0	832.7	138.5	232.7	113.7
2011	March		917.5	851.3	127.5	410.8	264.0
	April		923.4	819.4	154.0	403.4	246.8
	May		906.8	840.3	135.7	451.6	344.0

1: Mixed vetch and oat hay were used to feed sheep in January and February; 2: Concentrate was used to feed sheep in January and February.

TABLE II
MEAN CONCENTRATES (MMOL /L) OF ALLANTOIN, URIC ACID, HYPOXANTHINE PLUS XANTHINE AND CREATININE IN SPOT URINE SAMPLES COLLECTED FROM GRAZING KARAYAKA SHEEP

Year	Grazing period Months	n	Allantoin	Uric acid	Hypoxanthine +Xanthine	Creatinine	Purine derivatives
	June	9	7.67±0.37	1.76±0.18	0.65±0.06	6.39±0.19	10.08±0.44
	July	9	7.71±0.38	1.40±0,08	0.68±0.06	6.30±0.32	9.79±0.43
	August	10	6.17±0.18	1.25±0.06	0.61±0.06	6.75±0.23	8.02±0.18
2010	September	10	6.81±0.29	1.77±0.09	0.75±0.07	5.74±0.21	9.32±0.29
	October	10	8.62±0.58	1.59±0.12	0.68±0.04	6.49±0.28	10.90±0.66
	November	9	7.28±0.27	1.64±0.19	0.35±0.03	4.72±0.22	9.27±0.31
	December	10	5.04±0.22	1.96±0.10	0.32±0.02	5.44±0.27	7.32±0.21
	January	9	4.84±0.18	0.87±0.05	0.42±0.04	5.00±0.16	6.13±0.20
	February	8	6.31±0.31	1.29±0.13	0.53±0.03	6.64±0.28	8.12±0.31
2011	March	10	5.64±0.28	0.59±0.04	0.50±0.05	5.60±0.27	6.73±0.30
	April	10	5.12±0.20	1.29±0.09	0.54±0.06	5.61±0.15	6.95±0.24
	May	9	5.55±0.33	1.28±0.12	0.48±0.05	5.46±0.20	7.31±0.35
	Significance of main effects						
	Linear		***	***	NS	NS	***
	Quadratic		***	NS	***	***	***
	Cubic		***	NS	***	NS	***

***P<0.001, NS:Non Significant, L:Linear, Q:Quadratic, C:Cubic

TABLE III
MEAN LEVELS OF PURINE DERIVATIVES INDEX (PDC INDEX), PURINE DERIVATIVES EXCRETION, PURINE DERIVATIVES ABSORPTION, MICROBIAL-N SUPPLY AND DOMI IN SPOT URINE SAMPLES COLLECTED FROM GRAZING KARAYAKA SHEEP

Grazing period		n	PDC index	PD excretion (mmol/d)	Purine absorption (mmol/d)	Microbial-N supply		DOMI g/d
Year	Months					(g of N/d)	(g of N/kg of DOMR)	
	June	9	32.29±1.51	16.24±0.76	19.28±0.92	14.02±0.67	20.87±0.73	1249.44±90.31
	July	9	31.90±1.02	16.04±0.52	19.06±0.62	13.86±0.45	20.86±0.78	1226.26±61.17
	August	10	24.36±0.74	12.25±0.37	14.48±0.45	10.53±0.33	21.06±0.70	776.13±44.04
2010	**September**	10	33.27±1.17	16.74±0.59	19.89±0.70	14.46±0.51	20.98±0.70	1308.28±69.59
	October	10	33.98±1.40	17.09±0.70	20.31±0.85	14.77±0.62	19.76±0.73	1350.41±83.61
	November	9	40.26±1.20	20.25±0.60	24.10±0.72	17.52±0.52	18.12±0.73	1725.41±71.46
	December	10	28.00±1.14	14.08±0.57	16.69±0.69	12.14±0.50	17.49±0.73	993.41±67.84
	January	9	24.90±0.69	12.53±0.35	14.81±0.43	10.77±0.31	21.24±0.70	808.77±41.49
	February	8	25.04±0.99	12.60±0.50	14.89±0.61	10.83±0.44	17.26±0.70	816.91±59.32
2011	**March**	10	24.71±0.92	12.43±0.46	14.69±0.56	10.68±0.41	17.29±0.70	797.47±54.68
	April	10	25.23±0.94	12.69±0.47	15.01±0.57	10.91±0.42	15.69±0.73	828.42±56.06
	May	9	27.14±0.96	13.65±0.48	16.18±0.59	11.76±0.43	19.34±0.70	942.39±57.47
Significance of main effects								
Linear			***	***	***	***	***	***
Quadratic			NS	NS	NS	NS	NS	NS
Cubic			***	***	***	***	*	*

***P<0.001, *P<0.05, NS: Non Significant, DOMR: Digestible organic matter fermented in the rumen calculated as 0.65xDOMI (g of N/kg of DOMR), DOMI: Digestible organic matter intake g/d.

IV. DISCUSSION

Measurement of microbial protein supply to sheep has been a major area of study in the context of their protein nutrition. An estimate of microbial protein contribution to the intestinal protein flow is incorporated into the new protein evaluation systems already being used in a number of countries. The supply of microbial protein to the animal per unit of feed ingested varied from 14 to 60g microbial-N/kg digestible organic matter fermented in the rumen [16]. This variation is due to the influence of various factors related to the diet or rumen environment.

As seen in Table I, CP levels of plant samples collected monthly did not fluctuate all year around. The lowest CP levels were observed in August and December. They are in agreement with estimated mean microbial-N and DOMI values (Figs. 1, 2) for Karayaka sheep.

