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Addition of Tannin-Containing Legumes to Native Grasslands: Effects on Enteric Methane Emissions, Nitrogen Losses and Animal Performance of Beef Cattle

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Abstract: Extensive cattle production on native grasslands is vital to the sustainability of the South American Pampas, and the inclusion of tannin-containing legumes can increase farm profitability, improve nitrogen (N) use and reduce greenhouse gas (GHG) emissions. This study quantified the effects of adding tannin-containing legumes to native grasslands on enteric methane (CH_4) emissions, animal performance and N balance in cattle. A crossover design trial was conducted with 22 beef heifers under two treatments: native grassland (NG) and native grassland with the addition of *Lotus uliginosus* and *L. angustissimus* (NG+L). The results showed that forage mass disappearance was similar between treatments; however, 54% of the forage disappearance in the NG+L corresponded with legumes, indicating that the heifers in this treatment consumed a better-quality diet. While individual CH_4 emissions were similar between treatments, heifers grazing the NG+L showed a higher average daily gain (ADG) and lower emissions intensity than heifers grazing NGs (0.25 vs. 0.58 g CH_4/g ADG, $p < 0.05$). Additionally, they also ingested 20% more N and were more efficient in its utilization. Incorporating tannin-containing legumes into native grasslands can improve animal productivity and N use efficiency while reducing the intensity of enteric CH_4 emissions.

Keywords: emissions mitigation; enteric methane; legume; nitrogen excretion; SF_6 tracer technique

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1. Introduction

The South American Pampas span approximately 100 million hectares across Uruguay, Argentina and Brazil [1]. Native grasslands currently cover 36% of this region and provide forage for around 60 million cattle raised in extensive livestock systems [2]. The forage species in these grasslands are well adapted to the region's soils. However, inadequate management, widespread nutrient deficiencies—particularly nitrogen (N)—and climate conditions often result in limited forage production [3]. This leads to reduced animal performance and increased enteric methane (CH_4) emissions [4–8]. Improving farmers' profits and the economic sustainability of livestock production remains a significant challenge, with a critical need to balance enhanced cattle performance and the reduction of greenhouse gas (GHG) emissions, which significantly contribute to climate change [9,10].

Methane losses represent between 2% and 12% of the gross energy intake of cattle, depending on the quantity and quality of the diet as well as the genetics and physiological state of the animals [11]. Enteric CH_4 is produced through anaerobic fermentation by a complex symbiotic system of microbial groups in the ruminant digestive tract, primarily in the rumen [12,13]. These microorganisms metabolize dietary carbohydrates into volatile fatty acids such as acetate, propionate and butyrate [14], with CH_4 forming when certain microorganisms reduce carbon dioxide (CO_2) using hydrogen as an energy source [15]. Since CH_4 emissions represent energy losses, they have been studied for over 50 years [16,17], with a current focus on CH_4's significant role as a GHG, which has 28 times the global warming potential of CO_2. Enteric fermentation is the principal source of CH_4 emissions, accounting for 39% of GHG emissions from the livestock sector and between 11 and 13% of global CH_4 emissions [9,18]. In key beef-producing countries in the Pampas biome, such as Uruguay, CH_4 emissions from livestock can account for up to 46% of the total GHG emission [19]. Consequently, the quantification and mitigation of livestock emissions have become a central research focus in countries such as Argentina, Brazil and Uruguay [6–8,20,21].

The sustainable intensification of livestock production systems is a key strategy for mitigating GHG emissions from this sector [22]. While N fertilization can boost forage production and improve animal productivity [3], excessive use may lead to nitrous oxide emissions, ammonia volatilization and a reduction in native grasslands species [10]. An alternative is the incorporation of forage legumes into grassland ecosystems [23–25], which enhance forage quality and increase available N through biological fixation, thereby reducing GHG emissions [26–28]. In addition to improving the nutritional value of the bovine diet, certain legumes contain secondary metabolites that can improve animal performance and reduce CH_4 emissions [29,30]. Research by Tavendale et al. [31] and Jayanegara et al. [32] suggests that tannins may inhibit methanogen activity in the rumen, reducing hydrogen production and feeding degradation. Tannin-containing legumes, such as *Lotus* species, have been shown to reduce CH_4 production by approximately 17% in beef heifers [33,34]. Additionally, tannins improve N use efficiency and decrease urinary N excretion, reducing environmental N losses [30,33,35,36].

