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Effect of urine collection period on the estimation of urinary volume and purine derivatives in crossbred goats

[Efeito do período de coleta de urina na estimativa do volume urinário e dos derivados de purina em caprinos mestiços]

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Abstract: The objective of the present study was to determine the appropriate adaptation period for metabolic evaluation and the ideal time for spot sample collection in goats. For this, an entirely randomized experimental design was used with four adaptation periods (9, 13, 17, and 21 days) and six sample collection times (4, 8, 12, 16, 20, and 24 hours). Ten Anglo-Nubian x SRD crossbred male goats, approximately 210 days old with an average initial body weight of 25 kg, were randomly distributed across the experimental treatments. The experiment lasted 50 days. The goats were fed twice a day with a diet consisting of 20% roughage and 80% concentrate, following the NRC (2007) recommendations for a daily weight gain of 180g/day. The diet was composed of Tifton-85 hay and a concentrate based on ground corn, soybean meal, and a mineral mix containing monensin (2.7mg/kg DM) or doses of piperidine alkaloids from the algaroba pod (APA) (9.2, 18.4, and 27.6mg/kg DM) as well as a control concentrate without additives. The adaptation periods were 9, 13, 17, and 21 days for evaluating urinary excretions (allantoin, xanthine, hypoxanthine, total purine derivatives, creatinine) by comparing total collection with spot and hourly samples. There was no significant effect (P>0.05) between adaptation days and collection times; however, to ensure greater data consistency, it is recommended to use at least 17 days of adaptation to enhance the dietary efficiency for the animals.

Keywords: Ionophores; microbial protein; ruminant nutrition.

Resumo: Objetivou-se no presente estudo determinar para caprinos o período de adaptação adequado para avaliação metabólica e o horário ideal para coleta spot. Para isso, utilizou-se delineamento experimental inteiramente casualizado com quatro períodos de adaptação (9; 13;17 e 21 dias) e seis horários de coleta (4, 8, 12, 16, 20 e 24). Dessa forma, dez caprinos mestiços Anglo Nubiano x SRD, machos não-castrados, com idade aproximada de 210 dias e peso corporal inicial médio de 25 kg foram aleatoriamente distribuídos nos tratamentos experimentais. O experimento teve duração de 50 dias. Os caprinos foram alimentados duas vezes ao dia, na proporção volumoso: concentrado de 20:80 conforme as recomendações do NRC (2007) para ganho de peso diário de 180g/dia. A dieta foi composta por feno de Tifton-85 e o concentrado a base de milho moído, farelo

de soja e mistura mineral contendo monensina (2,7mg/Kg MS) ou doses de alcaloides piperidínicos da vagem de algaroba (APA) (9,2; 18,4; 27,6mg/Kg MS) e um concentrado controle sem aditivos. Os períodos de adaptação foram 9, 13, 17 e 21 dias para avaliação das excreções de urinárias (alantoína, xantina, hipoxantina, derivados totais de purina, creatinina,) comparando coleta total com amostra spot e total horários. Não houve efeito significativo (P>0,05) para os dias de adaptação e horários de coleta, entretanto, a fim de garantir maior consistência dos dados, preconizar pelo menos 17 dias de adaptação para potencializar a eficiência de utilização da dieta pelos animais.

Palavras-chave: Ionóforos; nutrição de ruminantes; proteína microbiana.

1. Introduction

Microbial protein is a high-quality source of amino acids available for absorption [1]. It represents a significant portion of the protein digested in the small intestine of ruminants, accounting for up to 80% of the total absorbable protein. Therefore, accurate estimation of microbial protein synthesis (MPS) is fundamental, not only to optimize protein supplementation for ruminants, but also to minimize N losses to the environment and to develop optimized feeding strategies [2].

However, studies with animals are laborious and exhausting for those conducting them and can cause great stress for the animals. Therefore, due to concerns about animal welfare and comfort, there has been a search for less invasive, faster, and more efficient techniques that allow for the acquisition of consistent data without significantly interfering with the animals' homeostasis. Therefore, it is necessary to establish non-invasive and precise techniques for quantifying microbial protein. According to Chen and Gomes et al. [3] and Ma et al. [4], the quantification of microbial protein synthesis can be performed by the indirect method, using urinary excretion of purine derivatives.

