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Can Ammonium Nitrate Be a Strategic Tool by Replacing Urea as a Nitrogen Supplementation Source to Beef Cattle in Intensified Grazing Systems?

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Abstract: For cattle raised on tropical grass pastures, it is essential to explore strategies that circumvent climatic seasonality that affect forage availability and quality. We hypothesize that the intensification of grazing systems, with rotational and deferred methods, combined with ammonium nitrate or urea supplementation, are excellent strategies to increase ruminal efficiency and animal productivity. For this purpose, 8 cattle with cannulas were distributed in rotational and deferred grazing systems, supplemented with urea or ammonium nitrate, and evaluated throughout the four seasons of the year over a period of two years. Dry matter intake and digestibility were measured using indigestible neutral detergent fiber, titanium dioxide and chromium oxide markers. Ruminal kinetics and degradability of DM and nutrients were measured using the nylon bag technique. Urine parameters were used to estimate microbial nitrogen compounds synthesis and efficiency of microbial protein synthesis. The rotational grazing improves NPN intake, NDF and ADF digestibility, and gross energy. Ammonium nitrate supplementation showed improved efficiency in microbial protein synthesis without negatively affecting feed intake, positioning it as a valuable nitrogen source for grazing cattle.

Keywords: beef cattle; deferred grazing; non-protein nitrogen; rotated grazing

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1. Introduction

In tropical regions, the beef industry can rely on grazing systems as it is economically more attractive; however, it has some disadvantages regarding pastures' vulnerability to climatic seasonality. Alternatives have been studied for growing beef cattle in tropical grazing systems as a means to improve performance and mitigate the negative effects of seasonality [1–4]. The intensification of grazing systems by pasture management (stockpiling or rotation) and adoption of supplementation are the most used tools to overcome the seasonality of the tropics and ensure ideal beef cattle production [5]. Non-protein nitrogen (NPN) supplementation is used as a strategy to increase the proportion of protein into the diet and meet the requirement of nitrogen ammonia for microbial protein synthesis in the rumen.

Notably, urea is one of the most well-known NPN sources that can be efficiently used in beef cattle diets. Alternative sources, such as nitrates, have also been used [6–9]; however, to our understanding, most of the research has been performed with cattle in feedlot systems, and fewer results are found in the literature with cattle in grazing systems being supplemented with nitrate. NPN supplementation can be nutritionally favorable, especially in tropical regions, where forage quality and availability vary greatly through the year [10], displaying lower protein content and increased lignification during the dry season, which are enough to directly affect feed intake and the digestion of structural carbohydrates in the rumen [11].

Nitrate metabolism leads to a higher release of negative Gibbs free energy compared to other NPN sources, potentially supporting greater microbial activity [12]. Coupled with fomenting bacteria growth, nitrate has the capability to lower methane production, and thus to play an important role in the mitigation of greenhouse gases emissions to the atmosphere [4,13] while preserving the energy that can be used by the animal.

Nonetheless, nitrate bitterness can be a limiting factor affecting feed intake [14,15], which might elicit lower digestibility of the diet's nutrients of cattle in tropical grazing systems and thus negatively compromise the synthesis and efficiency of microbial protein in the rumen. This effect can even be potentialized through the seasons of the year if any supplementation is adopted, as forage quality and its availability changes through the year [10].

Several nitrates can be used as NPN sources for ruminants, such as ammonium nitrate, calcium nitrate, sodium nitrate, and potassium nitrate, including encapsulated formulations with more than one N source [16–19]. In the rumen, bacteria can utilize H_3 as an electron donor to reduce nitrate to nitrite and ammonia. Furthermore, nitrite is toxic to methanogenic microorganisms [19]. When compared to urea, nitrates undergo a slower metabolism in the rumen, which reduces the risk of ammonia toxicity. In the bloodstream, nitrite converts hemoglobin to methemoglobin, causing methemoglobinemia. Therefore, careful dietary adaptation and not exceeding the upper supplementation limit are important [19,20].

Therefore, the hypothesis of this study is that the intensification of grazing systems through the adoption of rotational and deferred grazing methods, associated with non-protein nitrogen supplementation, has a positive effect on beef cattle ruminal metabolism. Furthermore, we hypothesize that the use of ammonium nitrate will favor the synchronization of nutrient utilization by rumen microorganisms, presenting an alternative to urea supplementation.

2. Materials and Methods

The study was conducted at the College of Veterinary Medicine and Animal Science (FMVZ/USP) in Pirassununga, Sao Paulo State, Brazil, at coordinates 21°56′47″ S 47°27′05″ W, over a two-year period.

The experiment began in June 2019 and ended in June 2021, with the second year being considered a repetition of the first year. The samples were taken two months after the beginning of each season (spring, summer, autumn, and winter). The study was approved by and followed the guidelines of the Committee for the Use and Care of Institutional Animals of the College of Veterinary Medicine and Animal Science of the University of São Paulo (No. 2347040422). This article was written based on the observations of the lead author in his doctoral research [21].

2.1. Experimental Design, Pasture System, and Treatments

The experimental area has Köppen climate classification: Cwa (Monsoon-influenced humid subtropical climate), with precipitation of approximately 1200 mm per year and an average annual temperature of 23 °C, at an altitude of approximately 590 m above sea level (more details on precipitation and average temperature can be found in Supplementary Materials File S1). The area was planted in 2018 with *Urochiloa brizantha* cv. Marandu, receiving liming and nitrogen fertilization of 110 kg of N per ha per year throughout the experiment, divided into two applications (January and March).

The experiment used a total area of 14.4 ha, divided into eight grazing units, in four of which a grazing rotated system was installed, again divided into six paddocks of 0.3 ha with a fixed grazing period of 7 days and a rest period of 35 days (Figure 1). The other four undivided units with 1.8 ha each were used to install the grazing deferred system. An auxiliary area of approximately 7 ha was used to keep the experimental animals of the grazing deferred treatment during the pasture rest period of 84 days, from April to June (Figure 1).