There are few studies evaluating the effects of including tannin-containing legumes on CH_4 emissions, N pathways (N-urine vs. N-feces) and beef cattle performance under grazing conditions. The objective of this study was to quantify the effects of adding tannin-containing legumes (*Lotus uliginosus* and *L. angustissimus*) to native grasslands on enteric CH_4 emissions, animal performance and N balance in cattle. This objective is based on the hypothesis that the improvement of forage quality and the presence of adequate levels of tannins through the inclusion of legumes in native grasslands can improve animal performance and reduce the environmental impact of extensive livestock production.

2. Materials and Methods

2.1. Experimental Site

The study was carried out at the INIA Glencoe Experimental Unit (Uruguay, 32°00′24″ S; 57°08′01″ W). The Campos region (south of Brazil, east of Argentina and central-north of Uruguay) has a humid subtropical climate (cfa in Köppen's classification), where the mean of the coldest month (July) is 11.7 °C and the mean of the hottest month (January) is 23.8 °C [37]. The annual rainfall averages between 1300 and 1400 mm [38]. Periods of water deficit are common in summer.

Climate conditions, including rainfall, temperature and evapotranspiration, were monitored using data from the on-site climate station throughout the trial. Additionally, a water balance was estimated to assess potential impacts of water availability on the analyzed variables (Supplementary Figure S1). The experimental period experienced generally favorable hydric conditions, with the exception of a small water deficit that developed during the final two weeks. This deficit (less than 50 mm) occurred too late in the trial to significantly affect the quantity or quality of the available herbage mass.

The vegetation of the experimental area was dominated by native warm-season grasses (*Andropogon ternatus*, *Axonopus affinis*, *Bothriochloa laguroides*, *Mnesithea selloana*, *Paspalum dilatatum*, *Paspalum notatum*, *Paspalum plicatulum*, *Schizachyrium microstachyum* and *Steinchisma hians*), although some cool-season grasses were also present (*Nassella charruana*, *Nassella mucronata* and *Piptochaetium stipoides*) [39]. In 2016, part of the experimental paddocks was oversown with *L. uliginosus* cv E-Tanin, *L. uliginosus* cv INIA Gemma and *L. angustissimus* cv INIA Basalto, while the rest of the area remained native grassland. Soil quality was standardized across paddocks through phosphorus (P) fertilization, with the soil P levels maintained at 10 ppm.

2.2. Animals, Treatments, Design and Grassland Management

The study was conducted with Hereford heifers born between 13 September 2021 and 21 November 2021, weighing 198 ± 3.5 kg at the beginning of the experiment, applying a crossover statistical experimental design, which consisted of two treatments, 22 replications (animals) and two 15-day evaluation periods (Figure 1). Two groups of 11 heifers, blocked by live weight and paternal genetics, were randomly assigned to each of the two diet treatments: native grassland (NG) and native grassland with the presence of legumes containing tannins (NG+L). After the first 15-day evaluation, animals were crossed over into the experimental treatments for a second period of evaluation. To ensure consistent conditions, the experiment was conducted in spring, when dry matter (DM) production peaks [40]. The first period extended from 12 to 25 October 2022, and the second, from 26 October to 13 November 2022.

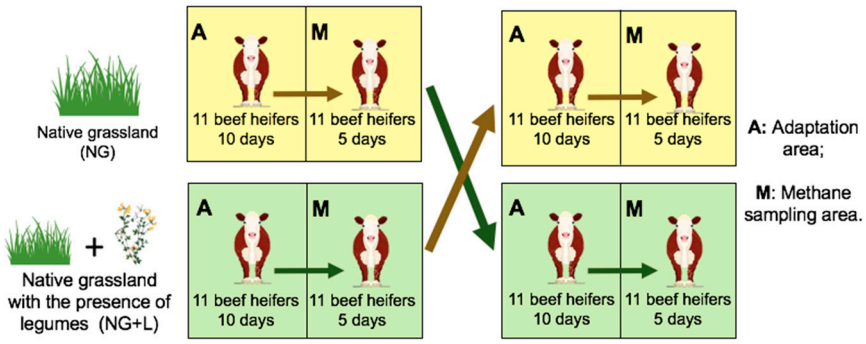

Figure 1. Crossover experimental scheme to evaluate enteric emissions from beef cattle on different types of pasture.

The total experimental area consisted of 5 hectares (ha), with 3.3 ha allocated to native grasslands, NG, and 1.7 ha to NG+L. Each treatment area was divided into four paddocks: two paddocks were used for animal adaptation to the diet for a 10-day period, and the other two paddocks were used for the 5-day sampling period [6,41]. Immediately after the first 15-day period, the treatments were crossed over, and animals were reallocated to ungrazed paddocks for the second 15-day period. Animal access to the paddocks was restricted 30 days prior to the experiment to ensure adequate herbage allowance.

