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Effects of rumen-undegradable protein supplementation on performance, nitrogen metabolism, and mammary gland development of grazing heifers

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ABSTRACT

This study examined the effects of supplementation with increasing levels of RUP on performance, nitrogen metabolism, and mammary gland development in grazing dairy heifers. Twenty-eight 5/8 crossbred Holstein × Gyr dairy heifers (initial BW of 278 ± 50.4 kg) grazed on *Megathyrsus maximus* cv. BRS Quênia grass pastures for an experimental period of 84 d, divided into 4 subperiods of 21 d each. The experiment followed a completely randomized design in a 4 × 3 factorial arrangement, with 4 supplementation strategies (control [CON], or supplementation with 30% [RUP30], 48% [RUP48], or 66% [RUP66] RUP) and 3 initial BW classes (light, medium, and heavy). The supplement was administered at 0.5% of the animals' BW with a consistent CP content of ~24% in all treatments. Sampling of pasture, feces, and urine was performed on 4 consecutive days in each period. On d 0 and 19 of each period, the animals were weighed and biometric measurements were recorded. Ultrasound images of the mammary gland and blood samples were taken on d 0, 42, and 84. On d 0, 42, and 84, the development of the reproductive tract was assessed by transrectal palpation using an ultrasound device. Liver tissue was sampled on d 0 and 84. Supplemented animals had higher DMI, DMI related to BW (g/kg of BW), and nutrient intake when compared with CON animals. An effect of RUP level was observed for supplement intake, pasture intake, and total DMI, with higher values noted in the RUP48 treatment. The supplemented animals had a significant increase in the digestibility of DM, CP, and OM. We observed a linear increase in NDF and OM digestibility across RUP level. Supplemented animals achieved higher BW, ADG, thoracic circumference, and rump height when compared with the CON animals. A quadratic effect of RUP level was observed for BW and ADG, with higher values in the RUP48 treatment. Supplemented animals had higher N intake, urinary and fecal N excretion, and microbial CP synthesis when compared with CON animals. A quadratic effect of RUP level was observed for N intake and fecal N excretion, with higher values in the RUP48 treatment. Supplemented animals had a lower pixel count in the mammary gland when compared with the CON animals, and no effect of RUP level was observed on this variable. Supplementation resulted in greater mean horn diameter and improved reproductive tract tone and score. An increasing linear effect was observed for uterine tone across the RUP levels. Nonsupplemented animals had higher liver expression of Glutamic-oxaloacetic transaminase 1 (GOT1) enzyme. Additionally, a quadratic effect was observed for GOT1 expression across the RUP levels, with lower expression in RUP48 and RUP66 treatments. In conclusion, a RUP level of 48% in the feed supplement is the optimal recommendation for grazing Holstein × Gyr crossbred dairy heifers, as it generally improves performance.

Key words: protein metabolism, protein supplementation, weight gain

INTRODUCTION

The rearing of dairy heifers is crucial in dairy production systems, as these animals are essential for herd renewal and expansion. However, the rearing phase accounts for a substantial proportion of the costs of dairy production systems and is often not given sufficient attention by farmers (Lowe et al., 2016). In this context, it is important to recognize that one of the most effective ways to reduce the production costs of rearing heifers is to ensure that these animals reach their first calving as early as possible (Hutchison et al., 2017; Machado et al., 2020). Because reproductive traits are closely correlated with BW, strategies to improve ADG and thus reduce age

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at first calving should therefore be implemented (Piantoni et al., 2012; Machado et al., 2019; Quirino et al., 2022).

In most tropical regions, pastures are the main source of forage due to their productive potential and biodiversity. In addition, in these regions, Holstein × Gyr cattle are one of the main crossbreeds used in this type of system because they are more resistant to grazing under high temperatures and to ectoparasites (Otto et al., 2018; Abreu et al., 2022; Quirino et al., 2022). This approach increases the proportion of forage in the animals' total diet, which leads to a reduction in feed costs (Lowe et al., 2016).

