Tropical Animal Health and Production (2024) 56:126
https://doi.org/10.1007/s11250-024-03963-7

REGULAR ARTICLES

Evaluation of internal markers in digestibility estimation for sheep and goats

Michel Alves da Silva[1] · Juliana Silva de Oliveira[1] · Edson Mauro Santos[1] · Gabriel Ferreira de Lima Cruz[2] · Gherman Garcia Leal de Araújo[3] · Márcia Maria Cândido da Silva[1] · Gilberto de Carvalho Sobral[1] · João Paulo de Farias Ramos[4] · Paloma Gabriela Batista Gomes[1] · Guilherme Medeiros Leite[1] · Maria Alyne Coutinho Santos[1] · Paulo da Cunha Torres Júnior[1] · Arinaldo Fernandes Matias Filho[1]

Received: 29 September 2023 / Accepted: 22 March 2024 / Published online: 16 April 2024

Abstract
The objective of this study was to compare the indirect methods of obtaining digestibility with the direct method of total fecal collection to estimate the apparent digestibility of nutrients in sheep and goats supplemented with non-protein nitrogen. Five goats and five sheep with no defined racial pattern were used, distributed in two 5×5 Latin squares, with split plots, considering the diets as plots and the apparent digestibility determination methodologies as subplots. The diets were composed of buffelgrass hay and the addition, via ruminal infusion, of increasing amounts of nitrogen supplementation in order to gradually raise the CP level of the basal diet in intervals of 2% points, that is, +2, +4, +6 and +8%. Samples of the feeds offered, and the leftovers were collected daily during the five days of collection to determine the nutrient intake, as well as the total collection of feces to determine the apparent digestibility of the nutrients. The amount of fecal dry matter excreted was estimated by the concentration of Indigestible Acid Detergent Fiber (ADFi), Indigestible Neutral Detergent Fiber (NDFi), Indigestible Dry Matter at 244 h (DMi 244 h) and Indigestible Dry Matter at 264 h (DMi 264 h). Among the evaluated markers, DMi 264 h had the lowest accuracy in estimating fecal excretion and nutrient digestibility. For the goat species, the markers ADFi and DMi 244 h proved to be able to adequately predict fecal excretion and digestibility indices, while NDFi stood out for both species. Among the evaluated markers, NDFi is the one that most accurately estimates the nutrient digestibility of the diet for goats and sheep.

Keywords Buffelgrass · Feces · Indigestible dry matter · Rumen-degraded protein · Urea

Introduction

Diet digestibility is one of the main ways used to express the nutrient content and available energy of ruminant feed. This is because the methodologies available for estimating digestibility are generally less complex and low cost when compared to other forms of obtaining the available energy of feed.

However, for estimating the digestibility in vivo it is necessary to obtain the amount of feces excreted daily by the animal. The standard procedure for obtaining the amount of excreted feces is their total collection, which is quite laborious. A less laborious alternative is the use of internal or external markers. Among these internal markers, the most used are indigestible neutral detergent fiber (NDFi) and indigestible acid detergent fiber (ADFi) (Detmann et al., 2004 and Watanabe et al., 2010).

✉ Gabriel Ferreira de Lima Cruz
g_ferreira_dm@hotmail.com

[1] Department of Animal Science, Federal University of Paraíba, Campus II, Areia-PB, João Pessoa CEP: 58397-000, Brazil

[2] Department of Animal Science, Federal University of Viçosa, Viçosa, MG CEP: 36570-900, Brazil

[3] Brazilian Agricultural Research Corporation—EMBRAPA Semi-arid Region, Petrolina, PE CEP: 56.302-970, Brazil

[4] Department of Animal Production, Federal Rural University of Rio de Janeiro, IZ, Seropédica, RJ CEP: 23.897- 000, Brazil

Markers must be indigestible or have constant digestibility. However, it is not yet known whether factors such as diet composition and animal can influence their concentration in feces (Lee & Hristov, 2013; Krizsan & Huhtanen, 2013). Thus, diets with different protein levels may influence the estimates of these markers.

Although NDFi and ADFi are widely used in the estimation of digestibility of ruminant diets, there are few studies with recommendations of these methods (Krizsan & Huhtanen, 2013), as well as for indigestible dry matter (DMi).

In experiments with sheep and goats, it has been verified that even with diets composed of medium- and high-digestibility feeds, such as those with high proportion of concentrate or superior-quality roughage, NDFi and ADFi tend to overestimate fecal production and consequently underestimate the apparent digestibility of the diets. For dairy cows, it was found that the NDFi underestimated the digestibility of protein-poor diets when used as a marker (Lee & Hristov, 2013). When evaluating silages from different sorghum cultivars for sheep, Gois et al. (2017) observed that NDFi underestimated the dry matter digestibility of the diets (55%), considering the high performance of the animals, which obtained weight gain of 200 g/day, predicted during feed formulation.

These imprecisions and uncertainties regarding the accuracy of the methods, especially for small ruminants, make this study important. Another factor to be considered is that there are no studies with comparison of digestibility determination methods using buffelgrass (*Cenchrus ciliaris*), a forage widely used in arid and semi-arid regions around the world, as well as no reports on how crude protein levels of the diet can interfere with the results obtained for goat and sheep species.