Although the two treatments differed in area, the total herbage mass per ha was significantly greater in NG+L than in NG. As a result, the paddocks were stocked continuously, with their size adjusted based on animal live weight, forage mass in the upper stratum, number of grazing days and expected forage accumulation rate. Herbage allowance was managed to allow for ad libitum dry matter intake, maintaining in both treatments and over the experimental period a target of 6 kg of dry matter per 100 kg of live weight per day [42].

2.3. Herbage Mass and Botanical Composition

Standing biomass (above 5 cm), sward height, legume proportion and forage chemical composition were determined pre- and post-grazing in each paddock. Before the animals entered and immediately after they left each paddock, ten herbage samples were collected

between 10:00 am and 3:00 pm along a 30 m transect. Using a 0.5 × 0.5 m square, all forage above 5 cm was collected by cutting with scissors [43]. Samples were stored in paper bags, transported to the laboratory and manually separated into grass and legume, then weighed (fresh weight), dried at 60 °C for 72 h and weighed again (dry weight). Herbage mass was considered to be the aboveground biomass of forage plants (grass and legume mass, according to treatment). Subsequently, the samples were ground to 1 mm to determine their chemical composition.

2.4. Forage Chemical Composition

Dry matter, ash and total N concentrations were determined according to AOAC [44]. The neutral (NDF) and acid (ADF) detergent fiber fractions were analyzed with heat-stable amylase and sodium sulfite, following Van Soest et al. [45], including residual ash. The ether extract fraction was determined by extracting the fat with petroleum ether extraction (AOCS AM 5-04, Ankom Technology Corp., Fairport, NY, USA). Organic matter was estimated as 100 minus ash concentration. The gross energy was determined with an adiabatic bomb calorimeter (Autobomb Gallenkamp; Loughborough, Leicester, United Kingdom). Condensed tannins in plant materials were determined by the butanol-HCl method according to Porter et al. [46]. Absorbance was read in a spectrophotometer (Agilent 84531, Agilent Technologies, Santa Clara, CA, USA) at 550 nm, and CT contents were expressed as g of leucocyanidin equivalent (g LEUE) per 100 g of dry sample (g LEUE/100 g).

2.5. Dry Matter Intake

Dry matter intake (DMI) was estimated from the total fecal production and the dry matter digestibility consumed by the animals. Total fecal production was estimated using titanium dioxide (TIO_2) as an external marker [47], dosed at 10 g/animal/day for ten consecutive days: five days of adaptation plus five days of sample collection directly from the rectum.

For TIO_2 supply and feces sampling, animals were taken to a stable located approximately 50 m from the paddocks. Fecal samples were collected once a day, between 9:00 and 12:00 am, and dried at 55 °C for 72 h. A composite sample per animal was prepared, ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1 mm sieve and then analyzed to determine the TIO_2 concentration, NDF and N [44,48].

Forage dry matter digestibility was calculated using the Lithourgidis et al. [49] equation from ADF content measured in consumed forage (weighted difference in ADF between pre- and post-grazing samples). Digestibility of the NDF fraction was calculated with the NDF content of fecal samples, total fecal excretion previously analyzed and individual intake of each animal [50].

2.6. Animal Average Daily Live Weight Gain

All heifers were weighed individually prior to each diet adaptation period and at the end of each sampling period. For each treatment, the average daily gain (ADG) was composed of two periods of 15 days and 11 observations for each period (n = 22 per treatment). Average daily gain was calculated as the weight on Day 15 minus initial weight divided by the total number of days in the period (15).

2.7. Determination of CH_4 Emissions

Enteric CH_4 emissions were determined using the sulfur hexafluoride (SF_6) tracer gas technique [51] adapted by Gere and Gratton [52]. One day before the diet adaptation period, each animal received an oral permeation tube filled with SF_6 using a dosing applicator. Soon after, the animals were assigned to the treatments and began to adapt to the corresponding pasture. The permeation rates (PRs) of SF_6 from tubes averaged 6.65 mg/day. Burped and exhaled air samples were collected for five consecutive days during the heifers' stay in the paddocks designated for the sampling period. The CH_4 collection containers for each animal consisted of two 0.5 L stainless steel cylinders, previously cleaned with high-purity

nitrogen gas (N_2) and pre-evacuated (<0.5 mb). Both cylinders were attached to a muzzle and placed on each side of a backpack fitted to the animal. Each cylinder was connected to an airflow regulator limited by a steel ball bearing that ended approximately 3 cm from the animal's nostril. The cylinders remained on the animals during the five sampling days of each event of the experimental period, and the inlet regulators were calibrated before each collection [6]. Three additional cylinders were placed adjacent to the treatment area to collect air samples representing ambient CH_4 and SF_6 concentrations (background samples). The cylinders' final pressure was measured immediately after removing the equipment from the heifers, with containers with pressure values of 400–600 mb considered valid, as they guarantee representative samples [52]. Five subsamples were extracted from each cylinder and stored in 6 mL vacutainers for determining CH_4 and SF_6 concentrations. At the end, the cylinders were emptied, flushed with N_2, evacuated and placed on the animals designated for cross-treatment for a second 5-day gas collection event.