Tropical pastures often supply excess energy but are deficient in protein, making supplementation necessary (Detmann et al., 2014a,b). Previous studies suggest that protein supplementation can improve performance in both beef cattle (Batista et al., 2016; Sotelo et al., 2019) and dairy heifers (Machado et al., 2019, 2020; Castro et al., 2023). Furthermore, protein supplementation increases the utilization of basal forage by stimulating rumen microbial growth and activity, thereby enhancing fiber digestibility (Machado et al., 2019). In addition, protein supplementation can increase the availability of amino acids to the animal's metabolism, potentially leading to greater synthesis of muscle tissue, higher nitrogen retention, and preventing body fat deposition (Bethard et al., 1997; Tomlinson et al., 1997; Bascom et al., 2007). This could lead to higher ADG without excessive fat deposition in the body and mammary gland of heifers (Silva et al., 2018a,b).

Studies on the supplementation of grazing dairy heifers are still limited. However, recent studies suggest that a supplement CP content of ~24% (DM basis) is the most suitable for Holstein × Gyr heifers at 350 kg of BW and 19 mo of age when grazing on *Megathyrsus maximus* cv. Mombaça pastures (Machado et al., 2020). In addition, Silva et al. (2018a,b) demonstrated that increasing the RUP content in the diet of feedlot heifers resulted in improved animal performance. With 51% RUP in the CP of the total diet, Silva et al. (2018a,b) found no negative effects on mammary gland development. In addition, this proportion improved the balance of nitrogenous compounds, resulting in lower nitrogen excretion into the environment.

Although these results are concise, there is a need to evaluate RUP-rich protein supplements to determine the most appropriate levels for grazing animals. We hypothesized that the use of a supplement with a CP content of 24% in combination with increased amounts of RUP would improve performance, protein metabolism, mammary gland development, as well as reduce nitrogen excretion in grazing dairy heifers. The aim of this study was to evaluate the intake, performance, mammary gland development, nitrogen metabolism, and liver expression of genes related to protein metabolism in grazing crossbred Holstein × Gyr dairy heifers supplemented with different levels of RUP.

Table 1. Dietary ingredients and chemical composition of supplements for grazing crossbred Holstein × Gyr dairy heifers fed supplements with different levels of RUP

Item	Treatment		
	RUP30	RUP48	RUP66
Supplement composition, g/kg of DM			
Corn grain fine ground	859	737	625
Heat-treated soybean meal	—	179	375
Soybean meal	106	67	—
Urea	35	17	—
Chemical composition, g/kg of DM			
DM, as-fed	920	914	915
OM	948	940	942
CP	233	247	251
Starch[1]	606	532	447
NDF	123	138	132
Indigestible NDF	19.5	16.5	17.6
Ether extract	45.7	39.6	36.6
NFC	610	578	513
RUP/CP[2]	286	479	668
Digestible energy,[1] Mcal/kg	3.45	3.55	3.64
MP/ME,[1] g/Mcal	27.9	38.6	51.9

[1]Estimated according to NASEM (2021).
[2]Amount of RUP in total CP (calculated using values obtained in the in situ trial).

MATERIALS AND METHODS

All animal management and procedures described in this study were approved by the Ethics Committee for the Use of Animals of Embrapa Gado de Leite (CEUA/EGL) under protocol number 2952051222. The experiment was carried out at the José Henrique Bruschi experimental field of Embrapa Gado de Leite in Coronel Pacheco, Minas Gerais, Brazil. The geographical coordinates are 21°33′22″S and 43°06′15″W. Temperature and precipitation data for the experimental period (January 19, 2023, to April 12, 2023) were obtained from meteorological station A557 in Coronel Pacheco, Minas Gerais, Brazil, using the National Institute of Meteorology (INMET) system (Figure 1).

Animals, Experimental Design, and Treatments

The study was conducted as a completely randomized design with 28 crossbred (5/8 Holstein × Gyr) heifers, arranged in a 4 × 3 factorial scheme with 4 supplementation strategies and 3 initial BW (**iBW**) categories (light, medium, and heavy). The animals were kept intermittently in paddocks with *Megathyrsus maximus* cv. BRS Quênia and subjected to one of 4 supplementation strategies: (1) the control group with no supplementation (**CON**; n = 7); (2) supplementation with 30% of the

