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Effects of amount and frequency of nitrogen supplementation on nutritional performance and metabolism in cattle fed medium-quality tropical forage

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Abstract
We aimed to evaluate how the amount and frequency of nitrogen (N) supplementation impact nutritional performance and metabolism in cattle fed medium-quality tropical forage. Five young Nellore × Holstein bulls (262 ± 20 kg) were used in a 5 × 5 Latin square design. The basal diet consisted of medium-quality signal grass hay, with the following treatments: (1) control, without supplementation; (2) daily supplementation with 30% of rumen degradable protein (RDP) requirements; (3) supplementation every three days with 30% of RDP requirements; (4) daily supplementation with 60% of RDP requirements; and (5) supplementation every three days with 60% of RDP requirements. Overall, there was no effect ($p \geq 0.38$) of N supplementation on voluntary intake, though infrequent supplementation reduced ($p \leq 0.04$) voluntary intake. N supplementation improved ($p \leq 0.03$) crude protein digestibility without affecting fibre digestibility ($p \geq 0.17$). Supplementation frequency did not affect ($p \geq 0.08$) digestibility characteristics. There were no effects ($p \geq 0.06$) of treatments on N retention or utilisation efficiency. However, interactions between treatments and supplementation cycle days were detected ($p \leq 0.01$) for both ruminal ammonia N (RAN) and serum urea N (SUN) concentrations, with RAN peaking on the first day for infrequently supplemented animals, while SUN peaked on the second day for cattle receiving 60% RDP infrequently. This decoupling between RAN and SUN concentrations suggests a potential mechanism for conserving dietary N with infrequent supplementation. Reducing N supplementation frequency decreases voluntary intake in cattle fed medium-quality tropical grass, but does not impair digestion, N utilisation, or metabolism.

Keywords Digestion, Forage intake, Nitrogen balance, Serum Urea nitrogen

1 Introduction

Tropical grasses rarely provide a balanced diet for grazing cattle in tropical regions. During the rainy season, forage quality generally improves; however, an excess of energy relative to available protein is often observed [1]. In such cases, protein-based supplements are recommended to optimize the utilisation of the forage's excess energy, thereby enhancing the utilisation efficiency of both energy and protein in metabolism [2]. However, effective supplementation programs require a comprehensive holistic approach, considering not only nutritional benefits but also costs related to labour, infrastructure, and on-farm logistics for supplement distribution [3]– [4]. To address these challenges, researchers have explored reduced supplementation frequency as a strategy to lower costs and streamline labour in beef cattle operations [5–7]. In tropical conditions, it has been reported that reducing protein supplementation frequency to as few as three times per week does not adversely affect cattle performance under grazing on tropical pastures [8].

While animal performance remains stable, few studies have investigated the physiological mechanisms underlying metabolism in infrequently supplemented cattle. Initial research suggests that nitrogen (N) recycling might serve as the primary nutritional mechanism supporting adequate N availability in animal metabolism on non-supplementation days [9]. However, recent studies in tropical conditions have revealed a range of physiological adaptations in response to reduced supplementation frequency, including changes in blood, liver, and kidney function in beef cattle [4, 10–12]. These adaptations likely represent a complex physiological response to altered dietary conditions, potentially explaining how animals maintain performance with less frequent supplementation.

On the other hand, infrequent supplementation can create metabolic challenges due to high substrate loads delivered at once, potentially testing buffering mechanism systems to their physiological limits. Negative effects of infrequent supplementation have been associated with reduced voluntary intake [10–12] and increased urinary N losses, leading to lower overall body N retention [4, 9, 13]. These impacts are typically attributed to large, single doses of rumen degradable protein (RDP) or energy substrates [11, 14] or to extended intervals between supplementation events [4].

Given the relative energy excess in medium- to high-quality tropical pastures, infrequent N supplementation might improve N status in cattle, thus optimizing the utilisation of surplus forage energy. Under these conditions, the potential drawbacks of infrequent high-RDP supplementation on forage intake, N utilisation, and body N retention could be minimized. Nonetheless, limited research has investigated the balance between the quantity of supplemental N and supplementation frequency in cattle fed medium-quality tropical grass.

Understanding the effects of varying N supplementation frequencies can provide valuable insights for improving N efficiency in ruminants, balancing N intake and retention, and reducing environmental N losses in supplementation programs. Hence, we aimed to evaluate the effects of the amount and frequency of N supplementation on nutritional performance and metabolism in cattle fed medium-quality tropical grass forage.

2 Materials and methods

The experiment was carried out at the Department of Animal Science at the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. All the animals used in this experiment were sourced from the herd of the Research and Extension Unit in Beef Cattle of the Federal University of Viçosa, and all animal care and handling procedures were approved by the Univesity's Ethics Committee on the Use of Production Animals (protocol number 17/2013).

2.1 Animals, treatments, and experimental desing

Five ruminally and abomasally cannulated young Nellore × Holstein bulls, averaging 241 ± 16 kg body weight (BW) and 19 ± 1 mo, were used in a 5 × 5 Latin square design. The bulls were housed in individual pens (2 × 5 m) with concrete floor, equipped with feeders and water dispensers. Bulls had *ad libitum* access to water and mineral mixture. The basal diet consisted of medium-quality signal grass (*Uruchloa decumbens* Stapf) hay (Table 1). Medium-quality forage applied here refers to the average forage quality typically available to beef cattle under continuous grazing during the rainy season in tropical regions [15]. Bulls were fed *ad libitum* twice daily at 0600 and 1800 h, with feed adjusted to leave approximately 100 g/kg in orts (as fed).

Five treatments were evaluated as follows: (1) control, without supplementation; (2) daily supplementation with 30% (110 g of crude protein - CP) of RDP requirements; (3) infrequent supplementation every three days with 30% (110 g CP) of RDP requirements; (4) daily supplementation with 60% (220 g CP) of RDP requirements; (5) infrequent supplementation every three days with 60% (220 g CP) of RDP requirements. To ensure similar daily CP amounts across supplemented treatments, bulls receiving infrequent supplementation at 30 and 60% RDP requirements were given 330 and 660 g of CP, respectively, on each supplementation day.