Gas Analysis and Calculation

Subsamples obtained were analyzed using a gas chromatograph (Agilent 7890A, Santa Clara, CA, USA) with a flame ionization detector (FID) and an electron capture detector (ECD) to determine the CH_4 and SF_6 concentrations. The maximum time period between the collection and the determination of the CH_4 and SF_6 concentrations was 20 days. After conducting a chromatographic analysis, the emissions of CH_4 per animal were calculated using the PR of each SF_6 capsule and the atmospheric (atm) and enteric (ent) concentrations of CH_4 and SF_6, considering the molecular weight (MW) of each one (Equation (1)).

$$CH_4\ (g/day) = SF_6\ PR\ (mg/day) \times [CH_4\ ent - CH_4\ atm\ (ppm)/SF_6\ ent - SF_6\ atm]\ (ppt)] \times [(16\ (MW\ CH_4))/(146\ (MW\ SF_6))] \times 1000 \quad (1)$$

The methane conversion rate (Ym) was calculated following the equation that was proposed by the IPCC Guidelines [53] based on the conversion efficiency value of the gross energy intake (GEI) of CH_4 (Equation (2)).

$$Ym\ (\%) = GEI\ (MJ/kg\ DM/day)/CH_4\ (g/day) \quad (2)$$

2.8. Fecal Nitrogen Excretion, Urine Production and Nitrogen Balance

The daily fecal N excretion per animal was quantified as the product of total fecal production times N concentration in feces.

Daily urinary N excretion was determined by multiplying urine volume by urine N concentration [54]. For this, spot urine samples (100 mL) were collected by vulvar stimulation at the same time as fecal sampling. Daily urine samples were grouped, and a composite sample per animal in each treatment was stored at −20 °C. A 20 mL aliquot of urine was acidified with 0.4 mL of concentrated sulfuric acid (H_2SO_4) (95%) for total N determination. Another aliquot of 12 mL of urine was diluted with 48 mL of 0.02 N H_2SO_4 for determination of creatinine, uric acid and urea [55]. Urine volume was estimated based on creatinine concentration in spot urine samples and estimated daily urinary creatinine excretion [54,56].

The N retained by the animal was estimated as the difference between N intake and N excretion in feces and urine, and N use efficiency was estimated as the ratio of N retained to N intake.

2.9. Statistical Analyses

The homoscedasticity and normality of residuals were tested using the BoxCox and Cramer–von Mises tests, respectively, using Nortest package of R software (R-3.6.3) [57].

Animal performance (DMI, ADG), CH_4 emissions per animal, per unit of DMI and per kg of ADG as well as the N balance components were analyzed using a mixed linear model. Treatments (NG and NG+L) were considered fixed effects, while measurement period, animal and paddocks were considered random effects. The forage composition and

production were tested considering the treatments and the pre- and post-grazing as fixed effects and the period, plots and paddocks as random effects. Means were compared with a Tukey test. The covariance structure of the error was included in the model to account for repeated measures in the same animal. The covariance structure with the lowest Akaike and Bayesian corrected information criteria was selected. Significant effects for treatment were stated at $p < 0.05$ and tendence at $p < 0.10$.

3. Results

3.1. Chemical Composition, Herbage Allowance, Canopy Height and Legume Proportion

Chemical analysis of the nutritional components is presented in Table 1. During the pre-grazing period, the NG was characterized by 88 g/kg DM of crude protein and 61% digestibility, while the NG+L was characterized by 101 g/kg DM of crude protein and 65% digestibility. After grazing, both treatments showed 83 g/kg DM of crude protein and 59% digestibility. The legumes (*L. uliginosus*, *L. angustissimus*) had 55 g/kg DM of condensed tannins. Thus, the condensed tannins in the NG+L and NG pastures were 22 and 13 g/kg DM, respectively.

Table 1. Chemical composition of native grassland (NG = control) and native grassland with inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*).