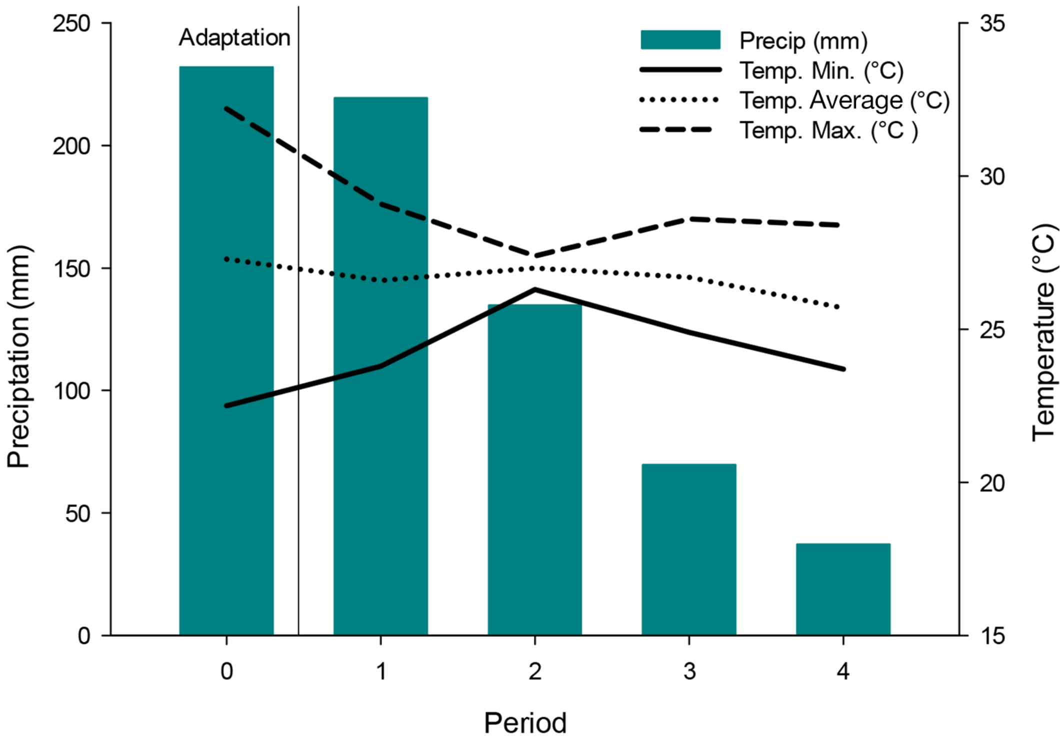

Figure 1. Precipitation (precip) data and minimum (min.), average, and maximum (max.) temperatures were recorded during the adaptation period (January 4 to 18) and the experimental subperiods: 1 (January 19 to February 8), 2 (February 9 to March 1), 3 (March 2 to 22), and 4 (March 23 to April 12). Source: National Institute of Meteorology (INMET). Retrieved from https://tempo.inmet.gov.br/TabelaEstacoes/A001.

supplement CP as RUP (**RUP30**; n = 7); (3) supplementation with 48% of the supplement CP as RUP (**RUP48**; n = 7); and (4) supplementation with 66% of the supplement CP as RUP (**RUP66**; n = 7; Table 1). For the iBW categories, light heifers averaged 238.6 ± 21.5 kg of BW and 12.2 ± 1.2 mo of age at the beginning of the experiment, medium heifers averaged 306.9 ± 12.5 kg and 16.2 ± 1.1 mo, and heavy heifers averaged 344.8 ± 10.3 kg and 18.5 ± 0.5 mo. Age and iBW were balanced across supplementation strategies. At the beginning of the study, all heifers were treated against ectoparasites with a fipronil-based product at a dosage of 0.1 mL/kg of BW. All animals had ad libitum access to water and a mineral supplement, which was placed in 2 covered troughs in the resting area. The experimental period lasted 84 d and was divided into four 21-d periods.

General Management

A 15-d adaptation period was carried out before the start of the study. During this period, the animals were placed on the *Urochloa decumbens* pasture and fed 1.5 kg/d of supplement. The supplement was provided in a feeder located in the management barn and consisted of 44.5% corn meal, 47.5% soybean meal, 6% mineral supplement, and 2% urea (all on a DM basis).