Thus, the objective was to compare the indirect methods for obtaining digestibility, using the markers NDFi, DMi incubated for 244 h, ADFi and DMi incubated for 264 h with the direct method of total feces collection to estimate the apparent digestibility of nutrients in sheep and goats supplemented with different protein contents.

Table 1 Chemical composition of forage and supplement components based on dry matter

Item	Buffelgrass	Urea	Casein	Ammonium Sulfate
		(g/kg of DM)		
Dry matter[1]	840.90	995.40	900.00	977.30
Organic matter	921.40	995.40	972.40	977.30
Mineral matter	78.60	4.60	27.60	22.70
Crude protein	55.00	2637.70	889.70	1426.00
Ether extract	11.10	0.00	3.20	0.00
NDFcp[2]	698.40	0.00	0.00	0.00
Non-fibrous carbohydrates	156.90	0.00	0.00	0.00
ADFcp[3]	312.20	0.00	0.00	0.00
Lignin	41.30	0.00	0.00	0.00
Cellulose	270.90	0.00	0.00	0.00
Hemicellulose	386.20	0.00	0.00	0.00
NDIP[4]	1.20	0.00	0.00	0.00
ADIP[5]	0.70	0.00	0.00	0.00

[1]Based on natural matter; [2]Neutral detergent fiber corrected for ash and protein; [3]Acid detergent fiber corrected for ash and protein; [4]Neutral detergent insoluble protein; [5]Acid detergent insoluble protein

Materials and methods

The experiment was conducted in the Goat Farming Sector, at the Center for Agricultural Sciences of the Federal University of Paraíba (UFPB), located in Areia, Paraíba, Brazil, and complied with the technical standards of biosafety and ethics, approved by the Ethics Commission on the Use of Animals (CEUA), Biotechnology Center (CBiotec) of the Federal University of Paraíba (UFPB) (protocol: 0209/2014).

Two simultaneous experiments were conducted, following the same methodological procedures, to evaluate the inclusion of different levels of non-protein nitrogen in the diet of the animals and the digestibility in vivo. For this purpose, five non-castrated sheep and five non-castrated goats, with no defined racial pattern (NDRP), weighing on average 45 ± 2.3 kg, were distributed in two 5×5 Latin squares, with split plots, considering the diets as plots and the apparent digestibility determination methodologies as subplots. Thus, there was rotation of the diets among the animals in each evaluation period and the estimation methods were applied as subplots in all evaluated animals and in all periods. All animals were fistulated in the rumen and kept in an intensive system. Each animal was housed in an individual Tie Stall-type metabolic cage, equipped with feeder and drinker.

The animals were supplemented with five protein levels in a diet based on deferred buffelgrass, with low protein value (Table 1).

The control treatment consisted of exclusive supply of hay and the others consisted of the addition, via ruminal infusion, of increasing amounts of nitrogen supplement in order to gradually increase the level of crude protein (CP) of the basal diet by percentage points, that is, + 1.94, + 3.89, + 5.83 and + 7.77%.

Thus, the animals consumed five CP levels in the basal diet based on dry matter (5.5, 7.44, 9.39, 11.33 and 13.27% CP). The supplement was composed of a mixture containing livestock urea, ammonium sulfate and casein in the proportions of 75:8.33:16.67, respectively (Table 1).

The experiment consisted of five periods, each lasting 20 days, with the first fifteen days for adaptation to the diets and the others for data collection, totaling 100 days of experimental period. The animals were kept confined and fed twice a day (7:30 h and 15:30 h) in equal proportion, and the feed was provided individually.

The diets were provided *ad libitum*, to generate at least 10% leftovers, with daily adjustments to ensure the correct level of feed supply. In the first two days of each adaptation period, one third of the supplement's full dose was infused. On the third and fourth days, two-thirds of the supplement was infused and, on the fifth, sixth and seventh days of the adaptation period, the full dose of the supplement was provided.

The amounts of hay (offered) and supplement (infused) daily were calculated considering the consumption of roughage from the previous day.

Samples of the feed offered and leftovers were collected daily along the five days of collection. These samples were then used to form composite samples for each experimental period, and their composition was determined according to the Association of Official Analytical Chemists - AOAC (1997), for dry matter (DM) (method 934.01), crude protein (CP) (method 954.01), ether extract (EE) (method 920.39), mineral matter (MM) (method 942.05) and lignin (method 973.18). The methodology of Van Soest et al. (1991) was used to determine neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the fiber analyzer from ANKOM (ANKOM200 Fibre Analyzer – ANKOM Technology Corporation, Fairport, NY, USA). NDF and ADF contents were corrected for ash and protein, with incineration of their residues in a muffle furnace at 600 ºC for 4 h; the correction for protein was performed based on neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP).

The concentration of non-fibrous carbohydrates (NFC) was estimated from the equation: NFC = 100 – (%CP + %EE + %ASH + %NDF), according to Van Soest et al. (1991).