	Treatment				
	NG		NG+L		
Chemical Composition	**Pre-Grazing**	**Post-Grazing**	**Pre-Grazing**	**Postg-Razing**	**s.e.m. (*n* = 4)**
Organic matter (g/kg DM)	908	906	911	918	10
Ash (g/kg DM)	92	94	89	82	5
NDF (g/kg DM)	587	612	539	612	10
ADF (g/kg DM)	361	382	356	379	10
Crude protein (g/kg DM)	88	83	101	83	4
Condensed tannin total (g/kg DM)	13	4	22	12	1
Dry matter digestibility (%)	61.0	59.2	65.2	59.4	0.5
NDF digestibility (g/kg DM)	620	-	640	-	30
Gross energy (MJ/kg)	17.0	18.1	17.0	17.7	2.9

NDF: neutral detergent fiber; ADF: acid detergent fiber.

The average amount of forage that disappeared during grazing was 416 and 506 kg/MS/ha in the NG and NG+L, respectively (Table 2). In the NG+L, 54% of this amount corresponded to legumes. Consequently, the amount of N that disappeared between pre- and post-grazing was higher in the NG+L than in the NG (12 vs. 6 kg N/ha, respectively; $p < 0.001$). In contrast, the total amount of NDF that disappeared was 37% lower in the NG+L than in the NG (139 vs. 220, respectively; $p < 0.001$).

Table 2. Composition and forage production of native grassland (NG) and native grassland with inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*) during pre- and post-grazing.

	NG		NG+L				
Forage Composition	**Pre-Grazing**	**Post-Grazing**	**Pre-Grazing**	**Post-Grazing**	**s.e.m.**	**Treatment**	**Pre and Post Grazing**
Herbage allowance (kg DM/100 kg of LW)	6.1	-	6.5	-	0.7	0.361	-
Canopy height (cm)	11.5	8.0	14.5	12.0	1	<0.001	<0.001
Total herbage mass (kg DM/ha)	1387	971	2372	1866	112	<0.001	0.043
Legume mass (kg DM/ha)	73	64	500	225	6	0.016	0.037
N total (kg/ha)	19	13	35	23	3	<0.001	0.051
Legume proportion (%)	6.5	5.7	24.6	13.7	1.5	0.031	0.005
N from legume (kg/ha)	2	2	13	6	1	0.004	0.055
NDF total in forage (kg/ha)	814	594	1280	1141	62	<0.001	<0.001
Condensed tannins (kg/ha)	19	5	51	28	4	<0.001	<0.001

DM: dry matter; LW: life weight; N: nitrogen; NDF: neutral detergent fiber. Significant difference $p < 0.05$ and tendence $p < 0.10$.

3.2. Animal Performance and Enteric Methane Emissions

The inclusion of tannin-containing legumes in NG did not affect the DMI and enteric CH_4 emissions of beef heifers ($p > 0.05$) (Table 3). The individual DMI ranged from 4.62 to 8.84 kg/day, corresponding to an average intake of 3.2% of LW (ranging from 2.2 to 3.5%). Enteric CH_4 emissions per unit of DM ingested also did not differ between treatments ($p = 0.113$) and ranged from 15.4 to 30.7 g CH_4/kg DMI. The ADG was 2.5 times higher in the NG+L treatment than in the NG treatment ($p = 0.003$), and enteric CH_4 emissions per ADG were significantly lower in the NG+L treatment than in the NG treatment, reflecting a lower emission intensity.

Table 3. Animal performance and methane (CH_4) emission variables of heifers grazing native grasslands (NG) and native grassland with the inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*) during the experiment.

	Treatment			
	NG	**NG+L**	**s.e.m.**	***p* Value**
DMI (kg/animal/day)	6.35	6.67	0.47	0.314
DMI (% LW)	3.1	3.3	0.2	0.435
ADG (g/animal/day)	235	581	100	0.003
CH_4 (g/animal/day)	139	148	6.1	0.113
CH_4 (g/kg DMI)	21.5	21.1	1.3	0.854
CH_4 (g/kg ADG)	0.58	0.25	0.10	0.007
CH_4 (g/g N intake)	1.40	1.23	0.07	0.052
Ym (%)	7.5	7.0	0.4	0.813

DM: dry matter; LW: life weight; ADG: average daily gain; N nitrogen; Ym: methane yield. Significant difference $p < 0.05$ and tendence $p < 0.10$.

3.3. Nitrogen Excretion and Balance

Heifers grazing the NG+L consumed more N than heifers grazing NG (Table 4). However, there was no evidence that this higher intake affected N urinary composition (Table 5). On the other hand, heifers grazing the NG+L excreted feces with a higher concentration of N than heifers grazing NG, resulting in higher daily N excretion in this form ($p = 0.054$). Approximately 26% of the N ingested by heifers from the NG was excreted in the urine, while from the NG+L, this percentage was 18% (Figure 2). Thus, N retention and N use efficiency in heifers grazing the NG+L were significantly higher than those in heifers grazing NG ($p < 0.001$).