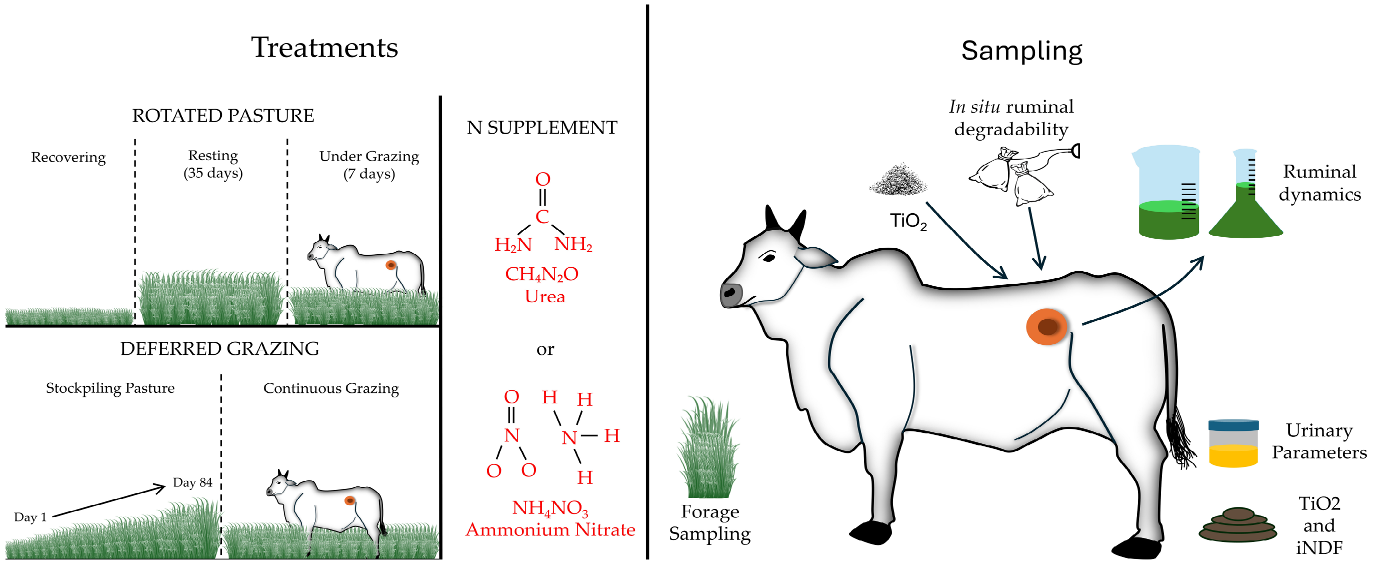

Figure 1. Schematic representation of treatments and sampling.

A total of eight Nellore female cows, with an average weight of approximately 551 ± 7.01 kg, were used as experimental animals for rumen fermentation data. The experimental animals were randomly assigned to 8 paddocks in a randomized block design, based on terrain location, over a two-year period and evaluated at each station. In total, 64 experimental units were used, a number considered adequate by the scientific community and the ethics committee on the use of animals for research with rumen-cannulated cattle in this type of experimental design.

The treatments involved the combinations of two grazing systems (deferred and rotational) and two types of supplementations of ammonium nitrate (NH_4NO_3) or urea: deferred grazing with NH_4NO_3, deferred grazing with urea, rotational grazing with NH_4NO_3, and rotational grazing with urea. Each block was divided by management corridors. A variable number of 'non-experimental' animals (regulators) were used to adjust the stocking rate using the "put and take" technique described by Mott and Lucas [22], aiming to maintain a specific intermediate pasture height (maximum of 45 cm and minimum of 30 cm) as an indirect assessment of forage mass availability [23] in each grazing unit.

Two different formulations of energy-protein supplement, with ammonium nitrate or urea, were adopted throughout the year to better meet the animals' nutritional requirements.

The animals received a concentrate (isoproteic) composed of ground corn, salt, mineral supplement, and either urea or ammonium nitrate. Urea was included at 13% and 22% of the total dry matter, while ammonium nitrate was included at 18% and 30% for the dry (autumn and winter) and rainy (spring and summer) seasons, respectively. Animals were gradually adapted to the urea and nitrate supplements over a 14-day period. Further details regarding supplement composition and feeding are provided in Section 2.3.

2.2. Hand-Plucking Technique to Estimate Forage Nutritional Value

The hand-plucking technique, adapted from Sollenberger and Sherney [24], was used to estimate diet nutritive value. The second month of each season was taken to perform all sampling. Hand-plucking was performed on the 1st, 3rd, and 7th days of the rotational grazing method, and in the same days, samples from the deferred method were also taken, dried at 65 °C for 72 h, and milled for subsequent analysis. The nutritional composition of forage can be seen in Table 1.

Table 1. Forage chemical composition of forage canopy collected by hand-plucking during the experimental period.

Fixed Effects		**Variables**								
Grazing	**Season**	**CP**	**NDF**	**ADF**	**LIG**	**EE**	**MM**	**NFC**	**GE**	**TDN**
		(%)	**(%)**	**(%)**	**(%)**	**(%)**	**(%)**	**(%)**	**(MJ/kg)**	**(%)**
Deferred		10.97	65.45	33.06	2.51	3.32	10.10	10.50	18.16	68.11
Rotated		11.09	64.41	34.16	3.08	3.28	9.99	10.46	18.22	70.43
	Winter	9.54	66.56	36.39	3.99	3.43	9.83	10.47	17.95	65.39
	Spring	10.69	66.81	34.21	3.54	3.32	9.60	9.43	18.17	67.55
	Summer	10.78	64.20	31.81	1.25	3.10	10.62	11.14	18.27	71.82
	Autumn	13.10	62.15	32.03 [c]	2.40	3.35	10.13	10.87	18.37	72.33
					Average data					
Average		10.89	65.36	33.61	2.80	3.33	10.05	10.48	18.18	69.27
SEM		0.24	0.33	0.35	0.18	0.10	0.09	0.13	0.05	0.73

CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; LIG: lignin; EE: ether extract; MM: mineral matter; NFC: non-fiber carbohydrates; GE: gross energy; TDN: total digestible energy (megajoules/kilogram); SEM: standard error of the mean.

2.3. Experimental Supplementation

The supplement was provided in measured amounts along the seasons due to toxicity risks. Due to the better nutritional quality of forage during the rainy season, a supplement containing approximately 6.2% N was used. In contrast, pastures during the dry season have higher fiber content and lower protein concentrations, which can limit microbial activity in the rumen and reduce fiber digestion. Therefore, during the dry season, a new formulation with a higher nitrogen inclusion (10.2% N) was used. The formulations and compositions of the supplement can be found in Table 2.

Table 2. Composition and proportion of each ingredient used to prepare supplements for the adaptation, rainy and dry season using urea or nitrate as nitrogen source.