The RDP requirements were calculated according to the BR-CORTE system [16], assuming a crossbred bull with 250 kg BW and 500 g/d of BW gain. The supplement was composed of pure casein (LabSynth, Diadema, São Paulo, Brazil), urea, and ammonium sulphate at 850, 135, and 15 g/kg as fed, respectively (Table 1). Supplements were divided into two equal portions, packed into paper bags, and delivered directly into the rumen concomitantly with the forage supply.

Table 1 Chemical composition of grass hay and supplement

Item[a]	Grass hay[b]	Supplement
DM, g/kg as fed	902 ± 2.4	894
OM, g/kg DM	945 ± 0.5	981
CP, g/kg DM	80.5 ± 2.6	1140
NDFap, g/kg DM	772 ± 4.1	–
NDIP, g/kg DM	46.8 ± 2.9	–
iNDF, g/kg DM	337 ± 12.0	–
DOM, g/kg DM[c]	463 ± 32.3	–

[a] DM, dry matter; OM, organic matter; CP, crude protein; NDFap, neutral detergent fibre assayed with a heat-stable amylase and corrected for contaminant ash and protein; NDIP, neutral detergent insoluble protein; iNDF, indigestible neutral detergent fibre; DOM, digested organic matter

[b] Mean ± standard error of the mean

[c] DOM was used as an indicator of the energy content of the diets. However, it was measured as an experimental response in this study. The value shown in this Table corresponds to the dietary DOM content obtained for the control treatment (i.e., only forage, without supplementation). Plese refer to the Results section for a more detailed presentation of this variable

2.2 Experimental procedures and sample collections

Each experimental period lasted 24 d, with the initial 9 d for adapting bulls to the treatments and the final 15 d for sample collections. We did not adopt a washout period between experimental periods. Our decision was base on the fact that basal diet – composed entirely of forage – was consistent across all treatments and accounted for 96 to 100% of the total diet. A washout period is typically necessary when the basal diet differs substantially between treatments, where apatation is expected to take longer [17], which was not the case in our study. Aditionally, the only component that varied between treatments was the supplemental N, which made up a maximum of 4% of the total diet. The residual effect of degradable N in the rumen are known to dissipate rapidly [18, 19], making a washout period less critical in this context. Lastly, we included a 9-d adaptation period (i.e., wash-in period [20]), at the beginning of each experimental period before any sampling began. This period was considered sufficient to dissipate any residual effects of the previous treatment and allow the animal to adapt to the new N supplementation scenario.

Bulls were weighed at the beginning and end of each experimental period to calculate average BW and relative voluntary intake. All evaluations were performed over at least one supplementation cycle (i.e., 3 d, or multiples of 3-d). The 3-day cycle is defined as follows: day 1, when both frequently and infrequently supplemented bulls received supplements; and days 2 and 3, when only frequently supplemented bulls received supplements. Infrequent supplementation commenced on the first day of each experimental period.

Voluntary forage intake was measured from d 10 to d 15 of each experimental period. The amount of hay offered from d 10 to d 15 and the orts collected from d 11 to d 16 were recorded to quantify voluntary intake. On each day, representative samples of both forage and orts were taken and ground through a 2-mm screen sieve using a knife mill. Half of each sample was then further ground to pass through a 1-mm screen sieve. Samples were pooled per animal and stored for subsequent chemical analysis. Supplement samples were collected directly from the feed mill during each production batch.

A total of 12 faecal grab samples were collected from d 11 to d 16 of each experimental period according to the following schedule: d 11 at 0400 and 1600 h; d 12 at 0600 and 1800 h; d 13 at 0800 and 2000 h; d 14 at 1000 and 2200 h; d 15 at 1200 and 2400 h; and d 16 at 0200 and 1400 h. Faecal samples were oven-dried at 55°C, ground and pooled as previously described for further analysis.

Digesta flow into the abomasum was estimated using the double-marker method, with indigestible neutral detergent fibre (iNDF) and cobalt-ethylenediaminetetraacetic acid (Co-EDTA) as particle and fluid markers, respectively. Bulls received 4 g/d of Co-EDTA, divided into four ruminal infusions at 0600, 1200, 1800, and 2400 h from d 8 to d 15 of each period. Abomasal digesta samples were collected from d 11 to d 16, according to the following schedule: d 11 at 0600 h; d 12 at 1000 h; d 13 at 1400 h; d 14 at 1800 h; d 15 at 2200 h; and d 16 at 0200 h. At each sampling point, approximately 1 L of digesta was collected and divided into two parts. The first aliquot of 750 mL was filtered through a nylon filter (100 µm; Sefar Nitex 100/44; Sefar, Heiden, Switzerland) to separate liquid and particulate phases for digesta flow estimation. The remaining 250 mL was used to isolate microorganisms associated with fluids and solids, following the procedures by

Reynal et al. [21]. Bacterial pellets (from both phases) were frozen (-80ºC), freeze-dried, and ground using a mortar and pestle.

From d 16 to d 18, urine was collected using funnel collectors attached to the bulls and secured with elastic straps across the bulls' backs. Funnels connected to hoses directed the urine into clean polyethylene containers kept in Styrofoam boxes filled with ice to minimize N losses. Urine collection began at 0600 h on d 16. At the end of each 24-h period, the total urine was measured, mixed, and two 50-mL aliquots per animal were filtered through four layers of cheesecloth. The first aliquot was used for total N, urea N, and creatinine analyses, and the second aliquot was frozen at -80ºC for subsequent analysis of 3-methylhistidine (3-MH).

From d 19 to d 21, rumen digesta samples were taken from the liquid-solid interface of the rumen mat at 0600 h, 1200 h, 1800 h, and 2400 h. Samples were filtered through three layers of cheesecloth and immediately assessed for pH using a digital potentiometer. Then, a 40-mL aliquot of ruminal fluid was fixed with 1 mL of a sulphuric acid solution (500 mL/L) and frozen at -20 °C for subsequent rumen ammonia N (RAN) analysis. Another 20-mL aliquot was fixed with 5 mL of metaphosphoric acid solution (250 g/L) and frozen at -20 °C for volatile fatty acid (VFA) analysis.