Table 4. The excretion and nitrogen (N) balance of heifers grazing native grasslands (NG) and native grassland with the inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*) during the experiment.

	Treatment			
	NG	**NG+L**	**s.e.m.**	***p* Value**
N intake (g/animal/day)	89.5	106.8	5.7	<0.001
Fecal production (kg DM/animal/day)	2.48	2.34	0.5	0.361
N concentration in feces (%)	1.88	2.30	0.1	<0.001
Fecal N excretion (g/animal/day)	45.7	53.2	2.4	0.054
Urine N excretion (g/animal/day)	23.4	19.3	2.7	0.457
N retention (g/animal/day) *	23.1	31.6	3.4	<0.001
N use efficiency (%) **	22.8	32.3	1.8	<0.001

* N retention = N intake − (fecal N excretion + urine N excretion); ** N use efficiency = N retention/N intake; significant difference $p < 0.05$ and tendence $p < 0.10$.

Table 5. The concentration of different Nitrogen-containing constituents in the urine of heifers grazing native grasslands (NG) or native grassland with the inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*) during the experiment.

	Treatment			
	NG	**NG+L**	**s.e.m.**	***p* Value**
Urinary volume (L/animal/day)	8.01	7.37	0.85	0.538
N total (g/L)	2.93	2.62	0.22	0.517
Urea (g/L)	0.87	0.89	0.11	0.649
Creatinine (g/L)	0.97	0.79	0.10	0.281
Uric acid (g/L)	0.25	0.24	0.02	0.998

Significant difference $p < 0.05$ and tendence $p < 0.10$.

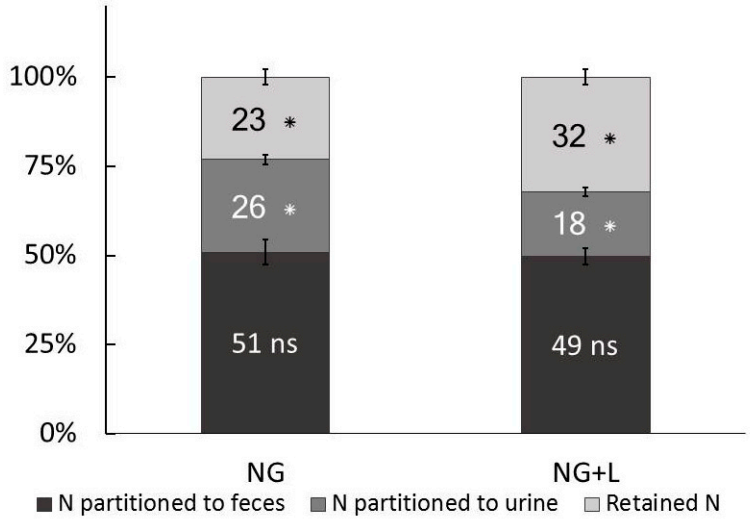

Figure 2. Nitrogen (N) balance (%) in Heifers grazing native grasslands (NG) and native grassland with the inclusion of tannin-containing legumes (NG+L; *Lotus uliginosus* and *L. angustissimus*) during the experiment. ns = no significance; * = significantly different ($p < 0.05$).

4. Discussion

The input of forage legumes in grassland ecosystems is a biological solution that can improve animal productivity and increase nutrient cycling and carbon sequestration in the soil without the use of synthetic fertilizers [26,28,58]. Thus, an ecologically diverse native grassland plus the presence of high protein and secondary compounds from tannin-containing legumes may offer ruminants a feed with the potential for synergisms that improve nutrition while reducing GHG emissions [59].

4.1. Chemical Composition and Forage Intake

The condensed tannin content observed in this study is comparable to that reported by Lagrange et al. [59] for forage legumes with moderate tannin levels (30–60 g/kg DM). The chemical composition of the forage during pre- and post-grazing and the data align with existing findings on grasslands with and without legume inclusion. Faverin et al. [60] reported similar crude protein values (90 g/kg DM) and dry matter digestibility (56%) for native grasslands in the Pampa biome, and Gonzáles et al. [34] found higher crude protein levels (115 g/kg DM) and digestibility (58%) in grasslands with the addition of *Lotus tenuis*. Nitrogen is a key limiting nutrient for pasture productivity [61], and its availability can be enhanced through biological N fixation, which improves N cycling within the soil–plant–animal system and boosts dry matter production per unit area [22,62].