Digestibility was determined according to the equation described by Berchielli et al. (2006), and the digestibility coefficient (DC), in g/kg, was calculated by:

$$DC = (\text{Ingested nutrient - Excreted nutrient})/\text{Ingested nutrient} \times 100$$

The energy value of the diets was quantified using the apparent digestibility data obtained in the experiment, applying the equation proposed by Weiss (1999):

$$TDN\,(\%) = NFCd + CPd + (EEd*2.25) + NDFd$$

Where "d" represents digestibility, TDN (g/kg) = Total digestible nutrients, CPd = Digestible crude protein; EEd = Digestible ether extract; NFCd = Digestible non-fibrous carbohydrates; NDFd = Digestible neutral detergent fiber.

In the last five days of each experimental period, total collection of feces was also performed. Feces were collected by attaching a feces collection bag to each animal. The collection, weighing and sampling of feces (10% of the total excreted after homogenization) was performed twice a day, at 7:00 h and 17:00 h.

The feces were analyzed for DM, OM, NDF, CP, EE and MM according to the previously described methodologies for feed and leftovers and used to estimate the apparent digestibility of nutrients in the diets.

On the 15th day at 6:00 h, 16th day at 9:00 h, 17th day at 12:00 h, 18th day at 15:00 h and 19th day at 18:00 h, feces were also collected from the final part of the rectum of each animal.

The amount of fecal dry matter excreted was estimated by the concentration of indigestible acid detergent fiber (ADFi), indigestible neutral detergent fiber (NDFi), indigestible dry matter at 244 h (DMi 244 h), indigestible dry matter at 264 h (DMi 264 h) obtained after in situ incubation of feed, leftovers and feces for periods of 244 and 264 h. Estimates of the amount of fecal dry matter excreted, obtained with the markers NDFi, ADFi, DMi 244 h and DMi 264 h, were also used to also determine the apparent digestibility of the diets.

For this, one bovine animal fistulated in the rumen (weighing on average 760 kg), receiving a diet *ad libitum* consisting of 70% roughage (elephant grass) and 30% concentrate supplement (based on soybean meal, wheat bran and corn), was used as recommended by Huntington and Givens (1995). The feed was provided twice a day, at 7:00 h and at 15:00 h, with *ad libitum* water supply.

After removal of the bags incubated in the rumen, they were washed with running water until the rinse water was completely clear and immediately transferred to a forced ventilation oven (55 ºC), where they were kept for 48 h. Subsequently, they were dried in a non-ventilated oven (105 ºC for 45 min), placed in desiccator (20 bags/desiccator) and weighed according to Detmann et al. (2001) to obtain undigested DM.

Undigested NDF and ADF were determined using the methodology proposed by Souza et al. (1999), with insertion of NDF and ADF solutions in test tubes in the digestor block. DMi content was obtained by the difference between the dry weight of the material before incubation and the dry weight of the residue after in situ incubation. NDFi and ADFi were determined by the difference between the dry weight of the material before incubation of the residue and after incubation and analysis of NDF and ADF, respectively.

The effect of digestibility methods was assessed by orthogonal contrasts to compare the indirect methods of

determination of apparent digestibility of nutrients in vivo (ADFi, NDFi and DMi) and in situ with the direct method of determination of apparent digestibility (total collection of feces) of nutrients in the different diets. When there was interaction, the comparison between the methods was performed for each crude protein level.

The procedures for comparison between the methods by total collection of feces and each marker were performed independently of the fixed effects of treatment and Latin square, by fitting a simple linear regression model of the predicted and observed values, testing the estimates of the regression parameters. Statistical analyses were carried out through the PROC REG procedure of the program SAS (2011), using Dunnett's test, adopting a significance level of $\alpha = 0.05$.

Results

There was no statistical difference between the total collection of feces (true) and the estimate of fecal excretion by the NDFi, regardless of the CP level used, for the two species studied ($p = 0.08$ for sheep; $p = 0.94$ for goats) (Table 2). For the sheep species, fecal excretion estimated by ADFi and DMi 244 h was overestimated at the CP level of 13.27% ($p = 0.035$ for ADFi; $p = 0.05$ for DMi 244 h) when compared with true fecal excretion. For both species, fecal excretion estimated by DMi 264 h differed from fecal excretion via total collection at all CP levels evaluated ($p = 0.0001$). The markers ADFi ($p = 0.09$) and DMi 244 h ($p = 0.344$) adequately estimated fecal excretion in goats.

For dry matter digestibility (DMD) in sheep, none of the null hypotheses were rejected ($p = 0.403$) for the internal marker NDFi (Table 3). On the other hand, the DMi 264 h did not adequately estimate DMD at any of the CP levels evaluated ($p = 0.0001$), while at the CP level of 13.27% the markers ADFi ($p = 0.0004$) and DMi 244 h ($p = 0.002$) did not correctly estimate DMD. For goats, the DMD was adequately estimated by the markers NDFi ($p = 0.59$), ADFi ($p = 0.106$) and DMi 244 h ($p = 0.06$), while DMi 264 h differed from the total collection at all CP levels ($p = 0.0013$).