Simultaneously to the rumen digesta sampling, blood samples were collected from the jugular vein using vacuum tubes with either coagulation accelerator gel (BD Vacutainer®, SST II Advance, Franklin Lakes, New Jersey, USA) or coagulation inhibitor gel (BD Vacutainer® K2, Franklin Lakes, New Jersey, USA). Tubes were promptly centrifuged to separate serum and plasma and then frozen at -20ºC for later analysis.

The ruminal fibre pool was measured at 1000 h (4 h post-morning feeding) on d 22 and at 0600 h (pre-morning feeding) on d 24 of each experimental period. Whole ruminal contents were manually evacuated via the ruminal cannula, placed in plastic containers, weighed, and thoroughly hand-mixed. A 50 g/kg sample was taken, and the remaining ruminal contents were promptly returned to the rumen. Samples were oven-dried (55 ºC), ground as previously described, and pooled by animal and period. To minimize animal stress, rumen evacuation was not performed during the 3-day supplementation cycle.

2.3 Laboratory analysis

Samples of hay, supplement, orts, abomasal digesta (liquid and particle phases), ruminal contents (collected from ruminal evacuation), and faeces, which were processed to pass through a 1-mm sieve, were analysed for dry matter (DM, dried over-night at 105ºC, method G003/1), organic matter (OM, combustion in a muffle furnace at 550ºC, method M-001/2), and CP (Kjeldahl procedure, method N-001/2) contents according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science - INCT-CA [22]. The neutral detergent fibre (NDF) analysis was performed using a thermostable α-amylase without sodium sulphite (method F-012/1; [22]). The NDF contents were expressed exclusive of ash and protein contaminants (NDFap).

Hay, orts, abomasal digesta, and ruminal content samples, processed to pass through a 2-mm screen sieve, were evaluated for iNDF content using F57 filter bags (Ankom Technology Corp., Macedon, New York, USA) and a 288-h in situ incubation procedure [23]. Potentially digestible NDF (pdNDF) was calculated as the difference between NDFap and iNDF.

Cobalt concentration in abomasal digesta samples (liquid and solid phases) was measured using an atomic absorption spectrophotometer (Avanta Σ; GBC Scientific Equipment, Braeside, Victoria, Australia). The RAN concentration was quantified using a colorimetric method (method N-006/1; [22]). The VFA concentration was measured by high-performance liquid chromatography (HPLC, Shimadzu SPD-10 A VP model) using a reversed-phase column with *o*-phosphoric acid (0.15 M) as the mobile phase and ultraviolet detection at 210 nm wavelength. The RAN, VFA concentrations, and ruminal pH values obtained at different sampling times were averaged per animal and period within each day of the supplementation cycle, resulting in a single representative daily average value.

Blood samples were pooled per animal and day of the supplementation cycle (equal volumes from each collection time) and analysed for serum urea N (SUN; enzymatic-colorimetric method; Human® 10505, Panama City, Panama), creatinine and triglycerides (alkaline picrate method; Human® 006, Panama City, Panama), and glucose (enzymatic-colorimetric method; Biosystems® A15, Barcelona, Spain).

Urine samples were analysed for total N, urea N, and creatinine contents according to previously described methods. Urinary 3-MH concentration was quantified by high-performance liquid chromatography in a commercial laboratory (Hermes Pardini Laboratory, Belo Horizonte, Minas Gerais, Brazil). Abomasal digesta samples (both liquid and solid fractions) and isolated microorganisms were analysed for purine bases [24] and CP (method N-001/2; [22]) contents.

2.4 Calculations

Faecal excretion was estimated by calculating the ratio of faecal iNDF concentration to daily iNDF intake. Abomasal flow was estimated using iNDF as an internal particle-phase marker and Co-EDTA as an external liquid-phase marker. The iNDF was assumed to be an ideal particle phase marker, while Co-EDTA was treated as a non-ideal liquid phase marker. The abomasal digesta reconstitution factor was calculated as stated by France and Siddons [25].

The intake and ruminal passage rates of NDF, pdNDF and iNDF were estimated by the ratio of intake and abomasum flow in relation to the rumen mass, respectively. The pdNDF degradation rate was obtained from the difference between the intake and passage rates [26].

The N balance (N retention) was calculated by subtracting faecal N excretion and urinary N excretion (UNE) from N intake. This calculation considered the average UNE across the three-day-supplementation cycle. Rumen N balance (RNB) was calculated by subtracting N abomasal flow from N intake.

Glomerular filtration of urea N (GFU) and fractional N-urea excretion were calculated as follows:

$$\mathrm{GFU} = \frac{\mathrm{UEC}}{\mathrm{SC}} \times \mathrm{SUN}, \tag{1}$$

$$\mathrm{FEU} = \frac{\mathrm{UUNE}}{\mathrm{GFU}}, \tag{2}$$

where GFU is the glomerular filtration of urea N (g/d), UEC is the urinary excretion of creatinine (g/d), SC is the serum creatinine (g/dL), SUN is the serum urea N (g/dL), FEU is the fractional excretion of N urea (g/g), and UUNE is the urinary urea-N excretion (g/d).

Microbial N synthesis in the rumen was quantified as the product of abomasal flow and microbial N concentration. The N_{RNA}:N_{total} ratio in microorganisms was used as a marker to estimate microbial production in the rumen.

2.5 Statistical analysis

The experiment was analysed according to a 5 × 5 Latin square design, including the fixed effects of treatments and the random effects of animal and experimental period. Treatments were evaluated according to a $2 \times 2 + 1$ factorial arrangement (two supplement amounts, 30 or 60% of the daily RDP requirements; two supplementation frequencies, daily or every three days; plus a control treatment). To ensure an orthogonal comparison among treatments, the control was compared against all supplemented treatments using one contrast. Results from this contrast are described hereafter as the general effect of N supplementation.

Analyses for variables related to rumen fermentation, blood characteristics, and urinary excretion were performed by considering the days of the supplementation cycle as repeated measures (fixed effect). The optimal (co)variance matrix structure was selected based on the Akaike's information criterion with correction. The degrees of freedom were estimated by the Kenward-Roger method. Statistical analyses were performed using the MIXED procedure of SAS 9.4 ($\alpha = 0.05$).