The animals were kept in paddocks with *Megathyrsus maximus* cv. BRS Quênia, covering a total area of ~2 ha. This area was divided into 10 paddocks of 2,000 m^2 each and a resting area of 430 m^2. Each paddock was fertilized with 42 kg of nitrogen and potassium fertilizer mix (20-00-20) 7 d before the start of the trial period. During the trial period, 42 kg of the same fertilizer was applied every 2 d after the animals had left the paddock. In total, 197.4 kg/ha of nitrogen and 197.4 kg/ha of potassium oxide were applied during the entire trial. The resting area was equipped with drinking fountains and feeders for mineral supplementation. Mineral composition was as follows: calcium (160 g/kg), phosphorus (60 g/kg), sodium (117 g/kg), sulfur (10 g/kg), magnesium (5 g/kg), iodine (86 g/kg), copper (1,010 mg/kg), manganese (1,150 mg/kg), selenium (22.5 mg/kg), zinc (3,965 mg/kg), cobalt (75 mg/kg), vitamin A (15,000 IU/kg), vitamin D (40,000 IU/kg), and vitamin E (1,000 IU/kg).

The 28 animals grazed simultaneously in the same paddocks in all experimental periods, to exclude effects related to the paddock. The stocking rate was 8.5 animal units per hectare, with an occupation period of 2 d until d 49 of the experimental period. For the remainder of the study, the stocking period was reduced to 1 d as the production of forage biomass in the paddocks decreased (Table 2).

Table 2. Pasture yield and chemical composition of *Megathyrsus maximus* cv. BRS Quênia assessed throughout the experimental periods

Item	Experimental period[1]			
	1	2	3	4
Pasture production				
Accumulated pasture, kg/ha	23,350	19,751	18,305	16,701
DM accumulated pasture, kg/ha	3,797	3,506	3,621	3,262
DM accumulated pasture, kg/ha per paddock	190	175	181	163
DM pasture allowance, kg/animal per day	6.78	6.26	5.68	5.27
Chemical composition,[2] g/kg of DM				
DM, as-fed	255	205	245	252
OM	909	909	913	912
CP	96	144	147	136
RUP,[3] g/kg CP	405	356	345	384
RDP,[3] g/kg CP	595	643	655	616
NDF	716	708	703	698
Indigestible NDF	166	132	154	122
Ether extract	14	14	11	12
NFC	84	42	52	66

[1]The number of grazing cycles was recorded throughout the experimental period.

[2]The chemical composition of the paddocks occupied by the animals during the consumption assessment period was analyzed.

[3]Calculated using a 16-h single-point in situ ruminal incubation.

The experimental supplement was administered at a level of 0.5% (5 g/kg BW, as-fed basis) of the average BW of the animals. The animals were weighed weekly to adjust the amount of supplement administered (Barbero et al., 2015; Camargo et al., 2022). The supplement was provided individually, and any remaining supplement was weighed. The animals were taken to the management area at 0900 h each day, where they received the supplement and were then moved to the paddocks. Each animal had a maximum of 30 min to consume the supplement. After this time, any remaining feed was removed and weighed. The animals in the CON treatment were treated in the same way as the others but were not given supplementary feed.

At the time of supplement preparation, samples were taken to determine their chemical composition. Forage mass and composition were estimated in each period by sward height measurements and sampling at the grazing stratum, following Gomide et al. (2007).

Performance, Intake, and Digestibility

At the start of the experiment and at the end of each evaluation period, the animals were weighed on a mechanical scale for 3 consecutive days before feeding (Machado et al., 2020). On the same day, morphometric measurements, including body length, chest circumference, withers height, and rump height were recorded using a hypometer (Machado et al., 2020). Additionally, on d 0, 42, and 84, the BCS of the animals was evaluated by 3 assessors. The BCS was rated on a scale from 1 to 5, in 0.25-point increments, where 1 indicated underweight animals and 5 indicated overweight animals (Ferguson et al., 1994).

Titanium dioxide was used as an external marker to estimate fecal excretion and was administered in encapsulated form at a dose of 15 g/d for 9 consecutive days, starting on d 9 of each period (Titgemeyer et al., 2001). The capsules were delivered directly into the esophagus using an applicator at 1000 h. To determine diet digestibility, 4 fecal samples were collected directly from the rectum on consecutive days of each period at 0600 h (d 15), 1000 h (d 16), 1400 h (d 17), and 1800 h (d 18; Sampaio et al., 2011). This staggered schedule was adopted to account for diurnal variation in titanium dioxide excretion and to obtain a representative average of daily output. The titanium dioxide content in the feces was quantified using the sulfur digestion method (method M-007/2; Detmann et al., 2025).

The fecal DM excretion was obtained based on the ratio between the amount of titanium supplied and its concentration in the feces, as follows:

$$\text{DMfecal} = \text{AOM}/\text{MCF},$$

where DMfecal is the daily fecal DM excretion (kg/d), AOM is the amount of marker ingested (g), and MCF is the marker concentration in fecal DM (g/kg of fecal DM).

