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RESEARCH ARTICLE

The Effects of Seasonal Variation on the Microbial-N Flow to the Small Intestine and Prediction of Feed Intake in Grazing Karayaka Sheep [1]

Mustafa SALMAN [1] Nurcan CETINKAYA [1] Zehra SELCUK [1] Bugra GENC [1]

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[1] Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Ondokuz Mayis University, TR-55139 Campuse/Kurupelit, Samsun - TURKEY

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Summary

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 the 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*

Merada Otlayan Karayaka Koyunlarında Mevsimsel Değişimin İnce Bağırsağa Geçen Mikrobiyal-N Miktarı ve Yem Tüketimine Etkisi

Özet

Merada otlayan Karayaka koyunlarında spot idrar örneklemesi ile idrarla atılan purin türevlerinden (ksantin, hipoksantin, ürik asit ve allantoin) ince bağırsağa geçen mikrobiyal-N miktarı ve sindirilebilir organik madde tüketiminin (SOMT) mevsimsel olarak belirlenmesi amaçlanmıştır. Araştırmada 2-3 yaşlarında 10 adet Karayaka koyunu kullanılmıştır. Hayvanlara yem materyali olarak 10 ay mera ve 2 ayda (Ocak, Şubat) kapalı ağıllarda fabrika yemi ve fiğ +yulaf otu ile beslenmiştir. Purin türevleri indeksi, idrarla atılan purin türevleri, absorbe edilen purin türevleri, mikrobiyel-N ve SOMT yönünden aylara göre bir değerlendirme yapıldığında gruplar arasında linear ve kübik (P<0.001) bir ilişki tespit edilmiştir. Mikrobiyal-N değerlerine bakıldığında koyunların protein yönünden yetersiz beslendikleri görülmüştür. Sonuç olarak spot idrar toplanarak denemenin yapıldığı benzer saha koşullarında otlatılan Karayaka koyunlarda protein beslenmesini tahmin etmekte idrarla atılan pürin türevleri ve Purin Türevleri: Kreatinin (PDC indeksi) kullanılabilir. Ortalama PT miktarı Karayaka koyunları için spot idrar toplamada en yüksek değerler Haziran, Temmuz ve Ekim aylarında belirlenmiştir. Bu aylardaki iklimsel değişikliklerden (yağış fazlalığı) dolayı meradaki koyunların protein beslenmesi etkilenmiştir. Ayrıca spot idrar toplama tekniği meradaki koyunların yem tüketimini belirlemede bir model olabilir. Bunun içinde farklı saha koşullarında farklı koyun ırklarıyla benzer çalışmaların yapılmasına ihtiyaç vardır.

Anahtar sözcükler: *Karayaka koyunu, Spot örnekleme, Idrar purin türevleri, PDC indeksi, Mikrobiyal-N, Yem tüketimi*

İletişim (Correspondence)
+90 362 3121919/2591
msalman@omu.edu.tr

INTRODUCTION

The Karayaka sheep is one of the indigenous breeds reared on the coastline of the Black Sea region of Turkey. Karayaka sheep are well adapted to the wet climate of the region and there is a total population of approximately one million [1].

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. 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.

MATERIAL and METHODS

The study was approved by the Local Ethics Committee on Animal Experiments of Ondokuz Mayis University (OMU, 30.12.2009, HADYEK/139).

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 47.6 kg, 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.

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 *Agropyron cristatum, 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 48 h. Ash content was determined by heating in a muffle furnace at 550°C [13]. Organic matter (OM) was calculated as DM–ash. Nitrogen (N) content was analyzed with the Kjeldahl method, with the use of a semi-automated N analyzer. Neutral detergent fibre (NDF) and acid detergent fibre (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 522 nm. 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 293 nm, 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 505 nm with that produced by a known amount of creatinine under the same conditions [5].

Mean monthly rainfall and air temperature (°C) in Bafra in 2010 and 2011 were collated by the Samsun Meteorological Office and are presented in *Fig. 1*.

Urinary Sample Collection

Spot urinary samples were collected from each sheep between 10:00 am and 12:00 pm (2 h 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 35 sec period. After collection, a 10 ml sub-sample was taken, acidified with 1 ml of 10% H_2SO_4 and diluted to 1:4 with distilled water. The samples were stored at -20°C until analysis for purine derivatives.

Estimation of Microbial-N Supply

The microbial-N supply was estimated using the following equation for sheep [5].