3 Results

3.1 Intake and digestibility

There was no effect ($p \geq 0.38$) of N supplementation on voluntary intake, except for CP intake, which increased ($p < 0.01$) with N supplementation (Table 2). No supplementation frequency × supplement amount interactions were observed ($p \geq 0.23$) on voluntary intake. Similarly, supplement amount had no effect on voluntary intake ($p \geq 0.41$), except for CP intake, which was higher ($p < 0.01$) in animals supplemented at 60% of RDP requirements. Although not statistically significant, infrequent supplementation tended to reduce forage intake (kg/d; $p = 0.09$), which ultimately led to lower ($p \leq 0.04$) digested organic matter (DOM) and digested NDFap intakes. Furthermore, infrequent supplementation reduced ($p \leq 0.04$) total DM, forage DM, OM, and NDFap intakes when expressed as g/kg BW.

Overall, N supplementation improved ($p \leq 0.03$) ruminal, intestinal, and total CP digestibility (Table 3), but had no effect on OM and NDFap digestibility or dietary DOM content ($p \geq 0.17$). Furthermore, supplementation frequency did not affect ($p \geq 0.08$) digestibility characteristics.

An interaction effect ($p < 0.04$) was observed between supplementation frequency and supplement amount on ruminal OM digestibility. However, the slicing procedure for this effect did not reveal differences ($p > 0.05$) among factor levels. In addition, supplementation at 60% of RDP requirements improved ($p < 0.02$) both ruminal and total CP digestibility compared to animals supplemented at 30% RDP requirements. Notably, positive values for ruminal CP digestibility were only observed when 60% of the RDP requirements were provided.

Table 2 Effects of amount and frequency of nitrogen supplementation on voluntary intake in cattle fed medium-quality tropical forage

Item[c]	**Treatments[a]**					**SEM**	***p*-value[b]**			
	Control	**30/D**	**30/I**	**60/D**	**60/I**		**C vs. S**	**A**	**F**	**A × F**
kg/d										
DM	4.98	5.08	4.79	5.54	4.77	0.416	0.84	0.46	0.090	0.42
DMF	4.98	4.99	4.70	5.36	4.58	0.416	0.81	0.67	0.090	0.42
OM	4.72	4.81	4.54	5.25	4.52	0.393	0.84	0.45	0.091	0.42
CP	0.40	0.52	0.49	0.65	0.58	0.032	< 0.001	0.001	0.088	0.50
NDFap	3.83	3.84	3.62	4.13	3.54	0.315	0.83	0.63	0.086	0.40
iNDF	1.64	1.60	1.52	1.76	1.53	0.178	0.72	0.41	0.15	0.49
DOM	2.27	2.44	2.30	2.71	2.20	0.175	0.38	0.57	0.041	0.23
DNDFap	2.01	2.10	1.98	2.24	1.82	0.149	0.85	0.95	0.043	0.24
CP: DOM[d]	176	213	216	238	273	–	–	–	–	–
g/kg BW										
DM	19.4	20.6	19.1	21.6	18.2	1.87	0.70	0.94	0.044	0.37
DMF	19.4	20.2	18.8	20.9	17.5	1.87	0.94	0.78	0.045	0.37
OM	18.4	19.5	18.1	20.5	17.2	1.78	0.70	0.95	0.043	0.35
NDFap	14.9	15.5	14.4	16.1	13.5	1.41	0.96	0.83	0.044	0.37
iNDF	6.4	6.5	6.0	6.8	5.8	0.76	0.83	0.88	0.096	0.54

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency

[c] DM, dry matter; DMF, DM from forage; OM, organic matter; CP, crude protein; NDFap, neutral detergent fibre assayed with a heat-stable amylase and corrected for contaminant ash and protein; iNDF, indigestible neutral detergent fibre; DOM, digested OM; DNDFap, digested NDFap

[d] g CP/kg DOM

Table 3 Effects of amount and frequency of nitrogen supplementation on total, ruminal, and intestinal digestibility in cattle fed medium-quality tropical forage

Item[c]	**Treatments[a]**					**SEM**	***p*-value[b]**			
	Control	**30/D**	**30/I**	**60/D**	**60/I**		**C vs. S**	**A**	**F**	**A × F**
Ruminal, g/g[d]										
OM	0.322	0.295	0.362	0.360	0.305	0.0423	0.77	0.89	0.82	0.036
CP	– 0.335	– 0.139	– 0.047	0.090	0.005	0.0687	< 0.001	0.019	0.94	0.11
NDFap	0.539	0.501	0.535	0.528	0.518	0.0319	0.33	0.76	0.45	0.20
Intestinal, g/g[d]										
OM	0.245	0.297	0.215	0.258	0.263	0.0529	0.67	0.86	0.18	0.13
CP	0.589	0.629	0.601	0.623	0.645	0.0360	0.030	0.17	0.80	0.080
NDFap	-0.014	0.092	0.011	0.051	0.003	0.0494	0.16	0.47	0.075	0.61
Total, g/g										
OM	0.489	0.509	0.505	0.526	0.496	0.0341	0.17	0.73	0.20	0.30
CP	0.454	0.587	0.589	0.657	0.655	0.0335	< 0.001	0.001	0.98	0.86
NDFap	0.532	0.547	0.543	0.553	0.525	0.0320	0.50	0.64	0.23	0.37
DOM[e]	463	481	478	499	470	32.3	0.17	0.69	0.21	0.30

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency

[c] OM, organic matter; CP, crude protein; NDFap, neutral detergent fibre assayed with a heat-stable amylase and corrected for contaminant ash and protein; DOM, digested OM

[d] Ruminal and intestinal digestibility were calculated as the fraction of the mass that entered the digestion site

[e] g/kg DM

3.2 Ruminal fibre kinetics

Overall, N supplementation had no effect ($p \geq 0.22$) on ruminal fibre kinetics (Table 4). Conversely, increasing the amount of supplemental N decreased ($p < 0.04$) ruminal iNDF pool, regardless of supplementation frequency. An interaction effect was observed ($p < 0.03$) between supplementation frequency and supplement amount on the ruminal NDF pool. The slicing procedure indicated a reduction ($p < 0.05$) in the ruminal NDF pool only in animals supplemented infrequently at 60% of RDP requirements. No treatment effects ($p \geq 0.21$) were found on ruminal pdNDF pool. Furthermore, no differences were found among treatments ($p \geq 0.05$) regarding fibre intake, degradation, and passage rates.