Ingredients		Dry * (Season)		Rainy * (Season)	
		Urea	Nitrate	Urea	Nitrate
		(%)	(%)	(%)	(%)
Ground corn		48	45	72	69
Salt (NaCl)		15	10	7	5
Mineral mixture [1]		15	15	8	8
Urea		22	-	13	-
Ammonium nitrate		-	30	-	18
Nutritional composition					
CP	(%)	66.34	61.13	43.01	43.34
TDN	(%)	42.02	39.46	63.13	60.5
EE	(%)	1.39	1.31	2.09	2.00
NDF	(%)	3.79	3.56	5.69	5.45
ADF	(%)	1.25	1.17	1.87	1.79
Ca	(%)	2.69	2.69	1.45	1.45
P	(%)	2.52	2.52	1.47	1.46
Na	(%)	5.86	3.91	2.74	1.96

[1] Minerthal® with estimated macro- and micromineral composition for the urea or nitrate supplement: 1.93 g/kg of potassium, 0.77 g/kg of magnesium, 3.29 g/kg of sulfur, 12.30 mg/kg of cobalt, 342.45 mg/kg of copper, 16.79 mg/kg of iodine, 402.90 mg/kg of iron, 291.00 mg/kg of molybdenum, 3.36 mg/kg of selenium, 812.70 mg/kg of zinc. * Rainy season (spring and summer) and dry season (autumn and winter). CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber, EE: ether extract, TDN: total digestible energy, Ca: calcium, Cl: chlorine, Na: sodium, P: phosphorus.

2.4. Dry Matter Intake of Forage and Supplement

The intake (forage and supplement) and feces excretion were determined using two internal markers (titanium dioxide [TiO_2] and chromium oxide [Cr_2O_3]) to determine feces excretion, and one internal marker (indigestible neutral detergent fiber [iNDF]) for forage intake. Feces were collected for marker analysis, and the dry matter intake of forage and supplement was calculated. Therefore, to assess forage intake, during 10 days of each experimental period, TiO_2 was administered (15 g/cow.day) directly into the rumen through the rumen cannula (Figure 1) twice a day at 8 a.m. (7.5 g) and at 4 p.m. (7.5 g), the first five days for adaptation, and the last five days for feces collection, twice a day (8 a.m. and 4:00 p.m.) [25].

Marker concentrations were obtained in ppm and subsequently converted to kilograms (kg) for the determination of feces excretion by means of a known amount of external marker administered (kg/day) and that found on the feces as follows:

$$\text{TDFE} = \frac{\text{MDiet}}{\text{MFeces}}$$

In which: TDFE: total daily feces excretion (kg); MDiet: marker in the diet (kg); MFeces: marker in the feces (kg).

Chromium concentration followed the methodology described by Almeida et al., [26], which used an energy dispersive X-ray fluorescence technique. Thus, for the determination of supplement dry matter intake, we used the following equation:

$$\text{DMIS} = \frac{(\text{TDFE} \times \text{IndFeces})}{\text{MIndDiet}}$$

In which: DMIS: dry matter intake of supplement (kg/day); IndFeces: indigestibility of feces (%); MIndDiet: indigestibility of diet (%).

Forage dry matter intake (DMI) was calculated by internal marker iNDF concentration (%) and determined by means of its incubation for 288 h in the rumen of cannulated animals kept in grazing pastures. The iNDF concentration on dried samples of forage and feces was used to calculate and estimate the forage feed intake as follows:

$$\text{DMIF} = \frac{(\text{TDFE} \times \text{IndFeces})}{\text{MIndForage}}$$

In which: DMIF: dry matter intake of forage (kg/day); IndFeces: indigestibility of feces (%); MIndForage: indigestibility of forage (%).

2.5. Total Apparent Digestibility of Dry Matter and Its Fractions

Apparent digestibility coefficients were calculated based on TiO_2 content in the diet and feces. Concentrations of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (Lig), ether extract (EE), and mineral matter (MM) in forage were determined using a near-infrared spectrophotometer (NIRS, model NIRFlex N-500 Solids, BÜCHI) with calibration developed and validated by Embrapa Southeast Livestock for samples of *Urochloa* spp., respectively: R^2 for CP = 0.979; R^2 for NDF = 0.930; R^2 for ADF = 0.970; R^2 for lignin = 0.943; R^2 for EE = 0.920; R^2 for MM = 0.960. Supplement and feces were dried for DM [27], milled to 1 mm, and analyzed for CP (micro-Kjeldahl [28]), EE (ANKOM XT15 Extractor® [29]), and MM (muffle furnace at 550 °C for 4 h). OM was calculated as 100 minus MM. GE of feces, supplement, and forage was determined by complete oxidation in a calorimetric bomb (C5000 control, IKA®, Staufen, Germany). The non-fiber carbohydrates (NFC) content of feed and feces was obtained by subtracting CP, EE, MM, and NDF percentages from 100. The total digestible nutrients (TDN) of feed and feces was calculated by summing the products of the apparent digestibility coefficients for CP, NDF, corrected EE, and NFC, divided by 100.

2.6. Ruminal Kinetics and Degradability of DM and Nutrients

The ruminal dynamics were assessed by total rumen emptying. Ruminal digesta was manually removed through the rumen cannula from each cow pressing the ruminal contents over a sieve (4 mm) to separate liquid and solid phases, to determine the disappearance rate (kt) in the rumen. Then, 1 kg of each solid and liquid sample were dried at 65 °C (forced-air oven) for 72 h. Ruminal solid and liquid mass data were used to calculate the solid disappearance rate using the equation suggested by Robinson et al. [30].

The ruminal degradability of DM, NDF, and CP was assessed following the Mehrez and Ørskov [31] methods, in which, nylon tissue bags with 50 µm pores in the dimensions of 10 × 20 cm were filled with 5 g of forage from each treatment, identified and then incubated in the rumen for 0, 3, 6, 12, 24, 48, 72, and 96 h. Degradability parameters were obtained and calculated using the NLIN procedure of SAS as suggested by Ørskov and McDonald [32].

2.7. Determination of Urinary Parameters

Microbial protein production was calculated by determining urinary volume through creatinine concentration, following Valadares et al. [33].

Urine samples were collected twice daily (8 a.m. and 4 p.m.) over five days via spontaneous urination. Each time, 10 mL of urine was preserved in 40 mL of 0.036 N sulfuric acid and stored at −20 °C for analysis of allantoin, uric acid, urea, and creatinine. Allantoin was measured using the colorimetric method of Chen and Gomes [34], uric acid by colorimetric enzymatic reaction with uricase and peroxidase (Bioclin® Kit Ref K139, Belo Horizonte, Brazil), and urea and creatinine by commercial kits (Bioclin® Ref K047 and

K067, respectively). Daily urinary creatinine excretion (CE) was estimated using Chizzotti et al. [35]:

$$CE = 0.345 \times EBW^{0.9491}$$

In which: CE: daily urinary creatinine excretion; EBW: empty body weight.