The purine derivative technique is considered an accurate estimator of microbial protein synthesized in the rumen and is easy to determine, as it overcomes the disadvantages of more direct methods [5-8]. This technique is based on two main premises: I - that the flow of nucleic acids in the small intestine is predominantly of microbial origin and II - that purine bases, after intestinal digestion, are absorbed, metabolized and excreted in the urine as purine derivatives (hypoxanthine, xanthine, uric acid and allantoin).

The metabolite creatinine is excreted in the urine, in a relatively constant function of body weight, suffering little or no influence from dietary factors [9,10]. Thus, it is assumed that creatinine from muscle metabolism is not related to the diet of animals, showing its use as a good indicator to estimate urine volume from "*spot*" urine collections. Regarding goats, studies to obtain this data are still scarce. Therefore, the objective was to evaluate whether the type of collection (*spot*, accumulated by time, and total) influences the estimation of urinary volume in goats, as well as whether the adaptation period and the time after feeding affect the excretion of purine derivatives for estimating microbial protein synthesis.

2. Materials and methods

2.1 Ethical considerations and experiment location

The procedures adopted with the animals in this work were in accordance with the ethical principles of animal experimentation, approved in protocol 115/2015 by the Ethics Committee on the Use of Animals of the State University of Southwest Bahia, and the experiment was conducted in the goat and sheep farming sector of the Juvino Oliveira Campus, belonging to State University of Southwest Bahia, which is located in the city of Itapetinga, BA.

2.2 Animals, period and experimental design

Ten crossbred Anglo-Nubian x mixed breed male goats, uncastrated, approximately 210 days old and with an average initial body weight of 25 ± 4.8 kg, were used. The experiment lasted 50 days, divided into two experimental periods (blocks) of 25 days each. These were subdivided into four adaptation periods (9, 13, 17, and 21 days) and six independent collection times (4, 8, 12, 16, 20, and 24 hours after feeding), designed in randomized blocks.

2.3 Experimental diets and handling procedures.

The animals gradually adapted to concentration until they reached an intake level of 80%, while simultaneously adapting to the metabolic cages. The diets were balanced for goats according to the requirements described in the NRC [11] for a daily weight gain of 180 g. In this study, the animals were fed diets with a roughage:concentrate ratio of 20:80, using Tifton 85 hay (Cynodon cv. Tifton 85) as the roughage (Table 1).

Table 1. Chemical composition of ingredients and total experimental diet and proportion of ingredients in the concentrate in a goat rehearsal.

Nutritional components (%DM)[1]	Tifton 85 Hay	Concentrate	Total Diet[2]
Dry matter	88.94	87.40	87.71
Crude protein	9.69	19.13	17.24
Ether extract	1.55	3.35	2.99
NDFap[3]	71.04	13.82	25.26
Acid detergente fiber	38.72	4.99	11.74
Total carbohydrates	82.36	73.27	75.09
Non-fibrous carbohydrates	11.32	59.45	49.82
Lignin	6.13	1.21	2.19
Total digestible nutrients	54.79	82.10	76.64
Ratio of ingredients in the concentrate (%)			
Ground corn		71.4	
Soybean bran		26.0	
Mineral salt		2.6	

[1]Nutrients as a percentage of dry matter; [2]Roughage:concentrate ratio 20:80; [3] Neutral detergent fiber corrected for ash and protein.

The diets were offered *ad libitum* daily at 7:00 AM and 4:00 PM, allowing for 15% leftovers, and the water troughs were washed and refilled daily. The animals were weighed at the beginning and end of the period (after fasting from solid food) to assess the variation in body weight and obtain the average body weight to correlate with daily creatinine excretion.

2.4 Urine sample collection (total and spot)

After feeding, urine samples were collected on the 9th, 13th, 17th, and 21st days of each experimental cycle. Three types of collection were performed, via spontaneous urination, as described below.

2.4.1 Total urine collection (24 hours)

A total urine collection (24 h) from each animal was performed using collection buckets positioned below each metabolic cage containing 100 ml of 20% (v/v) H_2SO_4 to acidify the sample, maintain the pH below 4, and thus prevent its deterioration. At the end of 24 hours, the urine was weighed, homogenized, filtered through layers of gauze, and a 50 mL aliquot was collected and stored.

2.4.2 Spot urine collection (every 4 hours for a period of 24 hours)

Spot urine samples were collected at 4-hour intervals over a 24 hour period. In this case, the urine sample was collected using collection bags which were placed on the prepuce of each animal at six specific times (4, 8, 12, 16, 20, and 24). 10 mL aliquots were diluted in 40 mL of 0.036N H_2SO_4, properly labeled, and stored.