Creatinine is produced from creatine in muscle, and is excreted in the urine. Its excretion is correlated with the muscle mass. When expressed as 'mmol/per kg W0.75', the daily excretion is relatively constant. The value is approximately 0.5mmol/kg W0.75/d in sheep [5]. The excretion of creatinine is affected minimally by the amount of protein and non-protein nitrogen consumed. In the current study, creatinine concentrations in the spot urine samples were higher in August, October and February than in other months but overall values were not significantly influenced by season (Table II). There was no linear or cubic relationship between creatinine excretion and month but a quadratic relationship was determined. It was also the case that season did not affect creatinine excretion in urine.

The method for estimating microbial-N production from urinary purine derivatives assumes that duodenal nucleic acids are mostly of microbial origin [15], [18]. It has been reported that after intestinal digestion and absorption, the purine base catobolites are proportionally recovered in urine, mostly as allantoin, but also as hypoxanthine, xanthine and uric acid 18. The present study reports 69-84%, 9-27% and 4-8%, for allantoin, uric acid and xanthine plus hypoxanthine, respectively. For the same catabolites, Chen and Gomes [11] reported the proportions to be 60-80%, 10-30% and 5-10% for allantoin, uric acid and xanthine plus hypoxanthine, respectively. Furthermore, allantoin excretion in urine was reported to be 80-85% of total purine derivatives. The profile of PD excretion in grazing Karayaka sheep was similar to that reported in previous studies [19]-[21]. The results obtained may indicate that the proportion of purine derivatives is independent of diet.

In the current study, the levels of allantoin in the urine of grazing sheep ranged from 4.84-8.62 mmol/L on a monthly basis across the sampling period. The study also determined a positive correlation (r=0.615, P<0.01) between the amount of allantoin excreted in urine and rumen microbial protein flowing into the small intestine, as described by the equation:

$$Y=0,378X+1,594$$

wherey is the amount of allantoin excreted in urine and x is rumen microbial protein flowing into the small intestine. This equation showed that allantoin excretion was able to enhance duodenal flow of microbial protein. Studies of cattle [22], [23] and sheep [7] have indicated a high correlation between the excretion of purine derivatives and rumen microbial protein flow into the small intestine (R2 = 0.97). In the present study, the average allantoin amounts in spot urine samples for sheep were lowest in January, April and December, whereas the highest values were determined in June, July and October. In 2010, allantoin amounts in June, July and October were higher than in other months (Table II). That phenomenon may reflect the impact of vegetation changes due to higher than average

rainfall during those months.

The higher excretion of PD in October and November clearly indicates enhanced microbial protein synthesis, since significant relationships have already been reported between urinary PD excretion and the levels of nucleic acid infused in the abomasums [4], [15] and duodenum [9], [24]. Orellana-Boero et al. [25] found that the excretion of purine derivatives in the urine increased linearly (r = 0.867) with digestible organic matter intake. The principle is that duodenal purine bases are efficiently absorbed in the small intestine [24], [26]. Urinary PD excretion is used to predict ruminal microbial protein synthesis. The daily excretion of purine derivatives and the microbial-N supply in grazing Karayaka sheep were found to be in the range of 12.25-20.25 and 10.53-17.52 mmol/d, respectively (Table III). These values for Karayaka sheep are within the range of those published for different sheep breeds [2], [10]. The urinary PD excretion values obtained in goats [3] and wethers are similar to those observed in sheep [10], [27], [28]. Hence, purine derivatives in spot urine samples may provide a practical indicator of microbial protein supply status in grazing ruminants.

The estimated average monthly DOMI values (Table III) in grazing Karayaka sheep were within the range of 776 to 1725 g/day. The estimation of DOMI from PDC index (Table III) through the use of spot urine sampling from grazing Karayaka sheep showed that this technique may be applied in grazing animals where DOMI cannot be measured directly. Many reports have confirmed a linear relationship between allantoin excretion and both the level of feed intake and flow of nucleic acids in the duodenum [29]. Laurent et al.[30] determined that allantoin excretion is correlated with digestible organic matter intake, and also that allantoin excretion ($r=0.54$, $P<0.01$) can be used as an index of rumen microbial protein synthesis, as also reported by Jetana et al. [31] and Laurent et al. [30].

The present study also determined that digestible organic matter fermented in the rumen was converted to a similar proportion of microbial-N in all months, ranging from 16 to 21 g N/kg of digestible organic matter apparently digested in the rumen (Figs. 1, 2; Table III), which was similar the range reported by Yu et al. [32]. The higher amount of urinary allantoin reported in the present study in June, July, September, October and November was due to the increased digestible organic matter intake. The present study also determined that there were linear and cubic relationship between the PDC index, urinary excretion of purine derivatives, microbial-N and DOMI and month (Table III), and that there was a seasonal influence on these parameters.

In the current study, the estimated microbial-N values appear insufficient for adequate protein nutrition. Monthly mean values of PDC index, microbial-N and DOMI were relatively stable. However, fluctuations were observed between June and December (Figs. 1, 2). The lowest values for DOMI, PDC index and microbial-N were observed in August and December (Figs. 1, 2). This is due to meadow and pasture conditions reflecting the driest period of the year (Fig. 1). In conclusion, protein nutrition of pastured sheep may be affected by weather changes. Spot urine sampling may serve as the basis for modeling their feed requirements. Furthermore, it may provide a basis for the preparation of balanced diets to meet the protein requirements of sheep by closely approximating the amount of rumen microbial-N flowing into the small intestine. However, further studies are required under different field conditions and with different breeds of sheep to develop the spot urine sampling technique into a model.

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