The crude protein digestibility (CPD) estimated by the NDFi was similar to that obtained by the total collection, regardless of the CP level adopted in goats ($p = 0.99$) or sheep ($p = 0.736$) (Table 4). For the ADFi and DMi 244 h markers, the results were also similar to that of true CPD in goats ($p = 0.907$ for ADFi; $p = 0.948$ for DMi 244 h), but with CPD results for goats underestimated by these markers at the CP level of 13.27% ($p = 0.008$ for ADFi; $p = 0.020$ for DMi 244 h). The estimate using 264 h DMi for goats was similar to the true CPD ($p = 0.09$), while for sheep,

Table 2 Estimates of daily fecal excretion in sheep and goats consuming diets with different crude protein (CP) concentrations through the internal markers of buffelgrass

	Markers[1]					Contrasts[2]			
	Total	NDFi	ADFi	DMi 244 h	DMi 264 h	SEM	Total x NDFi	Total x ADFi	Total x DMi 244 h	Total x DMi 264 h
	Feces, g/day									
% CP	Sheep									
5.5	211.52	173.20	252.85	233.39	341.74	46.572	ns	ns	ns	*
7.44	246.31	152.59	327.20	207.48	402.65	46.572	ns	ns	ns	*
9.39	227.85	154.59	304.00	209.83	370.84	46.572	ns	ns	ns	*
11.33	217.30	141.71	256.18	172.68	304.89	46.572	ns	ns	ns	*
13.27	146.88	188.99	269.35	260.23	305.74	46.572	ns	*	*	*
% CP	Goats									
5.5	183.57	183.72	255.74	162.35	117.26	41.932	ns	ns	ns	ns
7.44	223.12	175.66	240.91	156.63	75.54	41.932	ns	ns	ns	*
9.39	199.57	193.43	263.29	173.15	93.57	41.932	ns	ns	ns	*
11.33	192.11	202.91	259.36	182.52	93.12	41.932	ns	ns	ns	*
13.27	188.88	190.12	177.38	167.32	41.06	41.932	ns	ns	ns	*

[1]Total = true excretion, obtained by the total collection of feces; NDFi = indigestible neutral detergent fiber, ADFi = indigestible acid detergent fiber; DMi 244 h = indigestible dry matter incubated for 244 h; and DMi 264 h = indigestible dry matter incubated for 264 h; SEM = standard error of the mean. [2]Significant comparisons ($P < 0.05$) by Dunnett's test

Table 3 Estimates of dry matter digestibility (DMD) of diets with different crude protein (CP) concentrations in sheep and goats through the internal markers of buffelgrass

	Markers[1]						Contrasts[2]			
	Total	NDFi	ADFi	DMi 244 h	DMi 264 h	SEM	Total x NDFi	Total x ADFi	Total x DMi 244 h	Total x DMi 264 h
	DMD, g/kg									
% CP	DMS, g/kg					Sheep				
5.5	743.08	773.25	696.49	677.15	578.75	54.528	ns	ns	ns	*
7.44	737.08	733.73	665.13	782.94	587.39	54.528	ns	ns	ns	*
9.39	754.00	733.30	683.55	783.00	612.25	54.528	ns	ns	ns	*
	736.18	781.22	609.74	735.08	540.67	54.528	ns	ns	ns	*
	751.19	776.84	529.96	555.01	472.61	54.528	ns	*	*	*
	Goats									
5.5	744.30	821.60	705.06	842.90	894.28	63.555	ns	ns	ns	*
7.44	742.12	779.02	731.79	803.88	940.72	63.555	ns	ns	ns	*
9.39	769.48	779.00	708.04	802.26	904.72	63.555	ns	ns	ns	*
	770.22	748.84	687.34	774.30	890.52	63.555	ns	ns	ns	*
	746.04	746.70	744.47	776.90	944.02	63.555	ns	ns	ns	*

[1]Total = true excretion, obtained by the total collection of feces; NDFi = indigestible neutral detergent fiber, ADFi = indigestible acid detergent fiber; DMi 244 h = indigestible dry matter incubated for 244 h; and DMi 264 h = indigestible dry matter incubated for 264 h; SEM = standard error of the mean. [2]Significant comparisons ($P < 0.05$) by Dunnett's test

Table 4 Estimates of crude protein digestibility (CPD) of diets with different crude protein (CP) concentrations in sheep and goats through the internal markers of buffelgrass

Param-eters	Markers[1]						Contrasts[2]			
	Total	NDFi	ADFi	DMi 244 h	DMi 264 h	SEM	Total x NDFi	Total x ADFi	Total x DMi 244 h	Total x DMi 264 h
CPD, g/kg										
% CP			Sheep							
5.5	656.78	676.05	600.13	516.17	429.97	91.998	ns	ns	ns	*
7.44	682.32	700.15	602.14	736.93	508.44	91.998	ns	ns	ns	*
9.39	695.09	692.42	608.10	730.59	520.20	91.998	ns	ns	ns	*
11.33	681.21	639.50	534.66	682.26	451.52	91.998	ns	ns	ns	*
13.27	678.37	674.09	388.54	416.89	314.97	91.998	ns	*	*	*
			Goats							
5.5	669.91	602.86	682.02	641.33	700.20	130.731	ns	ns	ns	ns
7.44	706.77	736.30	710.25	765.40	956.97	130.731	ns	ns	ns	ns
9.39	713.36	740.11	643.85	767.32	878.76	130.731	ns	ns	ns	ns
11.33	592.90	526.49	446.42	574.93	806.52	130.731	ns	ns	ns	ns
13.27	644.96	668.48	679.16	708.65	937.31	130.731	ns	ns	ns	ns

[1]Total = true excretion, obtained by the total collection of feces; NDFi = indigestible neutral detergent fiber, ADFi = indigestible acid detergent fiber; DMi 244 h = indigestible dry matter incubated for 244 h; and DMi 264 h = indigestible dry matter incubated for 264 h; SEM = standard error of the mean; [2]Significant comparisons ($P < 0.05$) by Dunnett's test

regardless of CP level, CPD was underestimated when compared to the true value ($p = 0.0001$).