Supplement, forage, and feces samples were subjected to partial drying at 55°C for 72 h immediately after collection (method G-001/2; Detmann et al., 2025). They were then ground with a cutter mill (STAR FT 50/6, Fortinox), first through a 2 mm sieve and then through a 1 mm sieve. Samples ground to 1 mm were analyzed for DM (method G-003/2), CP (method N-001/3), and ash content (method M-001/3 method) according to the methods described by Detmann et al. (2025). In addition, the samples were analyzed for the NDF content corrected for ash (methods F-002/3 and M-002/2; Detmann et al., 2025). Samples ground to 2 mm were used to determine the concentration of indigestible NDF (**NDFi**; method F-009/3; Detmann et al., 2025). In brief, supplement, forage, and fecal samples were incubated in the rumen of 3 heifers via a rumen cannula for a period of 288 h, and the residual NDF was determined. The NDFi was used as an internal marker for estimating pasture intake, as follows:

$$\text{DMIpasture} = \frac{\left(\text{DMfecal} \times \text{NDFFi}\right) - \text{NDFSi}}{\text{NDFPi}},$$

where DMIpasture is the dry matter intake from pasture (kg/d); DMfecal is the daily fecal DM excretion (kg/d), NDFFi is the concentration of NDFi in the feces (kg/kg), NDFSi is the concentration of NDFi in the supplement (kg/kg), and NDFPi is the concentration of NDFi in the pasture (kg/kg).

Microbial Protein Synthesis and Nitrogen Balance

Urine samples were collected on the same days and sequentially at the same sampling times as fecal samples by stimulating urination through gentle vulva massage, and ~100 mL of midstream urine was obtained. After sampling, the urine was filtered through gauze, and a 50-mL aliquot was collected. Another 10-mL aliquot was collected and immediately diluted in 40 mL of 0.036 *N* sulfuric acid to prevent the degradation of purine derivatives. Both samples were stored at −20°C for further analysis.

Nondiluted urine samples were used to determine the total N concentration using the N-001/3 method (Detmann et al., 2025). The samples previously diluted in sulfuric acid were used to determine the concentrations of creatinine, urea, uric acid, and allantoin. Creatinine was determined using the Labtest colorimetric determination kit (Labtest Creatinine), and urea and uric acid concentrations were measured using Biotek EON microplate spectrophotometer with Gen5 software and Labtest determination kits. Finally, allantoin concentrations were determined using the technique described by Chen and Gomes (1992).

Daily urinary volume was estimated from creatinine excretion, calculated using the equation proposed by Chizzotti et al. (2008). Purine derivatives (allantoin + uric acid) were used to estimate absorbed purines (Prates et al., 2012), and microbial N synthesis was calculated according to Chen and Gomes (1992), considering an intestinal digestibility of microbial purines of 0.93 (Barbosa et al., 2011). Metabolizable protein of ruminal origin was obtained as microbial N × 6.25 × 0.65 (NASEM, 2021). The RDP intake was assumed to be equal to microbial CP, and digestible RUP was calculated as (CP intake − RDP) × 0.80 (NASEM, 2021). Total MP flow was the sum of microbial MP and digestible RUP, and N balance was determined as N intake minus fecal and urinary N excretion.

Mammary Gland Ultrasound, Blood Analysis, and Reproductive Assessment

Ultrasound images of the mammary gland were obtained on d 0, 42, and 84 using a B-mode device with a 6-MHz microconvex transducer (M6Vet, Mindray) following the standardized procedure described by Nishimura et al. (2011) and Albino et al. (2017). Images were analyzed with ImageJ (National Institutes of Health) calibrated at 100 pixels/cm. Three 16-mm^2 areas were randomly selected near the mammary ducts, and pixel intensity was quantified on an 8-bit grayscale (0–255), with higher values indicating adipose tissue and lower values indicating parenchyma (Albino et al., 2015; Silva et al., 2018a).

Blood samples were collected via coccygeal vessel puncture on d 0 and at the end of each period, 4 h after supplementation. Samples were centrifuged at 1,500 × *g* at 4°C for 20 min, and sera were stored at −20°C until analysis. Glucose, urea-N, albumin, and total protein were determined using commercial kits (Bioclin) and an automated analyzer (BS200, Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, China), whereas IGF-1 was quantified by chemiluminescence using the Liaison XL platform (Diasorin).