Total daily urinary volume (L/cow) was calculated by dividing daily urinary creatinine excretion by the observed creatinine concentration in spot samples. This volume estimated daily excretions of urea, allantoin, and uric acid.

Purine derivatives (PuD) excretion was calculated by multiplying daily urine volume by PuD concentration. Absorbed microbial purines (AP, mmol/day) were calculated from urinary PuD excretion using:

$$PuD = (0.85 \times AP) + (0.385 \times BW)$$

In which: PuD: purine derivatives; AP = absorbed microbial purines; BW: body weight.

Intestinal flow of microbial nitrogen compounds (MicN, g N/day) was calculated with:

$$MicN = \frac{70 \times AP}{0.83 \times 0.116 \times 1000}$$

In which: MicN = microbial nitrogen; AP = absorbed microbial purines.

Microbial nitrogen synthesis efficiency (EMNS) was determined by the ratio of microbial N production to digested organic matter (OM).

2.8. Statistical Analysis

Data were statistically analyzed using the online version of the software Statistical Analysis Systems—OnDemand for Academics SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Prior to statistical analysis, the data were assessed for the presence of disparate information ("outliers") and the normality assumption of the residuals was assessed by the Shapiro–Wilk test. When the normality assumption was not accepted, the logarithmic or the square root transformation was applied. Data were analyzed according to the mixed procedure (PROC MIXED), in which season was considered as a repeated variable (split-plot in time). A total of 15 different covariance structures were tested, and the chosen one was based on the lower value of the Corrected Akaike Information Criterion (AICC) [36]. The Variance Components (VC), Compound Symmetry (CS), and Heterogeneous Compound Symmetry (CSH) are the covariance structures most commonly used. The model includes the effect of grazing method, nitrogen source, period of the year (winter, spring, summer and autumn), and the interaction between grazing method, nitrogen source and season of the year. The effects of blocks were considered as random factors.

$$Y_{ijkl} = u + b_i + g_j + n_k + (gn)_{jk}\, e_{(1)ijk} + s_l + (sg)_{lj} + (sn)_{lk} + (sgn)_{ljk}\, e_{(2)ljk}$$

where Y_{ijkl}: experimental answer; u: constant; b_i: effect of the block; g_j: effect of grazing; n_k: effect of nitrogen source; $(gn)_{jk}$: interaction effect of grazing and nitrogen source; $e(1)_{ijk}$: random error; s_l: effect of season; $(sg)_{lj}$: interaction effect of season and grazing; $(sn)_{lk}$: interaction effect of season and nitrogen source; $(sgn)_{ljk}$: interaction effect of season, grazing and nitrogen source; $e(2)_{ljk}$: random error.

In the presence of interaction, the effects of one factor inside the other were evaluated using the SLICE command of the Mixed Procedure. All means were presented as least squares means and statistical differences by treatment effects were obtained by pairwise difference test (PDIFF) using the Fisher test, considering a significance of $p \leq 0.05$.

3. Results

Feed intake variables showed mainly significant effects for season. There was an effect of grazing method on NPN intake and microbial nitrogen synthesis (MicN: microbial nitrogen compounds synthesis; EMNS: efficiency of microbial protein synthesis), where animals in deferred grazing had the highest MicN and EMNS values compared to animals under rotational grazing (Table 3).

Table 3. Dry matter intake (forage, supplement, total, and NPN intake), rumen dynamics (disappearance), and microbial nitrogen synthesis (microbial nitrogen, efficiency of microbial protein synthesis) from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons of the year.

Fixed Effects			Variables							
			DMI				Rumen Dynamics		Microbial Nitrogen Synthesis	
Grazing	N Source	Season	Forage (kg/day)	Supplement (kg/day)	Total (kg/day)	NPN (kg/day)	Disappearance (%/h)	Disappearance (kg/h)	MicN (g/day)	EMNS (g/kg OM)
Deferred			7.46	0.39	8.06	0.054	5.44	0.34	529.41	170.30
Rotated			7.43	0.63	8.03	0.080	5.12	0.33	424.93	136.70
	Nitrate		7.64	0.41	8.14	0.045	5.21	0.33	478.25	153.85
	Urea		7.25	0.60	7.95	0.089	5.36	0.34	476.06	153.15
		Winter	5.98 [b]	0.52 [a]	6.84 [b]	0.071	4.59 [b]	0.30 [b]	461.51 [bc]	148.46 [bc]
		Spring	5.76 [b]	0.55 [a]	6.28 [b]	0.074	4.04 [b]	0.27 [b]	433.80 [c]	139.55 [c]
		Summer	9.26 [a]	0.34 [b]	9.59 [a]	0.045	6.17 [a]	0.40 [a]	499.30 [ab]	160.62 [ab]
		Autumn	8.78 [a]	0.62 [a]	9.47 [a]	0.078	6.33 [a]	0.38 [a]	514.06 [a]	165.37 [a]
Average data										
Average			7.68	0.50	8.20	0.232	5.30	0.26	495.59	159.42
SEM			0.312	0.050	0.317	0.028	0.341	0.012	10.60	3.410
Statistical Probabilities (*p*-value)										
Grazing			NS	NS	NS	0.0429	NS	NS	0.0362	0.0363
N source			NS	NS	NS	0.0008	NS	NS	NS	NS
Season			<0.0001	0.0163	<0.0001	NS	<0.0001	<0.0001	0.0210	0.0212
Grazing × N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing × Season			NS	NS	NS	NS	NS	NS	NS	NS
N source × Season			NS	NS	NS	NS	NS	NS	NS	NS
Grazing × N Source × Season			NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: standard error of the mean; DMI: dry matter intake; NPN: equivalent non-protein nitrogen; %/h: percentage per hour; kg/h: kilograms per hour; MicN: microbial nitrogen; EMNS: efficiency of microbial protein synthesis; OM: organic matter; NS: not significant.

In Table 4, we show that nitrate was the treatment with the highest digestibility coefficient for the NFC variable. With the exception of EE, all variables showed seasonal effects. EE showed a Grazing × Season interaction, and ADF showed an interaction between Grazing × N Source × Season.

A higher digestibility coefficient of EE was detected in spring and autumn for the rotational grazing method (Figure 2a).

ADF (%) digestibility was higher when animals were fed urea within deferred grazing during the spring season. The same pattern was detected in autumn but within rotational grazing, with animals fed urea having higher ADF digestibility. Yet, in Figure 2b, it is possible to notice that nitrate supplementation increased ADF (%) digestibility in the rotational grazing method, when compared to deferred. Meanwhile, in autumn, the same pattern of ADF (%) digestibility was observed, with greater digestibility in rotated grazing methods within urea supplementation.