Cattle in the NG+L selectively grazed areas with higher legume concentrations within the paddock, reflecting that a high percentage of legumes present in the total forage mass

consumed between pre- and post-grazing in this treatment (Table 2). The defoliation of forage plants by ruminants during grazing is selective, both in terms of plant and species [63]. Wallis de Vries and Daleboudt [64] suggested that cattle are more likely to select patches of grassland where forage plants have a better nutritional value. This selective grazing likely contributed to the reduction in forage quality post-grazing, especially in the NG+L (Table 1). Additionally, the content and quantity of condensed tannins in the NG+L also decreased during grazing, suggesting that heifers in this treatment had the opportunity to ingest tannins at a rate of 2% per kg DM (Table 1), a value similar to that recommended by Herremans et al. [35], which would be a sufficient amount to cause an effect on cattle digestion and not alter intake.

4.2. Animal Performance

The quantity and nutritional quality of forage available to cattle significantly influence the DMI and animal productivity [6]. Low nitrogen and high fiber content can limit bovine DMI. Thus, improving the nutritional quality of forage diets, especially with the inclusion of legumes, can increase individual animal DMI [30,65]. In the present study, despite the similar herbage allowance (6 kg DM/100 kg LW) in both treatments, the higher disappearance of N and lower disappearance of fiber (NDF) between pre- and post-grazing in the paddocks with legume inclusion (Table 2) suggest that the animals in this treatment ingested a better-quality diet. However, there was no evidence of the effect of legume inclusion in the native grassland on individual DMI (Table 3). These results are similar to those obtained by Berça et al. [66] and Gonzáles et al. [34], who reported a DMI of approximately 2.8% of live weight in young bovines on pastures with or without the inclusion of legumes, ($p > 0.05$). As recently reported by Cunha et al. [8], sward structure (i.e., sward height and herbage mass) appears to affect individual DMI more strongly than forage nutritional quality, and the effect of nutritional quality on animal productivity is more pronounced.

In this work, heifers grazing the NG+L showed an ADG twice as high as the average observed in the NG treatment. A similar trend was observed in other studies, where the authors reported approximately double the animal productivity in pastures supplemented with N (through fertilization or the use of legumes) compared to non-fertilized pastures [22,23,58]. The contrast in animal performance between the NG and NG+L treatments aligns with the predictions of Homem et al. [24] that a beef animal, initially weighing 234 kg and consuming approximately 6 kg of DM/day, would gain 0.611 kg/day if grazing *Brachiaria* grass mixed with legumes but 0.542 kg/day if grazing *Brachiaria* grass monocultures ($p < 0.05$). In livestock production systems, along with energy requirements, protein needs for maintenance and growth must be considered [67]. According to the NRC [68], the relationship between energy and protein can be expressed by the ratio between dietary energy and microbial crude protein production, with the efficiency of microbial crude protein synthesis estimated at 13% of total digestible nutrients in the diet. Therefore, the consequent live weight gain of the animal is established as a function of the rate of energy and protein intake. Since the gross energy content was similar in both treatments (Table 1), the higher protein intake, lower fiber intake and improved nutrient utilization efficiency in the NG+L (Table 2) likely contributed to the observed increase in the ADG [69].

4.3. Enteric Methane Emissions

In experiments conducted in Canada, McCaughey et al. [70] concluded that improving pasture quality by including legumes reduced enteric CH_4 emissions by 10% due to lower fiber content and increased gross energy and CP in the diet. In the present study, while pre-grazing forage in the NG+L had 13% more CP and 8% less NDF than in NG, no significant effect was found on absolute CH_4 emissions per animal or per kilogram of DMI (Table 3). However, treatment did affect emission intensity. These results were confirmed by enteric CH_4 emissions per ADG (g CH_4/kg ADG), which showed that emission intensity was approximately two times lower in heifers in the NG+L treatment than in heifers in the NG

treatment. According to Santander et al. [71] and Richmond et al. [72], better diet quality leads to improved production efficiency, which translates into lower emissions per unit of product (meat or milk) or production cycle.