3.3 Nitrogen balance and microbial synthesis

Overall, N intake mirrored the pattern observed for CP intake (Table 5). Conversely, no treatment effects were observed on faecal N excretion ($p \geq 0.14$), N retention ($p \geq 0.06$) or N utilisation efficiency ($p \geq 0.12$).

The N supplementation improved ($p < 0.01$) RNB compared to the control treatment. Likewise, increasing the amount of supplemental N enhanced RNB ($p < 0.01$), with no effect of supplementation frequency ($p > 0.99$). Notably, positive RNB values were observed only in animals supplemented at 60% of RDP requirements. No treatment effects ($p \geq 0.07$) were verified on microbial N synthesis or efficiency.

3.4 Ruminal fermentation characteristics

There was no effect ($p > 0.30$) of N supplementation on ruminal pH when compared to the control (Table 6). However, an interaction effect ($p < 0.02$) was observed between supplementation frequency and the supplement amount for ruminal pH. The slicing procedure showed that animals supplemented infrequently exhibited lower ruminal pH at 30% of RDP requirements, while those supplemented infrequently at 60% of RDP requirements had a higher ruminal pH compared to those supplemented daily. Despite

Table 4 Effect of amount and frequency of nitrogen supplementation on the resident fibre mass in the rumen and on the fractional rates of ruminal fibre dynamics in cattle fed medium-quality tropical forage

Item[c]	**Treatments**[a]					**SEM**	***p*-value**[b]			
	Control	**30/D**	**30/I**	**60/D**	**60/I**		**C vs. S**	**A**	**F**	**A × F**
g/kg BW										
NDF	17.7	17.7	18.0	17.8	15.8	1.64	0.47	0.048	0.10	0.028
pdNDF	4.1	3.4	3.5	4.2	3.3	0.52	0.26	0.39	0.31	0.21
iNDF	13.6	14.3	14.5	13.6	12.5	1.42	0.89	0.039	0.47	0.27
/h										
ki NDF	0.036	0.038	0.034	0.040	0.036	0.0051	0.76	0.31	0.067	0.88
ki pdNDF	0.099	0.112	0.106	0.104	0.101	0.0149	0.67	0.62	0.75	0.90
kd pdNDF	0.093	0.096	0.098	0.093	0.092	0.0143	0.88	0.73	0.96	0.92
kp pdNDF	0.007	0.016	0.008	0.010	0.009	0.0033	0.22	0.44	0.12	0.31
kp iNDF	0.020	0.021	0.018	0.023	0.020	0.0037	0.93	0.23	0.088	0.88

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency

[c] NDF, neutral detergent fibre; pdNDF, potentially digestible neutral detergent fibre; iNDF, indigestible neutral detergent fibre; ki, kp and kd rates of intake, passage and degradation, respectively

Table 5 Effect of amount and frequency of nitrogen supplementation on N retention and microbial synthesis in the rumen of cattle fed medium-quality tropical forage

Item[3]	Treatments[1]					SEM	*p*-value[2]			
	Control	30/D	30/I	60/D	60/I		C vs. S	A	F	A × F
N intake, g/d	63.9	82.4	77.8	103.2	93.1	5.11	< 0.001	0.001	0.088	0.49
Fecal N, g/d	35.3	34.1	31.9	35.8	23.6	4.08	0.40	0.50	0.14	0.77
N retention[c]										
g/d	3.3	9.6	7.1	17.4	9.0	3.90	0.063	0.15	0.12	0.38
g/g N intake	0.05	0.12	0.09	0.17	0.10	0.047	0.12	0.38	0.20	0.54
Rumen N balance	– 21.4	– 12.1	– 4.8	9.0	1.7	5.83	0.001	0.006	0.99	0.10
Microbial N synthesis										
g/d	31.1	40.5	44.8	53.6	42.6	7.81	0.073	0.42	0.61	0.26
g CP/kg DOM[4]	84.4	100.2	122.3	123.1	130.9	20.05	0.12	0.41	0.43	0.71

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30%RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency

[c] The N retention was calculated considering the average N excretion across the days of the supplementation cycle (Table 7).
[4] CP, crude protein; DOM, digested organic matter

Table 6 Effects of amount and frequency of nitrogen supplementation on ruminal fermentation characteristics in cattle fed medium-quality tropical forage

Item[c]	Treatments[a]					SEM	*p*-value[b]				
	Control	30/D	30/I	60/D	60/I		C vs. S	A	F	A × F	T × D
pH	6.30	6.29	6.21	6.19	6.31	0.098	0.30	0.96	0.66	0.027	0.65
RAN, mg/dL	6.8	13.8	16.4	21.3	28.0	1.85	< 0.001	< 0.001	0.009	0.19	< 0.001
VFA, mmol/dL	4.97	4.61	4.81	5.11	5.19	0.481	0.91	0.22	0.68	0.87	0.35
VFA, mmol/100 mmol											
Acetate	67.7	66.5	68.1	67.0	67.7	0.86	0.65	0.93	0.14	0.56	0.60
Propionate	20.6	21.6	21.3	21.2	20.7	0.38	0.12	0.12	0.17	0.72	0.25
Butyrate	11.6	11.9	10.6	11.8	11.6	0.66	0.79	0.43	0.19	0.31	0.57
A: P	3.30	3.09	3.22	3.19	3.31	0.098	0.31	0.26	0.16	0.91	0.42

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency, T × D, interaction between treatments and days of the supplementation cycle

[c] RAN, rumen ammonia N; VFA, volatile fatty acids; A:P, acetate-to-propionate ratio

these variations, ruminal pH values were generally similar across treatments, suggesting that these changes are unlikely to affect rumen function.

On average, N supplementation increased ($p < 0.01$) RAN compared to the control treatment. Among supplemented animals, N supplementation at 60% of RDP requirements raised RAN concentration ($p < 0.01$) compared to supplementation at 30% of RDP requirements. Likewise, on average, infrequent supplementation led to higher ($p < 0.01$) RAN concentration compared to daily supplementation. However, an interaction effect ($p < 0.01$) was observed between treatments and supplementation cycle days on RAN concentrations. Examination of this effect revealed that RAN concentration remained constant ($p \geq 0.05$) across supplementation cycle days for both the control and daily supplementation groups (Fig. 1). Conversely, with infrequent supplementation, RAN concentrations peaked on the first day of the supplementation cycle ($p < 0.05$) and gradually declined over the following days.