Table 4. Digestibility coefficients (%) of DM and nutrients from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons of the year.

Fixed Effects			Digestibility Coefficient							
Grazing	N Source	Season	DM (%)	CP (%)	NDF (%)	ADF (%)	EE (%)	NFC (%)	MO (%)	GE (Mj/kg)
Deferred			70.96	77.35	71.34	67.98	67.08	76.70	74.50	70.91
Rotated			72.77	78.10	73.94	69.64	72.11	76.92	75.87	73.05
	Nitrate		72.43	77.88	72.95	68.04	70.62	78.28	75.71	72.65
	Urea		71.30	77.57	72.33	69.57	68.56	75.35	74.67	71.31
		Winter	67.33 ^{b}	74.03 ^{b}	67.67	68.98	72.88	74.16 ^{b}	71.24 ^{b}	67.02 ^{b}
		Spring	69.24 ^{b}	77.04 ^{b}	71.26	63.07	71.04	65.53 ^{c}	72.97 ^{b}	69.25 ^{b}
		Summer	75.55 ^{a}	77.09 ^{b}	77.34	73.89	67.39	81.37 ^{a}	78.33 ^{a}	75.90 ^{a}
		Autumn	75.34 ^{a}	82.74 ^{a}	74.30	69.28	67.06	86.19 ^{a}	78.21 ^{a}	75.74 ^{a}
Average values										
Average			10.89	65.36	33.61	2.80	3.33	10.05	10.48	18.18
SEM			0.24	0.33	0.35	0.18	0.10	0.09	0.13	0.05
Statistical Probabilities (*p*-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	0.0283	NS	NS
Season			<0.0001	<0.0001	0.0001	<0.0001	NS	<0.0001	0.0003	0.0007
Grazing × N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing × Season			NS	NS	NS	NS	0.0447	NS	NS	NS
N source × Season			NS	NS	NS	NS	NS	NS	NS	NS
Grazing × N Source × Season			NS	NS	NS	0.0249	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: standard error of the mean; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; EE: ether extract; NFC: non-fibrous carbohydrate; GE: gross energy (megajoules/kilogram); NS: not significant.

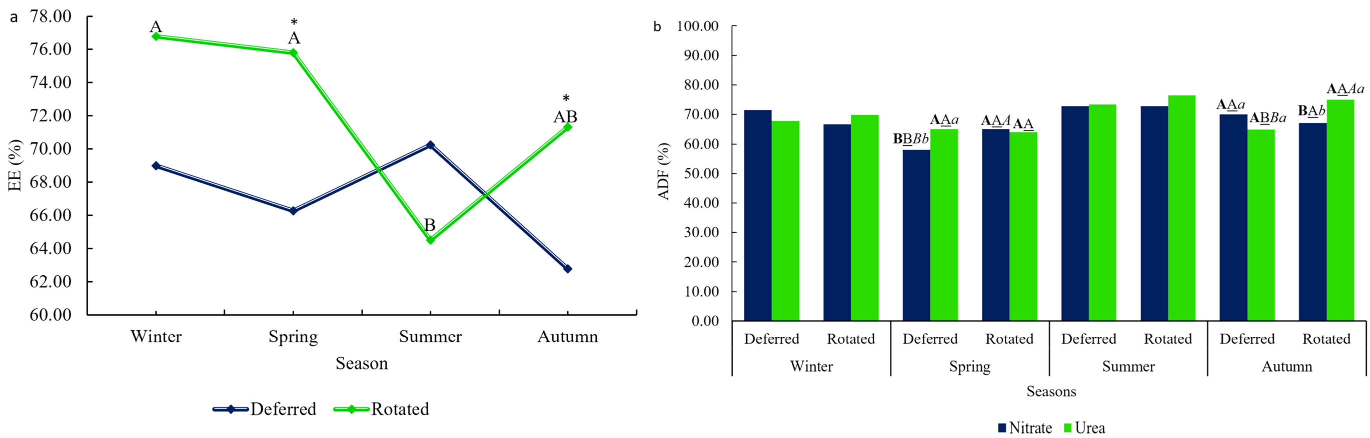

Figure 2. Decomposition of the Grazing × Season for EE digestibility coefficient (**a**) and decomposition of the Grazing × N Source × Season for ADF digestibility coefficient (**b**) from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation in different seasons of the year. Different capital letters indicate statistical differences among treatments in the same season ($p < 0.05$). * Indicates statistical difference within season at $p < 0.05$. In (**b**), different capital bold letters (**A**,**B**) within season indicate difference between nitrogen source at $p < 0.05$; different underlined capital letters (A,B) within season indicate difference between grazing method at $p < 0.05$; different italic capital letters (*A*,*B*) within nitrogen source indicate difference between grazing method within season at $p < 0.05$; different italic lowercase letters (*a*,*b*) within grazing method indicate difference between nitrogen source within season at $p < 0.05$.

Only the PD of DM (potential degradability of dry matter; a + b) had a significant effect, with an interaction for N source × Season (Figure 3), in which we can see greater potential digestibility of DM in spring when animals were fed nitrate as opposed to urea. The variables De5 and De8 (DM degradability at 5 and 8%) did not have a significant effect (Table 5), while the variables a, b, c, PD, and De2 differed between seasons (Table 5).

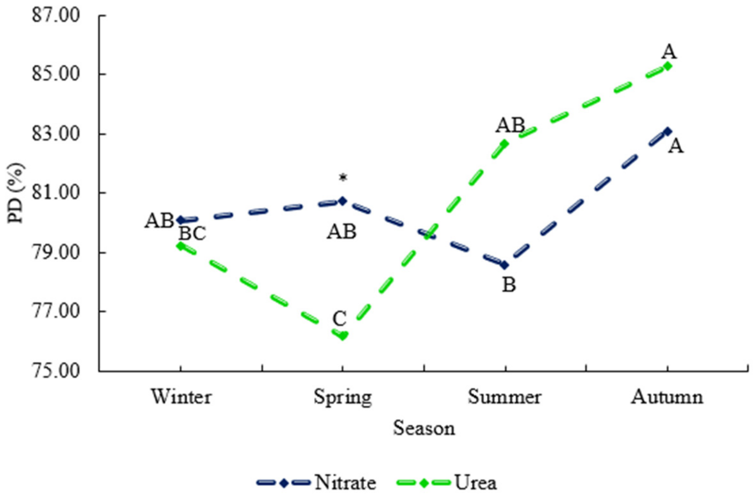

Figure 3. Interaction effect of season and nitrogen source on PD (%) of diet DM of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. Different capital letters indicate statistical differences among treatments in the same season ($p < 0.05$). * Indicates statistical difference within season at $p < 0.05$.