There was no significant difference ($p > 0.05$) between the true neutral detergent fiber digestibility (NDFD) and NDFi at all CP levels, regardless of species ($p = 0.581$ for sheep; $p = 0.99$ for goats) (Table 5). The same absence of effect was observed for the NDFD obtained by the markers ADFi ($p = 0.79$) and DMi 244 h ($p = 0.75$) in the goat species, while for sheep the DMi 244 h marker underestimated the NDFD at the CP level of 13.27% in the DM ($p = 0.014$). The NDFD estimated by the DMi 264 h marker differed from the true NDFD in sheep, when the CP levels of 5.5% ($p = 0.049$), 11.33% ($p = 0.016$) and 13.27% ($p = 0.0002$) were used in the diet, which were underestimated when compared to the true NDFD (Table 5). Regarding goats, DMi 264 h overestimated ($p = 0.001$) the NDFD and did not differ from the total collection ($p = 0.82$) only with the diet containing 5.5% of CP.

The estimate of total digestible nutrients (TDN) obtained by the NDFi marker was similar to the TDN obtained by the total feces collection method, at all CP levels and in both species ($p = 0.348$ for sheep; $p = 0.999$ for goats) (Table 6), and for the markers ADFi ($p = 0.802$) and DMi 244 h ($p = 0.760$) in goats. At the CP level of 13.27% for sheep, differences were observed in the estimates of TDN via ADFi ($p = 0.0002$) and DMi 244 h ($p = 0.001$), when

Table 5 Estimates of neutral detergent fiber digestibility (NDFD) of diets with different crude protein (CP) concentrations in sheep and goats through the internal markers of buffelgrass

Parameters	Markers[1]						Contrasts[2]			
	Total	NDFi	ADFi	DMi 244 h	DMi 264 h	EP	Total x NDFi	Total x ADFi	Total x DMi 244 h	Total x DMi 264 h
NDFD, g/kg										
% CP			Sheep							
5.5	748.81	776.16	704.94	677.40	589.50	63.637	ns	ns	ns	*
7.44	758.25	748.36	690.08	799.60	668.16	63.637	ns	ns	ns	ns
9.39	784.26	756.52	732.38	811.81	689.26	63.637	ns	ns	ns	ns
11.33	750.65	794.02	731.92	750.42	565.08	63.637	ns	ns	ns	*
13.27	747.81	780.38	528.28	559.21	473.70	63.637	ns	*	*	*
			Goats							
5.5	729.71	693.03	711.94	724.39	781.29	68.254	ns	ns	ns	ns
7.44	754.65	786.92	747.98	810.38	946.45	68.254	ns	ns	ns	*
9.39	769.12	780.49	718.38	803.73	911.92	68.254	ns	ns	ns	*
11.33	774.61	762.34	704.49	786.50	897.56	68.254	ns	ns	ns	*
13.27	758.09	768.82	779.38	796.66	952.20	68.254	ns	ns	ns	*

[1]Total = true excretion, obtained by the total collection of feces; NDFi = indigestible neutral detergent fiber, ADFi = indigestible acid detergent fiber; DMi 244 h = indigestible dry matter incubated for 244 h; and DMi 264 h = indigestible dry matter incubated for 264 h; SEM = standard error of the mean. [2]Significant comparisons ($P < 0.05$) by Dunnett's test

Table 6 Estimates of total digestible nutrients (TDN) of diets with different crude protein (CP) concentrations in sheep and goats through the internal markers of buffelgrass

Param-eters	Markers[1]						Contrasts[2]			
	Total	NDFi	ADFi	DMi 244 h	DMi 264 h	SEM	Total x NDFi	Total x ADFi	Total x DMi 244 h	Total x DMi 264 h
TDN, g/kg										
% CP			Sheep							
5.5	748.04	716.53	705.52	691.98	600.49	46.335	ns	ns	ns	*
7.44	721.47	808.68	655.51	762.82	585.45	46.335	ns	ns	ns	*
9.39	713.37	782.76	650.39	737.90	586.93	46.335	ns	ns	ns	*
11.33	692.82	734.56	581.63	692.96	518.83	46.335	ns	ns	ns	*
13.27	696.61	630.33	496.64	519.95	444.59	46.335	ns	*	*	*
			Goats							
5.5	702.20	664.19	688.22	694.24	746.93	67.969	ns	ns	ns	ns
7.44	711.64	746.59	706.19	768.27	889.30	67.969	ns	ns	ns	*
9.39	767.69	780.63	715.40	802.22	896.92	67.969	ns	ns	ns	*
11.33	757.48	744.94	678.64	768.43	875.43	67.969	ns	ns	ns	*
13.27	760.69	771.26	780.88	799.37	955.23	67.969	ns	ns	ns	*

[1]Total = true excretion, obtained by the total collection of feces; NDFi = indigestible neutral detergent fiber, ADFi = indigestible acid detergent fiber; DMi 244 h = indigestible dry matter incubated for 244 h; and DMi 264 h = indigestible dry matter incubated for 264 h; SEM = standard error of the mean. [2]Significant comparisons ($P < 0.05$) by Dunnett's test

compared to the true TDN, while for the DMi 264 h marker the estimates differed ($p = 0.0001$) from the average of true TDN at all CP levels.