2.4.3 Total cumulative urine (over a 24 hour period)

A collection bucket was positioned below each metabolic cage, and the volume of urine produced was accumulated and measured by weighing every four hours (4, 8, 12, 16, 20, and 24 hours). This was then compared to the volume estimated from daily creatinine excretion. A 10 mL aliquot of urine was collected at each time point and subsequently diluted in 40 mL of 0.036N sulfuric acid. At the end of the 24-hour period, the volume collected from the samples was counted for comparison with the estimated daily urine volume using the daily creatinine excretion, and all samples were stored in a freezer at -20ºC for subsequent laboratory analysis.

Urea and creatinine concentrations in urine were determined using commercial kits (Bioclin®, Belo Horizonte, MG, Brazil), and absorbance readings were measured using a UV-Visible Spectrophotometer (Single-Beam) Model UV-M51 – BEL. Allantoin, xanthine and hypoxanthine contents were determined by colorimetric methods, according to the methodology described by Chen and Gomes [3] and uric acid content was calculated based on xanthine and hypoxanthine concentration. Total purine derivative (PD) excretion was obtained by summing the amount of allantoin, uric acid, and xanthine and hypoxanthine excreted in the urine (mmol/L).

The estimated urine volume from the *spot* collection samples was calculated by dividing the average daily creatinine excretion (2.1 mg/L) found in the total urine collection by the concentration (mg/L) in the *spot* collection sample and multiplying by the animals' body weight at the time of collection. The estimated urine volume from the cumulative total urine samples was calculated using the average creatinine excretion, found by extrapolating the urine volume and creatinine concentration (mg/L) at each time point from the same samples, and dividing by the average body weight of the animals. The excretion of total purine derivatives (PD) was obtained by summing the amount of allantoin, uric acid, xanthine, and hypoxanthine excreted in the urine (mmol/L).

2.5 Statistical analyses

The results obtained were evaluated using the general procedure for linear models (PROC MIXED) in a factorial arrangement for a completely randomized design, and the means were compared using Tukey's test, with a probability level of 0.05 for type I error, in SAS 9.4 software (SAS Institute, Cary, NC, USA). The data were analyzed using the following model:

$$Yij(k) = \mu + Ti + Bj + eij$$

In what:

Yij = observed value of the variable in the treatment and block;

μ = overall average;

Ti = the fixed effect of the treatment (days or times of collection);

Bj = the fixed effect of the block.

eij = random experimental error.

3. Results

There was no effect of the evaluated adaptation periods on total urine volume, creatinine concentrations, uric acid, and allantoin in goats (P>0.05) (Table 2). Furthermore, the adaptation periods did not influence the concentrations of xanthine + hypoxanthine and total purines in goats (P>0.05). Therefore, from this point onward, only days 17 and 21 were used for evaluation purposes, aiming to reduce data variability and achieve greater metabolic stability.

In the present study, the urine volume estimated by *spot* collection and the total weighed urine volume did not differ statistically (P>0.05). However, the urine volume estimated by hourly total collection, for both days of adaptation evaluated, differed from the total weighed volume, with higher urine volume values observed in the samples estimated by hourly total collection when compared to the total weighed volume on both days 17 and 21 of adaptation. Furthermore, creatinine levels in mg/PC showed a significant difference (P<0.05) in the values found for the *spot* sample for both 17 and 21 days of diet adaptation (Table 3).

Table 2. Comparison of the days of adaptation to diets by urinary volume (kg/day), creatinine concentration in mg/BW and purine derivatives in mmol/day in goat urine.

Items	Adaptation				SEM[1]	P-value
	9	13	17	21		
Urinary volume (Kg/day)	1.06	1.09	0.96	0.96	0.056	0.4711
Creatinine (mg/BW)	4.49	5.59	5.05	5.07	0.067	0.4606
Uric acid (mmol/day)	0.41	0.40	0.44	0.42	0.015	0.8745
Allantoin (mmol/day)	5.07	4.91	3.50	4.36	0.322	0.2121
Xanthine + Hipoxanthine (mmol/day)	1.28	1.42	1.19	1.19	0.072	0.4122
Total purines (mmol/day)	6.79	6.88	5.21	6.21	0.347	0.2215

[1]Standard error of the mean.

Table 3. Comparison of urinary volumes and daily excretion of total vs. *spot* (4h) and total vs. hourly (4h) creatinine in urine samples from goats.