Table 5. In situ degradability of DM of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed Effects			Variables						
Grazing	N Source	Season	a	b	c	PD	De2	De5	De8
			(%)	(%)	(%/h)	(%)	(%)	(%)	(%)
Deferred			26.36	52.65	0.07	80.62	66.50	55.62	49.74
Rotated			28.03	53.43	0.05	81.02	65.41	54.10	48.88
	Nitrate		27.45	52.82	0.07	80.76	66.53	55.70	49.77
	Urea		26.94	53.24	0.07	80.76	65.38	54.02	49.77
		Winter	29.81 [a]	50.14 [c]	0.04 [b]	79.66 [b]	63.74 [b]	52.76	47.50
		Spring	28.68 [ab]	49.16 [c]	0.06 [ab]	78.39 [b]	65.15 [b]	54.75	49.28
		Summer	23.88 [c]	55.10 [a]	0.09 [a]	80.68 [b]	66.04 [a]	55.76	50.96
		Autumn	26.32 [b]	57.73 [b]	0.05 [b]	84.55 [a]	68.90 [a]	56.18	49.49
Average data									
Average			27.02	53.56	0.066	80.95	66.19	55.25	49.45
SEM			0.492	0.727	0.005	0.675	0.556	0.599	0.635
Statistics Probabilities (*p*-value)									
Grazing			NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS
Season			<0.0001	<0.0001	0.0188	0.0020	0.0020	NS	NS
Grazing × N source			NS	NS	NS	NS	NS	NS	NS
Grazing × Season			NS	NS	NS	NS	NS	NS	NS
N source × Season			NS	NS	NS	0.0380	NS	NS	NS
Grazing × N Source × Season			NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: standard error of the mean; a: interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly; b: potentially degradable fraction; c: rate of degradation of the potentially degradable fraction; PD: potential degradability (a + b); De2, De5 and De8%—rumen degradability rate 2, 5 and 8%; NS: not significant.

For in situ degradability of CP and NDF, we did not find isolated effects of Grazing and N source (Table 6). However, the season effect was detected for all CP variables and all NDF variables except De5 (Table 6).

Table 6. In situ degradability of CP and NDF of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed Effects			Variables													
			CP							NDF						
Grazing	N Source	Season	a (%)	b (%)	c (%/h)	PD (%)	De2 (%)	De5 (%)	De8 (%)	a (%)	b (%)	c (%/h)	PD (%)	De2 (%)	De5 (%)	De8 (%)
Deferred			49.98	41.77	0.053	91.51	78.90	70.37	65.83	11.99	68.37	0.046	80.60	59.54	45.27	37.31
Rotated			51.16	43.80	0.048	90.81	79.72	70.88	66.46	15.20	67.29	0.048	80.23	60.72	45.12	37.72
	Nitrate		49.93	44.41	0.051	91.25	79.56	70.48	65.73	12.84	67.34	0.048	79.73	59.99	45.48	37.53
	Urea		51.21	41.17	0.050	91.08	79.05	70.76	66.56	13.75	68.32	0.047	81.10	60.28	44.91	37.52
		Winter	53.66 a	37.92 b	0.055 a	89.30 b	79.79	72.56 a	68.98 a	20.25 a	58.49 c	0.044	78.90 b	59.98	46.53	40.58
		Spring	55.29 a	38.61 b	0.058 a	90.47 ab	81.89	74.63 a	70.54 a	16.43 b	61.75 b	0.047	75.94 b	57.92	44.34	36.90
		Summer	47.53 b	47.75 a	0.035 b	92.89 a	75.86	65.61 c	60.91 c	10.38 c	73.61 b	0.045	84.31 a	61.64	45.71	37.51
		Autumn	45.81 b	46.87 a	0.054 a	91.98 a	79.70	69.70 b	65.27 b	6.13 d	77.47 a	0.053	82.52 a	61.00	44.19	35.10
Average Data																
Average			48.93	43.36	0.05	91.31	79.12	69.95	65.35	12.82	68.30	0.046	80.89	59.98	45.19	37.74
SEM			1.36	1.30	0.003	0.59	0.52	0.77	0.89	0.839	1.121	0.001	0.561	0.494	0.533	0.584
Statistics Probabilities (*p*-value)																
Grazing			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Season			0.0022	0.0104	<0.0001	0.0075	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	0.0167	NS	0.0008
Grazing × N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing × Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	0.0034	0.0176	NS	NS	NS
N source × Season			NS	NS	NS	NS	0.0251	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing × N Source × Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: standard error of the mean; a: interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly; b: potentially degradable fraction; c: rate of degradation of the potentially degradable fraction; PD: potential degradability (a + b); De2, De5 and De8%—rumen degradability rate 2, 5 and 8%; NS: not significant.

Significant N source × Season interactions were observed in De2 of CP, with greater degradability within spring when animals were fed nitrate (Figure 4a). Significant Grazing × Season interactions were observed in c and PD of FDN (Table 6; Figure 4b,c).