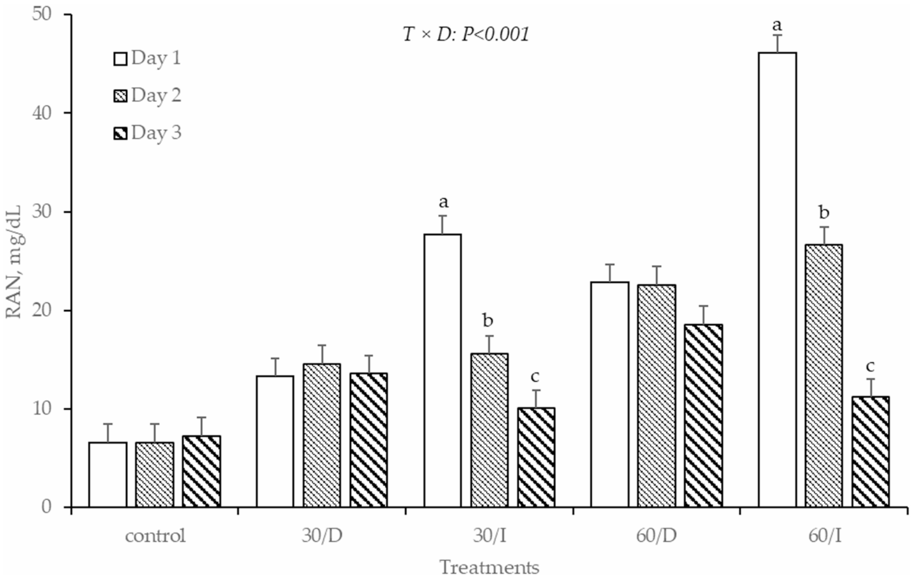

Fig. 1 Effect of amount and frequency of nitrogen supplementation on the average daily concentration of ruminal ammonia nitrogen (RAN) in cattle fed medium-quality tropical forage, according to different days of the supplementation cycle. [Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively. Means followed by different letters within a treatment are different at $p < 0.05$]

Table 7 Effects of amount and frequency of nitrogen supplementation on blood, urinary excretion characteristics, and kidney function in cattle fed medium-quality tropical forage

Item[c]	**Treatments**[a]					**SEM**	***p*-Value**[b]				
	Control	**30/D**	**30/I**	**60/D**	**60/I**		**C vs. S**	**A**	**F**	**A × F**	**T × D**
Blood characteristics											
Glucose, mg/dL	46.5	42.3	46.3	48.1	51.4	5.10	0.82	0.031	0.12	0.89	0.57
Triglycerides, mg/dL	15.7	16.5	15.7	16.7	12.6	2.12	0.79	0.18	0.038	0.13	0.89
SUN, mg/dL	7.0	12.0	11.3	15.9	17.1	1.49	< 0.001	< 0.001	0.66	0.22	< 0.001
Urinary excretion											
3-MH: creatinine, mg/g	105	107	97.8	103	94.1	10.36	0.52	0.62	0.18	0.92	0.27
UNE, g/d	25.6	39.0	45.2	49.9	52.7	4.95	0.001	0.078	0.36	0.72	0.62
UUNE, g/d	8.6	19.1	19.0	28.9	24.5	2.54	< 0.001	< 0.001	0.075	0.080	0.61
Kidney function											
GFU, g/d	18.6	33.5	34.4	45.7	48.9	4.58	< 0.001	< 0.001	0.42	0.65	0.12
FEU, g/g	0.467	0.571	0.560	0.682	0.537	0.0509	0.019	0.29	0.077	0.11	0.92

[a] Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively

[b] C vs. S, control vs. N supplementation; A, amount of supplement (30 vs. 60% RDP); F, supplementation frequency (daily vs. infrequent); A × F, interaction between supplement amount and supplementation frequency; T × D, interaction between treatments and days of the supplementation cycle

[c] SUN, serum urea N; 3-MH, 3-methylhistidine; UNE, urinary N excretion; UUNE, urinary urea-N excretion; GFU, glomerular filtration of urea N; FEU, fractional excretion of urea N

No treatment effects ($p \geq 0.12$) were found regarding VFA concentration or molar proportion.

3.5 Blood and urinary excretion characteristics, and kidney function

There was no effect ($p \geq 0.12$) of N supplementation or supplementation frequency on blood glucose concentration (Table 7). However, increasing the amount from 30 to 60%

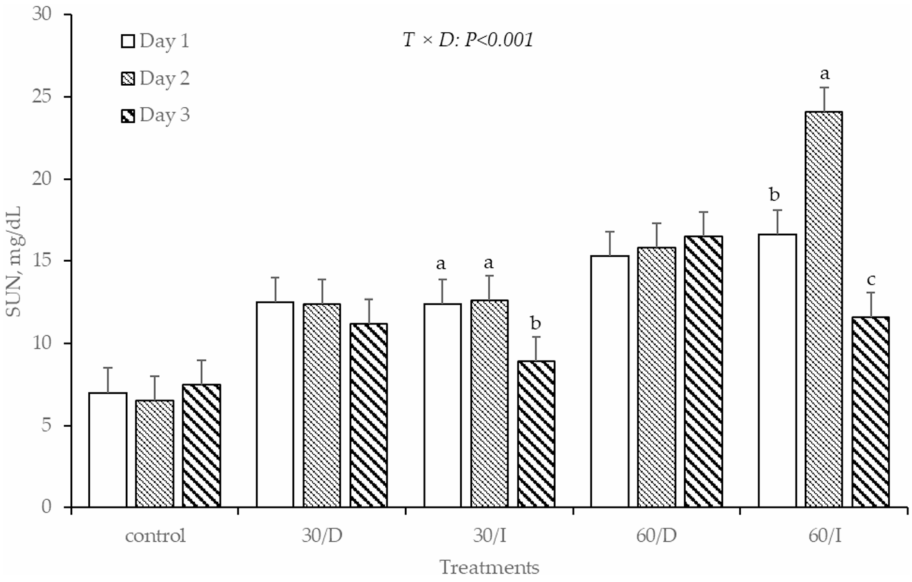

Fig. 2 Effect of amount and frequency of nitrogen supplementation on the average daily concentration of serum urea nitrogen (SUN) in cattle fed medium-quality tropical forage, according to different days of the supplementation cycle. [Control, without supplementation; 30/D and 30/I, daily and infrequent (every three days) supplementation with 30% RDP requirements, respectively; 60/D and 60/I, daily and infrequent (every three days) supplementation with 60% RDP requirements, respectively. Means followed by different letters within a treatment are different at $P < 0.05$]