Items	Collect			SEM[1]	P-value
	Total	*Spot*	Accumulated by time		
17 days of adaptation					
Urinary volumes (Kg/day)	0.96a	1.07a	1.48b	0.24	<0.0001
Creatinine (mg/BW)	2.68a	2.11a	2.07b	0.16	<0.0001
21 days of adaptation					
Urinary volumes (Kg/day)	0.96a	1.11a	1.75b	0.21	<0.0001
Creatinine (mg/BW)	2.27a	2.15a	2.01b	0.13	0.0465

Different lowercase letters in the line differ statistically at a 5% probability level according to Tukey's test.
[1]Standard error of the mean.

Regarding the collection times after the morning meal, there was no significant effect (P>0.05) of the collection times on daily creatinine excretion and estimated urine volume (Table 4). Furthermore, no effect (P>0.05) of the collection times was observed on the concentrations of allantoin, xanthine + hypoxanthine, uric acid, and purine derivatives (Table 4).

Table 4. Daily creatinine excretion, estimated urine volume and purine derivatives from *spot* urine samples collected from goats every 4 hours after the morning feeding.

Items	Tratament						SEM[1]	P-value
	4	8	12	16	20	24		
Creatinine (mg/BW)	2.15	2.06	2.14	2.06	2.11	2.10	0.12	0.9647
Estimated urine volume (Kg/day)	1.11	1.08	1.01	1.09	0.99	1.09	0.02	0.7274
Allantoin (mmol/day)	5.50	5.55	5.74	5.63	5.64	5.76	0.26	0.8950
Xanthine + Hipoxanthine (mmol/day)	1.32	1.35	1.31	1.35	1.44	1.30	0.05	0.9834
Uric acid (mmol/day)	0.52	0.53	0.48	0.49	0.44	0.51	0.01	0.3233
Total purine derivatives (mmol/day)	7.34	7.43	7.53	7.47	7.82	7.57	0.29	0.9187

[1]Standard error of the mean.

4. Discussion

Daily creatinine excretion and its concentration in urine can vary depending on several factors, such as urine volume, which is influenced by the glomerular filtration rate [12] which is dependent on water intake. In addition, diet quality also has a strong influence since its excretion can be altered if there is N deficiency in sheep, as observed by Naqvi et al. [13].

Considering these factors that affect creatinine variation, the use of daily creatinine excretion as an indirect tool to estimate urine volume per animal becomes relevant, given that its excretion is proportional to body weight [9] and is frequently related to the amount of muscle tissue [14]. In the present work, the daily creatinine excretion was not influenced by diet or adaptation days, allowing the average value obtained to be used to estimate urine volume from *spot* urine samples, single hourly total samples and, consequently, to calculate the excretion of purine derivatives in these samples.

Variations in creatinine concentration were expected throughout the day, with lower values in the afternoon and higher values at dawn. This expectation is based on the fact that, during the night, there is absorption of water and electrolytes [4], which can dilute the urine and influence the concentration of metabolites. In addition, Stkotnicka et al. [15] reported that, in mammals, urinary excretion tends to be lower at night than when they are active during the day.

However, according to Valadares et al. [9], creatine is synthesized in muscles, and its metabolite, creatinine, is excreted in the urine in a relatively constant manner, in proportion to body weight, which may limit the expected variation throughout the day. Therefore, urinary creatinine excretion can be used to obtain an estimate of daily urine production and purine derivatives, as well as bacterial protein production, from *spot* and hourly total samples.

The results of the present study demonstrated that the adaptation days do not influence the total urinary volume, nor the daily excretion of creatinine and total purine derivatives. Therefore, there was no influence on the estimate of microbial protein synthesis, corroborating with results found by Barbosa et al. [10] who, when evaluating the collection methodology for four categories of cattle subjected to two levels of concentrate, did not observe variations regarding the collection days for absorbed purines as well as for the use of *spot* sampling.

However, the absence of variation in the aforementioned variables as a function of the adaptation days implies that the collection methodology did not affect the metabolic parameters of the goats evaluated, suggesting that the evaluated days can be used without apparent harm to the animals' performance. However, as highlighted by Cholewińska et al. [16], the adaptation period of the animals to the management conditions and the diet provided can exert beneficial effects on the ruminal microbiota, contributing to the stability of the digestive environment and, consequently, to the prevention of metabolic disorders, such as acidosis or ketosis.