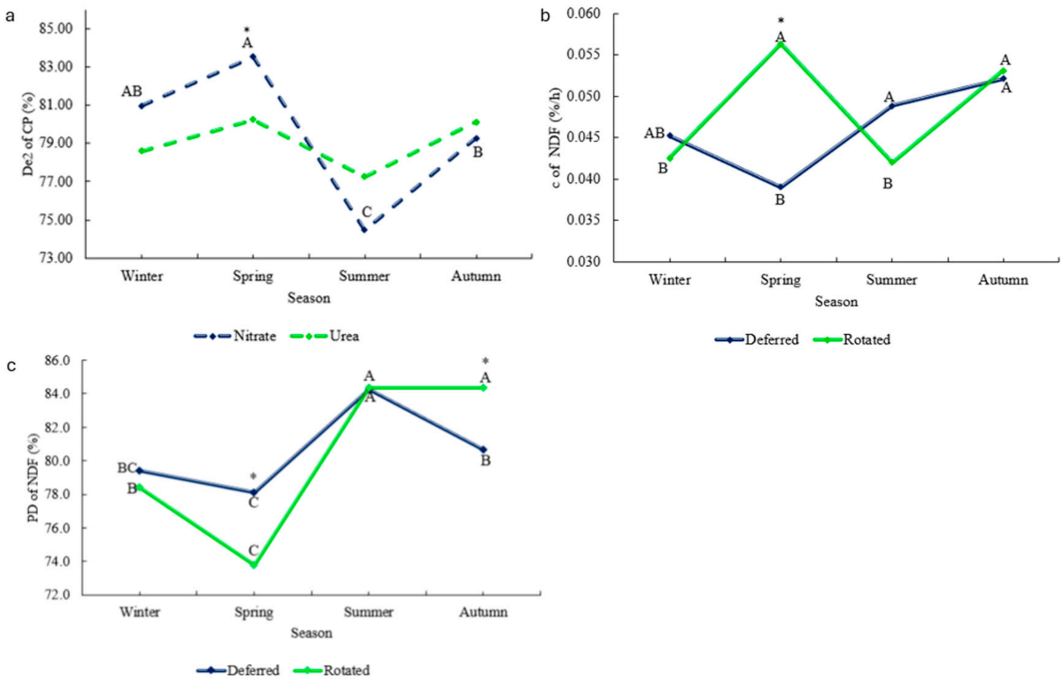

Figure 4. Decomposition of the N source × Season interactions for De2 of CP in situ degradability (**a**) and decomposition of the Grazing × Season interactions for c (%/h) and PD of NDF in situ

degradability (**b**,**c**) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. Different capital letters indicate statistical differences among treatments in the same season ($p < 0.05$). * Indicates statistical difference within season at $p < 0.05$.

Animals subject to the rotated grazing method had a greater rate of degradation of the potentially degradable fraction (c %/h) within spring; however, PD of NDF in situ degradability showed lower mean values for animals in rotated grazing within spring but greater values within autumn when compared to deferred grazing.

4. Discussion

This study used females of the Nelore breed. However, nitrate supplementation can be applied to any cattle on a low-protein diet (especially grazing cattle). The only limitations include the need for animal adaptation and the potential for very high dietary N levels to cause toxicity and increase nitrous oxide emissions on the farm [19]. It is important to emphasize that during the entire study period, not a single animal became ill or showed signs of intoxication. This indicates that the doses of N consumed by the animals were safe.

Nitrate resulted in lower NPN intake (kg/day), yet no significant effect on microbial protein synthesis or the efficiency of microbial N synthesis was detected. This lower intake of NPN may be associated with the bitterness of nitrate [14,15]; however, based on our findings, we understand that ammonium nitrate is a potential and suitable NPN source for beef cattle in the grazing system. The reduction of nitrate to ammonia represents a metabolic path that is thermodynamically favorable and incorporates more energy into the rumen by increasing the overall flow of microbial protein in the rumen coupled with reduction of methane emission [11]. On the other hand, grazing methods had an influence on MicN and EMNS with 19.72% lower values detected for animals under rotated grazing when compared to deferred grazing (Table 3). A possible reason for the lower EMNS (g/kg.OM) in rotated grazing is that animals under rotated grazing have access to pasture with a profile of forage with higher content of nutrients, and a higher concentration ratio of cell soluble compounds to structural compounds, which makes the forage highly concentrated in nutrients and, thus, more digestible, as observed in our findings (Table 4; Figure 2a,b).

No significant effect was detected on supplement intake. However, we expected that the animals would prefer harvesting new green leaf, a great source of PDR, to supplementation. Possibly, this led to a lower rate of conversion of protein to ammonia and thus resulted in a lower MicN when animals were in rotated grazing. The most likely and probable second reason that accounts for the lower MicN and EMNS is the greater rumen solids content expected from animals in rotated pasture, especially during the summer season [37]. Thereby, the pressure of fiber content on a full rumen into the rumen wall influences ruminal motility, which increases the disappearance rate by %.h and by kg/h. Theoretically, a greater amount of ruminal content leaving the rumen means lower retention of microbial protein, degradability, and consequently, lower EMNS as well [37].

Although nitrogen sources did not significantly affect feed intake or diet digestibility, N supplementation in beef cattle is intended to enhance these parameters by accelerating rumen passage and aiding forage cell wall breakdown, a result potentially influenced by the isoproteic nature of the diets. Martinez-Fernandez et al. [38] observed that supplementation in cattle grazing on tropical pastures increased the alpha diversity and Chao richness of the rumen microbial community. This increase is crucial for degrading forage cell components and improving overall rumen kinetics.

When studying the microbial community of pasture-fed steers and supplemented with encapsulated nitrate, Granja-Salcedo et al. [7] reported a reduction in *Methanobrevibacter* populations and a negative correlation between methane emissions and populations of

Proteobacteria, *Prevotellaceae*, *Selemonas*, and *Succinivibrio* spp. However, further studies are necessary to elucidate the microbiome and the modulation exerted by nitrate on the microbial community.

The higher disappearance rate by %/h and by kg/h over summer and autumn might be accounted for by the fact that in both seasons, pastures displayed overall higher forage availability and nutrients digestibility as well (Table 4). A greater content of dry matter in the rumen is expected in rainy seasons, which can lead to constant interaction and pressure into the rumen wall and pillars, where there are chemoreceptors that recognize the ruminal volume size and its chemical composition. This effect causes changes in the rumen motility, which ultimately causes the more dense and fine rumen content made up of concentrated and smaller degraded pieces of forage to leave the rumen, thus possibly increasing the disappearance rate by %/h and by kg/h.

It was observed that nitrate does not have a negative effect on forage, supplement, or total dry matter intake (kg/day). However, the pasture system impacted NPN intake (kg/day), which was 32.5% higher in animals grazing on rotational pastures compared to those on deferred pastures. This may be due to the synergistic effect of supplementation and the superior quality of rotational pastures, which led to higher NDF digestibility (Table 4). Rotational grazing systems typically have higher overall digestibility and nutrient concentration in leaves compared to deferred pastures, likely due to management practices that prioritize forage use at ideal plant height and maturity [39]. Our findings indicate that animals in rotational grazing systems had higher NDF (%) digestibility compared to those in deferred systems.

The ADF (%) digestibility was improved when animals were under rotated grazing and/or being supplemented with urea as the main source of non-protein nitrogen, as seen in Figure 2b. The effect might be related to the fact that in pastures of rotated grazing, animals can be selective and thus defoliation occurs primarily to high palatable leaves, the newly emerged ones, which have a lower concentration of lignified structural carbohydrates [40], therefore increasing fermentation activity and improving digestibility of the ADF (%).