Discussion

The DMi 264 h marker was highly ineffective in estimating fecal excretion for both species (Table 2). As the digestibility of nutrients is estimated from fecal dry matter production, the difficulty of the DMi 264 h marker in correctly predicting true excretion led to the same result for the digestibility of CP in sheep (Table 4) and digestibility of DM, NDF and TDN in both species (Tables 3, 5 and 6).

These problems may be related to the fact that the determination of DMi has contaminants, as detergents are not used after incubation, preventing the purification of cell wall residues and bacterial decontamination by anionic action, which may compromise the results (Huhtanen et al., 1994; Van Soest, 1994; Moreira Filho et al., 2017).

The level of contamination by residues does not seem to be constant among the materials, with greater variability of the results and consequently reducing the precision of fecal recovery via DMi (Casali et al., 2008; Sampaio et al., 2011). These results corroborate those of Sampaio et al. (2011); Valente et al. (2011a), so DMi should not be used as an internal marker in digestibility trials with goats and sheep.

It is also believed that this contamination was higher in the incubation of 264 h, when the DMi 244 h differed from the true fecal excretion only at the CP level of 13.27%, and in sheep (Table 2), emphasizing that ruminal incubation time is an influential variable on the representativeness of indigestible feed residues during in situ incubation procedures, with possibility of occurrence of substantial contamination after periods of more than ten days of incubation (Van Milgen et al., 1992).

The non-degradable fraction is a unique and exclusive characteristic of feeds/substrates (Ørskov, 2000), but the occurrence of variations in degradation rates may affect the time required for this fraction to be adequately estimated (Valente et al. 2011b), as observed by Reis et al. (2017), who found that the determination of NDFi and ADFi required a shorter period of ruminal incubation to be determined in cattle than in sheep, and the authors attributed these differences to the anatomical and physiological differences between the rumens of the two species.

As in the present study, the markers were obtained with in situ incubation in fistulated bovine animal, the possible interference of the species in the determination of the marker is avoided. However, the ruminal degradation rate may be affected by the animal diet (Souza et al., 2016) and, thus, a higher proportion of CP (13.27%) may have influenced the determination of fecal excretion in sheep through the DMi 244 h marker.

DMi 244 h accurately estimated fecal excretion and other digestibility indices in the goat species. Carvalho et al. (2013) reported an efficiency of DMi in estimating fecal excretion, with 240 h of incubation, for both species (sheep and goat). Casali et al. (2008) also indicated an incubation period of 240 h as adequate for obtaining accurate estimates of DMi and NDFi fractions for cattle.

Lee and Hristov (2013), when evaluating internal markers with high and low CP diets for lactating cows, observed that fecal production was overestimated and diet digestibility was underestimated with the adoption of NDFi as a marker; on the other hand, with the CP-rich diet, the production of fecal nutrients was underestimated, but with digestibility coefficients similar to those of the total collection. By contrast, in the present study, the NDFi did not differ from the total collection regardless of the CP level adopted, while the markers ADFi and DMi 244 h differed at the CP level of 13.27% for sheep, highlighting an influence of the diet on the adoption of these markers.

Although the ADFi proved to be a good indicator of fecal excretion and digestibility for goat species, the same did not occur with the sheep species, for which the ADFi was ineffective in estimating fecal excretion and digestibility of CP, NDF and TDN in diets with high CP content (13.27% CP). Reis et al. (2017) also observed that the ADFi did not accurately estimate these parameters in sheep and cattle consuming diets with high concentrate content.

Therefore, NDFi is more accurate than ADFi as a digestibility marker for both species, which results from the fact that ADFi is found at lower concentrations in feed, leftovers and feces, and consequently requires more careful analytical procedures in the laboratory to increase the precision of the results (Detmann et al., 2007; Sampaio et al., 2011) and avoid the accumulation of methodological errors in sequential analyses that are necessary until its measurement (Detmann et al., 2001; Figueiredo et al., 2019).

Barros et al. (2009) pointed out that, among the internal markers, NDFi determines a more accurate estimate of fecal production, because it has the lowest values for total bias (referring to the sum of short-term and long-term bias) and long-term bias, represented by failures in the recovery of the marker due to problems in obtaining representative samples of feces, in situ contamination of samples or errors related to analytical procedures.

Carvalho et al. (2013), Kozloski et al. (2009) and Detmann et al. (2007), in studies with small ruminants, suggested that ADFi is inefficient to estimate fecal excretion in digestibility studies with confined animals. On the other hand, the adoption of the NDFi marker as effective in estimating fecal excretion has already been widely reported (Carvalho et al., 2013; Detmann et al., 2007; Sampaio et al., 2011; Figueiredo et al., 2019), and the results obtained prove its capacity as an indicator of this variable.