In addition to the good nutritional quality of forage legumes, there are other factors inherent to these plants that can reduce methanogenesis, such as the presence of tannins [73]. Hydrolyzable tannins have been shown to be toxic to methanogenic microorganisms [74], while condensed tannins inhibit the binding of these microorganisms to hydrogen, reducing its availability for CH_4 production [18,31]. According to Stewart et al. [30], Angus heifers fed small burnet (45% tannins) emitted less CH_4 than heifers fed alfalfa (no tannins) (180 vs. 227 g/animal/day, $p < 0.05$). However, these authors did not find a significant difference in emissions per kg of DMI and per ADG, indicating that emissions were reduced due to lower animal performance. These findings are consistent with the results of Berça et al. [66], where heifers performing on tropical pastures of grasses mixed with forage peanut (*Arachis pintoi*; condensed tannins = 1.7%) and grasses fertilized or not with mineral N emitted 132, 140 and 115 g CH_4/animal/day, respectively ($p > 0.05$). Furthermore, they also found no significant difference in emission intensity and concluded that the heifers probably ingested a small amount of tannins (approx. 0.25% of ingested dry matter).

The amount of condensed tannins shown to reduce CH_4 production in several studies ranges from 11 to 20 g/kg DM [74], which is similar to the levels observed in both the NG and NG+L treatments (Table 1). Therefore, the lower emission intensity presented in the NG+L treatment could be associated with the higher presence of tannins with respect to the NG treatment [25]. However, the lack of a control treatment with equal crude protein content and the difficulty of measuring the precise amount of legumes ingested by grazing cattle generate some uncertainty about this finding. Future studies should address these aspects by extending the experimental duration or incorporating more evaluation periods to capture long-term effects, which would improve the representativeness and reliability of the data.

The results of the CH_4 conversion rate (Ym) were above the range proposed by Hristov et al. [75], between 5.5 and 6.5% in North America and Eastern Europe, and within the IPCC guidelines [53], a range between 6.5 and 7.5%, for cattle in tropical and subtropical conditions. Although not verified in the present study, Blaxter and Chapperton [76] argue that the introduction of legumes in grasslands tends to increase animals' voluntary consumption, making post-ruminal digestion more efficient and reducing the loss of dietary energy in the form of CH_4.

4.4. Nitrogen Balance

An alternative way to evaluate the effect of the presence of tannin-containing legumes in native grasslands on enteric CH_4 emissions would be by studying N compounds in urine, feces and N balance [77].

In the present study, heifers grazing the NG+L ingested more N and were more efficient in its use, excreting a lower proportion of urinary N and a greater amount of fecal N (Figure 2). Typically, with a low crude protein diet in ruminant production, feces are the main route of N excretion, whereas high crude protein levels increase urinary N excretion [35,78,79]. A high concentration and increased degradability of proteins, combined with an insufficient concentration of energy in the rumen environment, often result in an accumulation of ammonia and elevated urinary urea excretion [80,81]. However, tannins in the bovine diet can promote the formation of complexes with various dietary components, particularly proteins, altering ruminal fermentation [35]. This reduces ammonia concentration and enhances amino acid absorption [82], potentially explaining the improved N utilization efficiency and higher ADG observed in NG+L heifers. Additionally, the tannin–protein complexes formed in the rumen are stable and resist microbial degradation [83], increasing fecal N concentration and excretion [36].

According to the study by Zhou et al. [36], beef heifers fed low crude protein (11.1%) and high crude protein (13.6%) diets on average ingested 98 and 120 g N/animal per day,

presenting daily urinary N excretion per animal of 26 and 45 g ($p < 0.05$) and fecal N of 38 and 48 g ($p > 0.05$), respectively. However, when hydrolyzable tannins were added to the low and high protein diets, the daily averages observed per animal were 19 and 31 g urinary N and 48 and 49 g fecal N ($p < 0.05$), respectively. These results are similar to those obtained by Stewart et al. [30] and Lagrange et al. [69], who reported an increase in the proportion of N excreted in feces and a decrease in urine in beef cattle grazing legumes containing tannins.

5. Conclusions

Native grasslands with the inclusion of tannin-containing legumes did not affect the DMI per animal, increased ADG and reduced enteric CH_4 emission intensity (g CH_4/kg ADG). Heifers grazing native grasslands with tannin-containing legumes ingested more nitrogen and were more efficient in its use in addition to excreting a smaller proportion of urinary nitrogen and a high amount of fecal nitrogen. Incorporating tannin-containing legumes in native grasslands can help increase animal productivity and N use efficiency in extensive livestock systems, with proportionally smaller impacts on their CH_4 emissions. Further studies with extended experimental durations or additional evaluation periods are needed to assess the long-term impacts on total GHG emissions, which would enhance the representativeness and reliability of the data.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16209135/s1, Figure S1: Water balance indicating measured reference evapotranspiration (ETo, continuous line), estimated evapotranspiration (ET, dash line) assuming a soil with 60 mm maximum available water, and the resulting cumulative water deficit (ETo–ET, dash-dot line). In light grey, the experimental period is indicated, in dark grey, the two periods in which measurements were made.

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