Reproductive tract development was evaluated by transrectal ultrasound (M6Vet, Mindray; 6-MHz linear transducer) on d 0, 42, and 84, according to Rosenkrans and Hardin (2003) and Stevenson et al. (2008). Ovarian and uterine structures were recorded and a reproductive tract score was assigned on a scale of 1 to 3, where 1 = prepubertal (no tone, follicles <8 mm), 2 = peripubertal (slight tone, follicles 8–10 mm), and 3 = pubertal (uterine tone, follicles >10 mm, and corpus luteum present). The left and right uterine horns, the diameter of the corpus luteum (if present), and the tone of the uterus (scored on a scale of 1 to 5) were also evaluated.

Liver Tissue Collection and Gene Expression

Liver biopsies were obtained from 5 heifers per treatment at the beginning and end of the trial using a Tru-Cut needle (Becton, Dickinson and Company) between the 11th and 12th ribs (Miranda et al., 2010; Mølgaard

Table 3. Genes (amplicon base pairs), primer sequences, and access number used for liver tissue samples

Genetic symbol (bp)[1]	Sequence	NCBI
IGFBP3 (74)	Forward: TTACAAGAAAAAGCAGTGCCGC Reverse: TGCCCGTACTTATCCACACAC	NM_174556.1
IGF1 (91)	Forward: TGCACTTCAGAAGCAATGGGA Reverse: GGCATCTTCACCTGCTTCAA	NM_001077828.1
CPS1 (87)	Forward: GGCTTAACCAATGTGACGGC Reverse: TGTGTGCTGTTTGTGCCTTG	NM_001192258.1
GOT1 (85)	Forward: GGCTTAACCAATGTGACGGC Reverse: TACTCAACCTGCTTGGGGTTC	NM_177502.2
GAPDH (85)	Forward: AAGGTCGGAGTGAACGGATTC Reverse: ATGGCGACGATGTCCACTTT	NM_001034034.2

[1]*IGFBP3* = insulin-like growth factor-binding protein 3; *IGF1* = insulin-like growth factor; *CPS1* = carbamoyl phosphate synthetase; *GOT1* = glutamic transaminase-oxaloacetic (aspartate aminotransferase); *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase. NCBI = National Center for Biotechnology Information.

et al., 2012). Samples were stored in cryovials and frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Thermo Fisher Scientific), quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA), and integrity was verified by agarose gel electrophoresis. The cDNA was synthesized using the High-Capacity Reverse Transcription Kit (Applied Biosystems) and diluted to 5 ng/μL. Quantitative PCR was conducted in duplicate with a CFX96 Real-Time System (Bio-Rad) using SyBR Green (Promega) according to the manufacturer's instructions. Amplification efficiency (80%–100%) was confirmed with serial dilutions. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the endogenous control. Target genes evaluated included insulin-like growth factor-binding protein 3 (IGFBP3), IGF-1, carbamoyl phosphate synthetase (CPS1), glutamate transaminase-oxaloacetic acid (GOT1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 3).

In Situ Degradability Trial

To estimate dietary RDP and RUP, an in situ rumen trial was conducted with 2 lactating rumen-cannulated Holstein cows (680 kg BW). Feed samples (corn meal, soybean meal, and rumen-protected soybean meal) ground to 2 mm were incubated in nylon bags (15 × 8 cm; 50-μm pore size) for 16 h in triplicate (Calsamiglia and Stern, 1995; Paz et al., 2014). After incubation, residues were washed, dried at 55°C, and analyzed for DM (method G-003/1) and CP (method N-001/3; Detmann et al., 2025). The RDP was calculated as CP disappearance and RUP as the difference between CP and RDP.