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

Where X (mmol/d) is absorption of purines (X mmol/d)

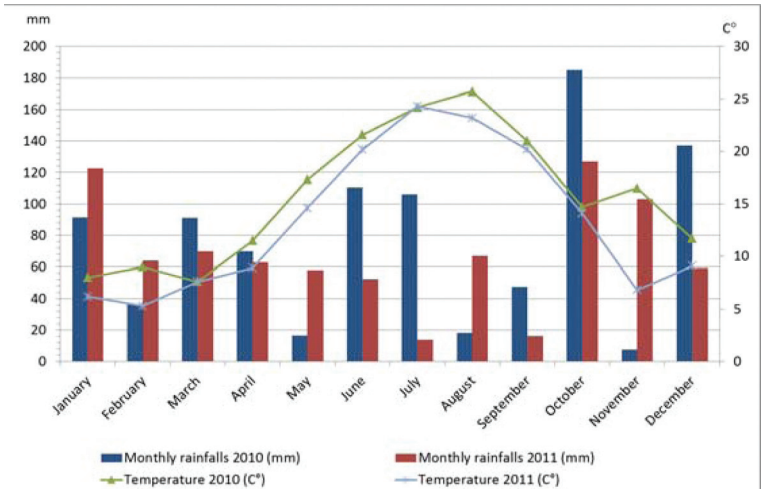

Fig 1. Mean monthly rainfall and air temperature (°C) in Bafra in 2010 and 2011

Şekil 1. Bafra'da 2010 ve 2011 yıllarında ortalama aylık yağış ve sıcaklık miktarları (°C)

and Y (mmol/d) is PD excretion in the urine. The calculation of X based on the above non-linear equation can be performed by means of the Newton Raphson iteration process, as shown below:

$X_{(n+1)} = X_n - [f(X_n) / f'(X_n)]$; where $f(X) = 0.84\ X + 0.150\ W^{0.75}\ e^{-0.25X} - Y$, and the derivative of f(X): $f'(X) = 0.84 - 0.038\ W^{0.75}\ e^{-0.25X}$

An initial value of $X_1 = Y \div 0.84$ is fed into the above equation to calculate X_2, and after the process is repeated for 4 iterations, X_5 should have reached a constant value.

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 (gN/d) = [X (mmol/d) x 70] / [0.116 x 0.83 x 1000]=0.727X

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].

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:

$$PDC\ index = (PD/C) \times W^{0.75}$$

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

PD excretion (mmol/d) = PDC index × C where C is the daily creatinine excretion (mmol/kg $W^{0.75}$) 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 503 µmol/kg $CA^{0.75}$ for sheep [5].

Estimation of Digestible Organic Matter İntake (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].

DOMI (g /d) = 59.7xPDC-678

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.

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 1*. Levels of creatinine and allantoin, uric acid, hypoxhanthine and xanthine in spot urine samples are shown in *Table 2*. The purine derivatives index (PDC index), purine derivatives excretion, purine derivatives absorption, microbial-N and DOMI for spot urine samples are shown in *Table 3*. Monthly variations in both mean values of the PDC index and microbial-N *(Fig. 2)* and mean values of DOMI are presented in *Fig. 3*.

Table 1. *The nutrient composition of meadow and pasture samples collected monthly (g/kg DM)*
Tablo1. *Aylık olarak toplanan çayır ve mera örneklerinin besin madde kompozisyonu (g/kg KM)*

Period			DM	OM	CP	NDF	ADF
Years	Months						
2010	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
	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
2011	January	1	902.1	829.4	118.0	613.0	469.6
	February	2	896.0	832.7	138.5	232.7	113.7
	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 2. *Mean concentrates (mmol /L) of allantoin, uric acid, hypoxanthine plus xanthine and creatinine in spot urine samples collected from grazing Karayaka sheep*
Tablo 2. *Merada otlayan Karayaka koyunlarından toplanan spot idrar örneklerinde allantoin, ürik asit, hipoksantin + ksantin ve kreatinin ortalama değerleri (mmol/L)*

Grazing Period		n	Allantoin	Uric Acid	Hypoxanthine +Xanthine	Creatinine	Purine Derivatives
Year	Months						
2010	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
	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
2011	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
	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*

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 60 g 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 1*, CP levels of plant samples collected monthly did not fluctuate all year around. The lowest CP levels were