Rotational grazing increased EE (%) digestibility, both independently and in interaction with the season (Figure 2a). Our findings indicate higher EE (%) digestibility in rotational grazing compared to deferred grazing during spring and autumn. This is likely due to the slightly higher nutrient concentration in rotational pastures and the selective grazing behavior, where animals consume the most digestible parts of the plants. Properly managed rotational pastures offer forage with higher nutrient concentration, digestible energy, and water-soluble carbohydrates [40], as defoliation occurs at the optimal stage of height and maturity. Over the two-year trial, rotational grazing also led to higher crude energy digestibility compared to deferred grazing.

Degradability parameters for forage-based diets naturally vary with the season, reflecting changes in the diet's composition and availability. In our study, nitrogen source interacted with the season and had an effect on the potential degradability (PD %) of DM, which was higher in animals fed nitrate during spring compared to those fed urea (Figure 3). This higher PD (%) with nitrate in spring may be due to improved overall digestibility and nutrient degradability as animals had higher supplement feed intake during spring than in summer (Table 3). The CP degradability rate at 2% (Figure 4a) showed the same effect of supplementation. Nitrate supplementation reduces it to ammonia, directly contributing to microbial protein synthesis. Additionally, the metabolic reduction of nitrate to ammonia generates a higher flow of negative Gibbs energy, which supports microbial growth, substrate transport, mobility in the rumen and improved degradation of nutrients fractions [41].

There was a notable difference for the variable b, the potential degradable fraction, which had lower values in winter and spring and higher values during summer and autumn. Surprisingly, summer had the lowest rate of degradation (3.5%/h) of the fraction b (potential degradable fraction), while no major effect was noticed among the other seasons. The decrease of 40.67% in the supplement dry matter intake over the summer and increase of 38.55% for forage DMI (Table 3) could be a reason for the lowest rate of degradation (3.5%/h) of the fraction b, since a greater proportion of the CP during that season came from forage.

In rotated grazing systems, the ruminal degradation rate of fraction b for NDF was 30.35% higher than that of deferred systems within the spring season, which might be a direct effect of the greater intake of NPN (kg/day) from animals kept in the rotated method, and the higher digestibility of the NDF (%) content from those experimental units as well. The intake behavior of animals in rotated grazing might play a role in this too. When animals are rotated to a "deferred" grazing area, there is great availability of new leaves and that is the main target of the cattle, as they harvest the foliage's tips, which are more digestible and nutritious [15,22,23]. In the same experiment that our study was carried out, Lelis [41] evaluated forage production and quality on deferred and rotated grazing methods and during the spring season; the authors observed that rotated grazing pastures had 40% more leaves and 27.7% more CP when compared to deferred. It is possible that the higher apparent digestibility of the NDF for the rotated grazing method (Table 4) and the higher availability of CP (27.3%) could have led to higher fermentation activity by the rumen bacterial community, which consequently can affect the rate of degradation of the potential degradable fraction of NDF, culminating in the results obtained. This trend does not happen in the deferred grazing method since it entails continuous grazing, where most of the forage leaves were already under harvesting.

The behavior of cattle in continuous grazing is different, and since they have full time availability of forage for grazing, the more digestible and palatable leaves are constantly taken. This, associated with extrinsic factors that do not contribute to forage growth along the seasons, can lead to the pasture having a lower content of new and nutritious leaves, and a higher content of more lignified and recalcitrant forage compounds that tend to take a greater time within the rumen. Therefore, we suggest further studies to elucidate methane emissions, the ruminal microbial community, and the productive performance of cattle raised in intensified grazing systems and on different sources of N in supplements.

5. Conclusions

The study demonstrates that the rotational grazing method improved NPN intake and the digestibility of NDF, ADF, and gross energy. The absence of negative effects of ammonium nitrate on parameters such as the digestibility of DM, nutrient intake, and ruminal kinetics demonstrate that it is a reliable source of N, like urea. The deferred system as a food reserve strategy in critical seasons (autumn and winter), when combined with NNP supplementation, promoted an improvement in microbial efficiency, which may contribute to animal performance. In addition, the use of ammonium nitrate was shown to be a safe strategy, demonstrating that it is an interesting option as a source of N for beef cattle in a grazing system. Although the initial results are promising, further studies investigating the ruminal microbial community, metabolism, gas production, and animal performance are essential to confirm the real benefit of ammonium nitrate in different grazing systems. Moreover, for a comprehensive understanding of its applicability, research on economic viability, nitrogen use efficiency, and the capacity to mitigate methane emissions is necessary. In this context, the judicious application of nitrate emerges as a strategy within climate-smart agriculture, aiming for sustainability in livestock production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11050261/s1, File S1: Graph of precipitation and average temperature during the experimental period.

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Institutional Review Board Statement: The study was approved by and followed the guidelines of the Committee for the Use and Care of Institutional Animals of the College of Veterinary Medicine and Animal Science of the University of São Paulo (No. 2347040422).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: Authors Alexandre Berndt and Patricia Perondi Anchão Oliveira were employed by the company Embrapa Southeast Livestock. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

a	Interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly
ADF	Acid detergent fiber
AICC	Corrected Akaike Information Criterion
AP	Absorbed microbial purines
b	Potentially degradable fraction
Ca	Calcium
CE	Daily urinary creatinine excretion
Cl	Chlorine
CP	Crude protein
Cr_2O_3	Chromium oxide
Cwa	Monsoon-influenced humid subtropical climate
De2%	Rumen degradability rate 2%
De5%	Rumen degradability rate 5%
De8%	Rumen degradability rate 8%
DM	Dry matter
DMI	Dry matter intake
DMIF	Dry matter intake of forage
DMIS	Dry matter intake of supplement
EBW	Empty body weight
EE	Ether extract

EMNS	Microbial nitrogen synthesis efficiency
FMVZ/USP	College of Veterinary Medicine and Animal Science
GE	Gross energy
iNDF	Indigestible neutral detergent fiber
IndFeces	Indigestibility of feces
kt	Disappearance rate
LIG	Lignin
MDiet	Marker in the diet
MFeces	Marker in the feces
MicN	Microbial nitrogen
MIndDiet	Indigestibility of diet
MIndForage	Indigestibility of forage
MM	Mineral matter
N Source	Nitrogen source
Na	Sodium
NDF	Neutral detergent fiber
NFC	Non-fiber carbohydrates
NH_4NO_3	Ammonium nitrate
NIRS	Near-infrared spectrophotometer
NPN	Non-protein nitrogen
NS	Not significant
OM	Organic matter
P	Phosphorus
PD	Potential degradability (a + b)
PDIFF	Pairwise difference test
PuD	Purine derivatives
SEM	Standard error of the mean
TDFE	Total daily feces excretion
TDN	Total digestible nutrients
TiO_2	Titanium dioxide

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