Furthermore, the use of the urinary excretion of purine derivatives technique for determining the synthesis of microbial nitrogen compounds, proposed by Topps et al. [17], demonstrated a direct relationship between urinary PD excretion and microbial nitrogen production in sheep and cattle. This occurs because, in ruminants, most of the PD excreted in the urine results from the partial metabolism of nucleic acid of microbial origin absorbed in the duodenum. [18].

However, this excretion is not determined solely by microbial production and can be modulated by nutritional factors. Rennó et al. [19] observed variation in the excretion of purine derivatives, more specifically allantoin excretion, proportional to dry matter intake and the level of concentrate in the diet, increasing as the animals' feed intake increases.

The methodology for urine collection, especially when performed non-invasively, represents a relevant aspect to be considered, particularly in field conditions where total urine collection becomes operationally limited and difficult to perform. Therefore, in order to evaluate the viability of alternative methods, *spot* samples and hourly total samples were compared with total urine collection (Table 4).

In the present study, the results indicated that the urinary volume estimated from *spot* samples showed good agreement with the total urinary volume obtained by weighing, both after 17 and after 21 days of adaptation to the diet, with no significant differences between the periods evaluated. On the other hand, the methodology based on accumulated samples by time overestimated the urinary volume, proving inefficient for estimating daily creatinine excretion and, consequently, for the accurate calculation of urinary volume.

These findings reinforce the superiority of the *spot* technique, since both the urine volume estimated by this approach and the total volume measured corroborated the ineffectiveness of hourly total samples in determining daily creatinine excretion. The underestimation observed in these samples compromises the accuracy of the urine volume estimate. On the other hand, *spot* samples proved to be consistent and adequate for this purpose, regardless of the time of adaptation to the diet, with no significant variation between the days evaluated.

Considering that the urinary volume estimated from *spot* samples is calculated based on daily creatinine excretion, and that no significant differences ($P > 0.05$) were observed for this variable, it is expected that the same behavior will be reflected in the urinary volume of goats (kg/day). The lack of effect of collection time on daily creatinine excretion was also observed in previous studies by Dos Santos et al. [20], Ma et al. [6], Valadares et al. [21] and Chizzotti et al. [9]. These results corroborate the findings of Chen et al. [5], Chen et al. [22] and George et al. [23], who reported that the creatinine concentration in *spot* urine samples remain relatively constant throughout the day.

The excretion of allantoin, xanthine + hypoxanthine, and uric acid in mmol/day and total purine derivatives in mmol/day BW did not show a significant difference ($P>0.05$) as a function of the time of sample collection after the morning meal. Therefore, it is possible to state that the

absence of significance (P>0.05) for linear or quadratic effects shows that the time of collection does not influence the estimated urinary volume, the estimated daily creatinine excretion, and the estimates of purine derivative excretion (allantoin, xanthine + hypoxanthine, and uric acid), nor total purine derivatives.

The lack of significance (P>0.05) for collection times is a relevant finding, since it is possible to choose times closer to feeding without compromising the accuracy of the estimates, thus reducing field time for collection and animal stress. Furthermore, it allows for the minimization of animal stress and promotes better comfort and well-being, aspects that are increasingly valued in experimental protocols.

5. Conclusion

Based on the data presented, although no significant differences were observed between the adaptation days, aiming to reduce data variability and increase metabolic stability, at least 17 days of adaptation is recommended for metabolic evaluation in goats. Furthermore, spot samples taken at any time after feeding are effective for estimating microbial protein synthesis using the purine derivative excretion method.

Conflict of interest statement

The authors declare no conflicts of interest.

Data availability statement

The data are available upon request.

Author contributions

Conceptualization: Silva, H. G. O., Pereira, M. L. A.; Data curation: Pereira, M. L. A., Mendes, R. B.; Formal analysis: Silva, H. G. O., Mendes, R. B., Oliveira, G. R. S.; Investigation: Mendes, R. B., Oliveira, G. R. S., Gonçalves, W. C., Santos, M. L. S., Lima, C. L. S., Correia, G. S.; Methodology: Silva, H. G. O., Pereira, M. L. A.; Validation: Mendes, R. B.; Writing—original draft: Mendes, R. B., Oliveira, G. R. S., Santos, M. L. S., Lima, C. L. S., Araújo, M. L. G. M. L.; Writing—review and editing: Silva, H. G. O., Pereira, M. L. A.

Generative AI use statement

The authors did not use any generative artificial intelligence tools or technologies in the creation or editing of any part of this manuscript.

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