of RDP requirements enhanced ($p < 0.04$) blood glucose. Additionally, infrequent N supplementation decreased ($p < 0.04$) blood triglycerides concentration compared to daily supplementation. Conversely, there were no effects ($p \geq 0.13$) of N supplementation or supplement amount on blood triglycerides concentrations.

On average, N supplementation raised ($p < 0.01$) SUN concentrations compared to control treatment. Similarly, SUN concentration was enhanced by increasing the supplemental N amounts ($p < 0.01$) but was not affected by the supplementation frequency ($p > 0.66$). However, an interaction effect ($p < 0.01$) between treatments and supplementation cycle days was observed for SUN concentrations. Analysis of this effect revealed that SUN concentrations varied ($p < 0.05$) across supplementation cycle days only with infrequent supplementation (Fig. 2). Specifically, for infrequent supplementation at 30% of RDP requirements, SUN concentrations remained stable ($p > 0.05$) during the first two days of supplementation cycle but declined ($p < 0.05$) on the third day. In contrast, for infrequent supplementation at 60% of RDP requirements, SUN concentrations peaked ($p < 0.05$) on the second day of the supplementation cycle before decreasing on the third day.

No treatment effects ($p \geq 0.18$) were observed on urinary excretion of 3-MH. However, N supplementation increased ($p < 0.01$) both UNE and UUNE compared to control. Even though no effect was detected on overall UNE ($p > 0.07$), increasing the supplemental N amount increased UUNE ($p < 0.01$). Additionally, no effects ($p \geq 0.07$) of supplementation frequency were found on UNE and UUNE.

Overall, supplementation increased GFU and FEU compared to control treatment ($p < 0.02$). Similarly, increasing the supplemental N amount increased GFU ($P < 0.01$), although it had no effect on FEU ($p > 0.29$). No effects ($p \geq 0.07$) of supplementation frequency were observed on renal function characteristics.

4 Discussion

In this study, we observed no effect of N supplementation on voluntary forage intake. In tropical environments, increased forage intake is typically associated with the availability of N compounds for rumen microorganisms and the sufficiency of absorbed nutrients for metabolic demands [1]. However, the basal diet in the current study had a CP content close to the minimal level required for optimal microbial growth on fibre substrates (80–100 g CP/kg DM; [1, 27]). Given these conditions, N supplementation would not expect to impact fibre utilisation. This statement is further supported by the absence of overall effects of N supplementation on fibre digestibility and ruminal kinetics.

On the metabolic level, however, N supplementation did increase N availability. This is evidenced by the elevated CP: DOM ratio with N supplementation. This ratio serves as an indicator of intake adequacy by reflecting both ruminal and metabolic events [1, 28]. In tropical regions, it has been suggested that optimal forage intake occurs when the CP: DOM ratio reaches approximately 220 g/kg [29]. In this study, N supplementation increased the CP: DOM ratio from 176 to 235 g/kg. Consequently, an increase in forage intake might be expected under these conditions, yet this was not observed. This finding is consistent with several studies that reported no effects of N supplementation on intake or digestion characteristics in cattle fed medium- to high-quality tropical forage [10, 30, 31].

Although not statistically significant ($P = 0.063$), it is noteworthy that N supplementation increased N retention compared to control, without affecting N utilisation efficiency. On average, supplemented animals retained 10.8 g N/d, while control animals retained 3.3 g N/d. At first glance, this increase could be attributed to a greater metabolisable protein supply, potentially resulting from improved microbial N synthesis with N supplementation. Indeed, we observed that N supplementation tended ($P = 0.073$) to increase microbial N synthesis from 31.1 g/d to 45.4 g/d. However, studies conducted in the tropics suggest that the increase in microbial N supply from supplementation typically accounts for only about 20% of the total increase in N retention [32]. This suggests that most of the effects of supplemental N are likely tied to post-ruminal or metabolic changes.

The metabolic impact of supplemental N can, to some extent, be assessed by examining both RAN and RNB values. We found negative RNB values for both the control and animals supplemented at 30% RDP requirements. In tropical conditions, several studies have reported negative RNB in cattle fed medium- to high-quality tropical forage [10, 12, 30, 31]. This pattern indicates that the N flow from the rumen to the abomasum exceeds N intake. Under these circumstances, a larger proportion of dietary N is directed toward N recycling, which increases the contribution of microbial N derived from recycled N [31, 33, 34]. Consequently, less N is available for other functions, such as tissue synthesis and deposition, reducing N retention and utilisation efficiency [2]. Under tropical conditions, RNB is positively associated with dietary N availability (e.g., dietary CP concentration and RAN). Detmann et al. [1] have indicated that a minimum RAN of 9.2 mg/dL is necessary to achieve a balanced N input and output in the rumen (i.e., zero RNB) and to ensure adequate N availability for other metabolic demands. In this study, we observed that N supplementation increased RAN to levels above 9.2 mg/dL, supporting an improvement in RNB and explaining the enhanced N retention observed in supplemented animals. Notably, positive RNB values were achieved only in animals supplemented at 60% RDP.

Additionally, we observed greater blood glucose concentrations in animals supplemented at 60% RDP compared to those at 30% RDP. This finding further supports the enhanced metabolic effects due to N supplementation. Ruminants can synthesize part of their glucose demanded through gluconeogenic amino acids [35]. However, when N availability is low, hepatic demand for amino acids for urea synthesis increases [36]. Therefore, the additional N supply may reduce reliance on gluconeogenic amino acids for intermediary N metabolism, thereby increasing the availability of precursors for glucose synthesis. Overall, our results indicate that the primary impact of N supplementation is on metabolic processes, with minimal changes observed in intake, digestion characteristics, or microbial growth.