It is known that the markers may vary according to diets, and a certain marker is suitable or not to a given roughage source, because the fiber constitution of each type of roughage is variable, which modifies the rate and extent of degradation (Berchielli et al., 2005). For diets based on buffelgrass with protein supplementation, NDFi proved to be adequate to estimate digestibility in goats and sheep, standing out for being a marker closely associated with dietary fiber and because its fecal recovery function as an ideal marker (Huhtanen et al., 2010; Reis et al., 2017).

The different estimates using NDFi, ADFi and DMi reinforce the need for further studies to identify possible influences of CP variation in the diet with other forage sources for both species, also allowing a methodological standardization that fits a given animal species.

Conclusions

Indigestible neutral detergent fiber (NDFi) is the marker that accurately estimates nutrient digestibility in buffelgrass-based diets with different CP contents for goats and sheep.

Author contributions All authors contributed to the study conception and design. Material, data collection and analysis were performed by

Michel Alves da Silva, Gabriel Ferreira de Lima Cruz, Márcia Maria Cândido da Silva, Gilberto de Carvalho Sobral, Paloma Gabriela Batista Gomes, Guilherme Medeiros Leite, Maria Alyne Coutinho Santos, Paulo da Cunha Torres Júnior, Arinaldo Fernandes Matias Filho. The first draft of the manuscript was written by Michel Alves da Silva, Edson Mauro Santos, Juliana Silva de Oliveira, João Paulo de Farias Ramos and Gabriel Ferreira de Lima Cruz, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The research was supported by the Brazilian National Council for Scientific and Technological Development (CNPq).

Data availability The datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Ethical approval This study was performed and approved by the Ethics Committee of Federal University of Paraiba (protocol number: 0209/14).

Consent to participate not applicable.

Consent to publish not applicable.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

References

AOAC INTERNATIONAL, 1997. Official methods of analysis of the Association Analytical Chemists Association of Official Analytical Chemists, Gaithersburg. (AOAC International, Arlington).

Barros, E.E.L., Fontes, C.A.A., Detmann, E., Vieira, R.A.M., Henriques, L.T., Fernandes, A.M. 2009. Vícios na estimação da excreção fecal utilizando indicadores internos e óxido crômico em ensaios de digestão com ruminantes. Revista Brasileira de Zootecnia., 38(10), 2015–2020.

Berchielli, T.T., Oliveira, S.G., Garcia, A., 2005. Considerações sobre os principais indicadores utilizados em estudos de nutrição com ruminantes. Arquivo de Ciência Veterinária e Zoologia, 8(2), 205–211.

Carvalho, G.G.P., Garcia, R., Pires, A.J.V., Silva, R.R., Detmann, E., Oliveira, R.L., Ribeiro, L.S.O., 2013. Long-term Bias of Internal Markers in Sheep and Goat Digestion Trials. Asian-Australasian Journal of Animal Science, 26(1), 65–71.

Casali, A.O., Detmann, E.E., Valadares Filho, S.C., Pereira, J.C., Henriques, L.T., Freitas, S.G., Paulino, M.F. 2008., A. Influência do tempo de incubação e do tamanho de partículas sobre os teores de compostos indigestíveis em alimentos e fezes bovinas obtidos por procedimentos in situ. Revista Brasileira de Zootecnia, 37(2), 335–342.

Detmann, E., Paulino, M.F., Zervoudakis, J.T., Valadares Filho, S.C., Euclydes, R.F., Lana, R.P., Queiroz, D.S. 2001. Cromo e indicadores internos na determinação do consumo de novilhos mestiços, suplementados, a pasto. Revista Brasileira de Zootecnia, 30, 1600–1609.

Detmann, E., Zervoudakis, J.T., Cabral, L.S., Rocha Júnior, V.R., Valadares Filho, S.C., Queiroz, A.C., Ponciano, N.J., Fernandes, A.M., 2004. Validação de equações preditivas da fração indigestível da fibra em detergente neutro em gramíneas tropicais. Revista Brasileira de Zootecnia, 33(6), 1866–1875.

Detmann, E., Souza, A.L., Garcia, R., Valadares Filho, S.C., Cabral, L.S., Zervoudakis, J.T., 2007. Avaliação do vício de "tempo longo" de indicadores internos em ensaio de digestão com ruminantes. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 59, 182–188.

Figueiredo, M.R.P., Saliba, E.O.S., Barbosa, S.S.C., Silva, F.A., Nunes, A.N., Silva, C.R.M., Moreira, G.R., 2019. Use of indigestible markers to estimate the apparent dry matter digestibility of diets containing a cocoa by-product. Semina: Ciências Agrárias, 40(6,) 2771–2782.

Gois, G.C., Santos E.M., Sousa W.H., Ramos J.P.F., Azevedo P.S., Oliveira J.S., Pereira G.A., Perazzo A.F., 2017. Qualidade da carne de ovinos terminados em confinamento com dietas com silagens de diferentes cultivares de sorgo Arquivo Brasileiro de Medicina Veterinária e Zootecnia 69(6), 1653–1659. https://doi.org/10.1590/1678-4162-9231.