Table 3. *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*

Tablo 3. *Merada otlayan Karayaka koyunlarından toplanan spot idrar örneklerinde PDC indeksi, pürin türevleri ekskresyonu, pürin türevleri absorpsiyonu, mirobial-N ve DOMI ortalama değerleri*

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)	
2010	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
	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
2011	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
	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*

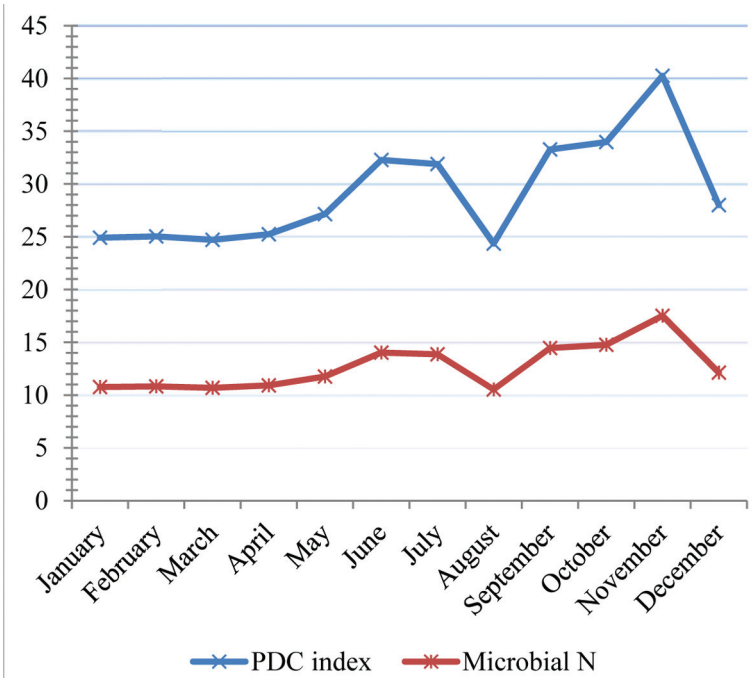

Fig 2. Monthly changes of mean values of PDC index and Microbial-N (g N/d) for grazing Karayaka sheep

Şekil 2. Merada otlayan Karayaka koyunlarında ortalama PDC index ve Mikrobial N değerlerinin aylık değişimi

observed in August and December. They are in agreement with estimated mean microbial-N and DOMI values *(Fig. 2* and *3*) 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 $W^{0.75}$', the daily excretion is relatively constant. The value is approximately 0.5 mmol/kg $W^{0.75}$/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

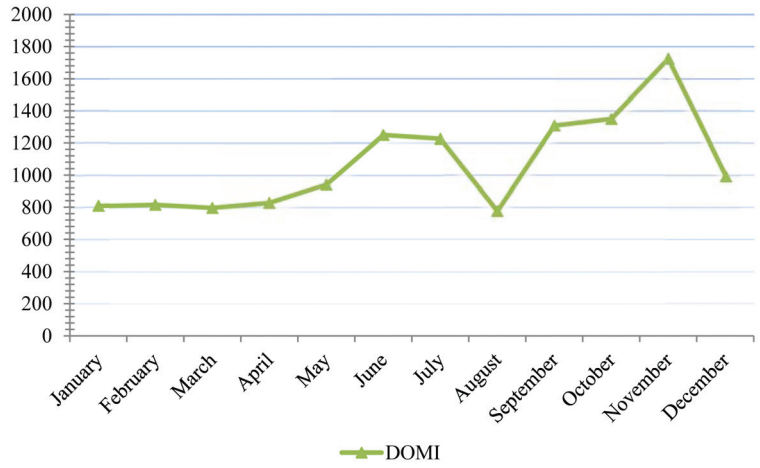

Fig 3. Monthly changes of mean values of DOMI (g/d) for grazing Karayaka sheep

Şekil 3. Merada otlayan Karayaka koyunlarında ortalama DOMI (g/gün) değerlerinin aylık değişimi

influenced by season *(Table 2)*. 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]. 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

Where y 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 (R^2 = 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 2)*. 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 abomasum [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 3)*. 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 3)* in grazing Karayaka sheep were within the range of 776 to 1725 g/day. The estimation of DOMI from PDC index *(Table 3)* 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.

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 (*Fig. 2* and *3*; *Table 3*), which was similar the range reported by Yu *et al.*[31]. 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 3)*, 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 (*Fig. 2* and *3*). The lowest values for DOMI, PDC index and microbial-N were observed in August and December (*Fig. 2* and *3*). 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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