Among supplemented animals, infrequent supplementation decreased voluntary forage intake. This reduction in forage intake was accompanied by lower DOM and digested NDF intakes, an effect directly related to decreased forage intake since digestibility coefficients were unaffected by supplementation frequency. Although no interaction was observed between supplementation frequency and supplement amount, the most prominent numerical decrease in forage intake occurred with infrequent supplementation at 60% of RDP requirements. This finding is further supported by the reduced ruminal NDF pool in animals supplemented infrequently at 60% RDP.

Several authors have shown negative effects of infrequent N supplementation on voluntary intake in cattle fed medium- to high-quality forage [10, 12, 37]. These effects are generally attributed to the large amounts of supplemental N provided at once, potentially leading to an excess of N in animal metabolism [10]. In our study, the impact of excess N was evident from elevated RAN concentrations with infrequent supplementation. On average, infrequent supplementation raised rumen RAN from 17.6 to 22.2 mg/dL compared to daily supplementation. This elevation likely resulted from a sharp ammonia peak on the first day of the supplementation cycle, reaching 46.1 mg/dL in animals supplemented at 60% RDP. Such high concentrations seem to exceed the rumen's capacity for ammonia uptake [1] and have been associated with decreased voluntary intake in tropical conditions [15]. Excess ammonia can reduce intake due to potential hepatic ATP deficiencies from increased urea cycle activity [38], increased body heat production [28], and discomfort due to excess ammonia in the bloodstream and within cells [39]. It is important to emphasize that our primary objective was to investigate the effects of infrequent N supplementation alone (i.e., without an additional energy source). Recent studies indicate that supplying both protein and energy can mitigate the adverse effects of infrequent supplementation on voluntary intake in cattle [12].

One of the primary concerns with infrequent, high-amount RDP supplementation is the potential for increased urinary N losses, which could ultimately lead to decreased N retention [9, 13]. However, despite the negative impact on forage intake, we did not observe any effects on N retention or N utilisation efficiency. Given the high ammonia concentrations detected in animals receiving infrequent supplementation (averaging 17.5 mg/dL for daily supplementation and 22.2 mg/dL for infrequent supplementation), one might expect an increase in urinary N excretion. Surprisingly, we found no effect of supplementation frequency on urinary N excretion. This finding suggests that animals supplemented infrequently may have employed physiological mechanisms to conserve dietary N in response to reduced supplementation frequency, potentially explaining the observed lack of effect on N utilisation efficiency.

The aforementioned arguments can be supported by assessing RAN concentrations throughout the supplementation cycle. Despite a gradual decrease in RAN levels over the days, infrequently supplemented animals maintained greater RAN concentrations than non-supplemented animals on days 2 and 3 (i.e., days without supplement access). This indicates that infrequently supplemented animals were able to conserve dietary N on days without supplementation, likely due, at least partially, to N recycling mechanisms to the rumen. In fact, N recycling has been recognized as a primary physiological mechanism explaining the maintenance of N status in the metabolism of infrequently supplemented animals [9, 10].

In tropical conditions, indirect evidence of increased N recycling can be inferred from decreases in RNB [1]. However, we did not observe any effects of supplementation frequency on RNB. Nevertheless, a combined evaluation of both RAN and SUN concentration patterns may provide insights into N conservation in infrequently supplemented animals. Typically, RAN and SUN concentrations are linearly associated [40, 41]. Notably, maximum RAN concentrations were observed on the first of the supplementation cycle, while peak SUN concentrations were recorded on the day after supplementation, particularly with infrequent provision of 60% of RDP requirements. When high ammonia detoxification is required, the liver's capacity for urea synthesis may become overloaded, allowing some ammonia to be absorbed by perivenous hepatocytes as glutamine. This process can potentially cause delays in urea synthesis over time [42]. Some researchers have suggested that this delay may be associated with a lag in enzyme expression within the ornithine cycle [43]. Such a pattern has been consistently observed in infrequently supplemented animals fed both low- [4, 11, 44] and medium- to high-quality forages [10, 12] and it appears to play a crucial role in maintaining adequate N status in animal metabolism, even on non-supplement days.

Furthermore, we observed that the peak in SUN on the second day of the supplementation cycle was not followed by an increase in urinary urea N excretion, an atypical pattern. A similar observation has been reported in studies with animals subjected to infrequent supplementation and fed high-quality tropical forage [10]. In theory, these results could be attributed to either an increase in SUN directed toward N recycling pathways or changes in the kidneys' fractional rates of N excretion. The lack of effects of supplementation frequency on GFU and FEU suggests that increased N recycling is the more likely explanation. Collectively, these observations could explain the absence of any effect from infrequent supplementation on urinary N excretion and, consequently, on N retention.

Given that N retention is closely linked with productive performance, this implies that infrequent supplementation would not adversely affect animal performance. Indeed, studies conducted in tropical conditions have indicated that reducing protein supplementation frequency to three times weekly does not impact the performance of grazing cattle, regardless of pasture quality [8]. This suggests that similar physiological mechanisms are involved in maintaining N status in metabolism, irrespective of basal forage quality.

5 Conclusions

Reducing the frequency of nitrogen supplementation decreases voluntary intake in cattle fed medium-quality tropical grass. However, infrequent supplementation does not impair digestion, nitrogen utilisation, or metabolic processes in cattle fed medium-quality tropical grass.

Author contributions

L.M.A.R., investigation, writing - original draft; J.P.P.R., investigation, writing - original draft M.O.F.: Writing – review and editing; N.S.A.L., writing – review and editing; L.C.O.S., writing – review and editing; C.B.S., writing – review and editing; E.D., writing – review and editing, conceptualization, formal analysis, supervision, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data generated during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All animals used in this experiment were sourced from the University's own herd, and the care and handling procedures were approved by the Ethics Committee on the Use of Production Animals of the Federal University of Viçosa (protocol number 17/2013). The study was conducted in accordance with the Brazilian Guide for the Production, Maintenance, and Use of Animals for Educational or Scientific Research Activities, issued by the National Council for the Control of Animal Experimentation (CONCEA), Brazil.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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