Huhtanen, P., Kaustell, K., Jaakkola, S., 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Animal Feed Science and Technology, 48,.211–227.

Huhtanen, P., Ahvenjarvi, S., Broderick, G.A., Reynal, S.M., Shingfield, K.J., 2010. Quantifying ruminal digestion of organic matter and neutral detergent fiber using the omasal sampling technique in cattle - A meta-analysis. Journal of Dairy Science, 93, 3203–3215.

Huntington, J.A., Givens, D.I., 1995. The in Situ Technique for Studying Rumen Degradation of Feeds: A Review of the Procedure. Nutrition Abstracts and Reviews, 63–65,1995.

Kozloski, G. V., Mesquita, F.R., Alves, T.P., Castagnino, D.S., Stefanello, C.M., Sanchez, L.M.B., 2009. Avaliação do uso de frações indigestíveis do alimento como indicadores internos de digestibilidade em ovinos. Revista Brasileira de Zootecnia, 38, 1819–1823.

Krizsan, S. J., Huhtanen, P., 2013. Effect of diet composition and incubation time on feed indigestible neutral detergent fiber concentration in dairy cows. Journal of Dairy Science, 96(3), 1715–26.

Lee, C., Hristov, A. N. Short communication: Evaluation of acid-insoluble ash and indigestible neutral detergent fiber as total-tract digestibility markers in dairy cows fed corn silage-based diets. Journal of Dairy Science, 96(8), 5295–5299. doi:https://doi.org/10.3168/jds.2012-6442. 2013.

Moreira Filho, M.A., Alves, A.A., Garcez, B.S., Moreira, A.L., Azevêdo, D.M.M.R., Parente, H.N., 2017. Digestibility markers of sheep diets containing hydrolyzed sugarcane-top hay. Revista Brasileira de Saude e Producao Animal., 18(1), 38–49.

Ørskov, E.R. The in situ technique for the estimation of forage degradability in ruminants. In: GIVEN, D.I.; OWENS, E.; AXFORD, R.F.E. et al. (Eds.). Forage evaluation in ruminant nutrition. Wallingford: CAB International, 175–188, 2000

Reis, M. J., Santos, S.A., Prates, L.L., Detmann, E., Carvalho, G.G.P., Santos, A.C.S., Rufino, L.M., Mariz, L.D., Neri, F., Costa, E., 2017. Comparing sheep and cattle to quantify internal markers in tropical feeds using in situ ruminal incubation. Animal Feed Science and Technology, 232, 139–147.

Sampaio, C. B., Detmann, E., Valente, T.N.P., Souza, M.A., Valadares Filho, S.C., Paulino, M.F., 2011. Evaluation of fecal recovering and long term bias of internal and external markers in a digestion assay with cattle. Revista Brasileira de Zootecnia, 40, 174–182.

SAS. Statistical Analysis Systems User's Guide. Version 9.2. SAS Institute, Cary, NC, 2011.

Souza, G.B., Nogueira, A.R.A., Sumi, L.N., Batista, L.A.R., 1999. Método alternativo para a determinação de fibra em detergente neutro e detergente ácido. São Carlos: Embrapa Pecuária Sudoeste. 21 p.

Souza, M.A., Detmann, E., Rocha, G.C., Oliveira Franco, M., Batista, E.D., Valadares Filho, S.C., Berchielli, T.T., 2016. Collaborative study to evaluate the indigestible neutral detergent fiber and indigestible acid detergent fiber contents in feeds by in situ procedure. Semina Ciências Agrárias, 37, 2589–2599.

Valente, T.N.P., Detmann, E., Valadares Filho, S.C., Cunha, M., Queiroz, A.C., Sampaio, C.B., 2011a. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. Revista Brasileira de Zootecnia, 40, 666–675.

Valente, T.N.P., Detmann, E., Queiroz, A.C., Valadares Filho, S.C., Gomes, D.I., Figueiras, J.F., 2011b. Evaluation of ruminal degradation profiles of forages using bags made from different textiles. Revista Brasileira de Zootecnia, 40(11), 2565–2573.

Van Milgen, J., Roach, M.L., Berger, L.L., Murphy, M.R., Moore, D.M., 1992. Technical note: mineral deposits on Dacron bags during ruminal incubation. Journal of Animal Science, 70, 2551–2555.

Van Soest, P.J. Nutritional ecology of the ruminant. 2. ed. Ithaca: Cornell University Press, 1994. 476p.

Van Soest, P.J, Robertson, J.B, Lewis, B.A. 1991. Methods for dietary fiber, and nonstarch polysaccharides in relation to animal nutrition' Journal of Dairy Science, 74(10), 3583–3597.

Watanabe, P.H., Ezequiel, J.M., Galati, R.L., Biagioli, B., Silva, O.G.C.H., 2010. Indicadores internos indigestíveis para a estimativa das digestibilidades de dietas à base de coprodutos. Revista Brasileira de Saúde e Produção Animal, 11(3), 849–857.

Weiss, W.P., 1999. Energy prediction equations for ruminant feeds. In: Cornell Nutrition Conference for Feed Manufacturers, 61. Proceedings. Cornell University, Ithaca, pp. 176–185.