Statistical Analysis

The experiment was conducted using a completely randomized design in a 4 × 3 factorial arrangement of treatments and analyses were performed using the GLIMMIX procedure in SAS (SAS OnDemand for Academics, SAS Institute Inc., 2024). For intake, digestibility, serum hormones and metabolites, and mammary gland development, the effect of sampling period was included in the model as a repeated measure over time. The selection of the (co)variance matrix was based on the corrected Akaike information criterion using the following model:

$$Y_{ijklm} = \mu + SUP_i + BW_j + (SUP \times BW)_{ij} + \delta_{ijk} + P_l + (SUP \times P)_{il} + (SUP \times BW)_{jl} + (SUP \times BW \times P)_{ijl} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the dependent variable; μ is the overall mean; SUP_i is the fixed effect of supplementation level (CON, RUP30, RUP48, and RUP66); BW_j is the fixed effect of initial BW categories (light, medium, and heavy); $SUP \times BW_{ij}$ is the fixed effect of the interaction between supplementation level and initial BW categories; δ_{ijk} is the random error representing the variance between animals within treatments, which is equal to the covariance between repeated measurements within animals; P_l is the fixed effect of sampling period; $SUP \times P_{il}$ is the fixed effect of the interaction between supplementation level and sampling period; $BW \times P_{jl}$ is the fixed effect of the interaction between initial BW categories and sampling period; $SUP \times BW \times P_{ijl}$ is the fixed effect of the interaction between supplementation level, initial BW categories, and sampling period; and ε_{ijklm} is the random error.

Performance variables and gene expression were analyzed using a completely randomized design. Measurements taken at baseline were included as covariates in the following model:

$$Y_{ijk} = \mu + SUP_i + BW_j + (SUP \times BW)_{ij} + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean; SUP_i is the fixed effect of supplementation level (CON, RUP30, RUP48, and RUP66), BW_j is the fixed effect of

Table 4. Nutrient intake and apparent digestibility of grazing crossbred Holstein × Gyr dairy heifers (n = 7) that were either not supplemented or supplemented with different levels of RUP and with different initial BW

	Treatment[1]					BW[2]				Probability[3]				
Item[4]	CON	RUP30	RUP48	RUP66	SEM	L	M	H	SEM	S	L	Q	B	S × B
Intake, DM basis														
Supplement DM,[5] kg/d	—	1.28	1.42	1.41	0.037	1.09[b]	1.47[a]	1.53[a]	0.039	—	0.02	0.09	<0.01	0.43
Pasture DM, kg/d	5.69	5.43	5.80	5.06	0.215	5.02[b]	5.69[a]	5.78[a]	0.161	0.29	0.23	0.03	<0.01	0.96
DM, kg/d	5.69	6.17	7.23	6.47	0.224	5.84[b]	6.80[a]	6.93[a]	0.168	<0.01	0.45	0.02	<0.01	0.82
Pasture, g/kg BW	18.7	17.9	18.1	16.1	0.77	20.1[a]	17.2[b]	15.8[b]	0.58	0.12	0.10	0.25	<0.01	0.59
DM, g/kg BW	18.7	21.9	22.5	20.5	0.76	23.3[a]	20.5[b]	19.0[b]	0.57	<0.01	0.19	0.19	<0.01	0.54
OM, kg/d	5.18	6.02	7.12	6.18	0.293	4.86[b]	6.62[a]	6.91[a]	0.347	<0.01	0.70	<0.01	<0.01	0.11
CP, kg/d	0.74	1.03	1.10	1.01	0.032	0.86[b]	1.01[a]	1.04[a]	0.023	<0.01	0.72	0.04	<0.01	0.49
NDF, kg/d	4.02	3.98	4.28	3.76	0.152	3.65[b]	4.16[a]	4.22[a]	0.11	0.95	0.29	0.03	<0.01	0.95
RDP, g/d	338	401	495	442	33.2	386	411	459	41.7	0.01	0.37	0.09	0.24	0.48
RUP, g/d	387	631	608	573	45.3	467[b]	602[a]	580[ab]	34.5	<0.01	0.37	0.92	0.03	0.87
MP, g/d	532	769	812	750	26.8	629[b]	753[a]	767[a]	20.4	<0.01	0.62	0.13	<0.01	0.81
CP:DOM, g CP/kg DOM	220	302	238	269	19.9	325[a]	224[b]	223[b]	14.9	0.03	0.25	0.05	<0.01	0.09
Apparent digestibility, g/kg														
DM	575	666	611	707	4.88	626[b]	649[a]	644[a]	4.28	<0.01	0.37	0.75	<0.01	0.11
OM	652	658	692	687	10.65	622[b]	696[a]	698[a]	8.37	0.04	0.07	0.15	<0.01	0.21
CP	682	744	725	735	7.59	710[b]	714[ab]	740[a]	5.78	<0.01	0.41	0.13	<0.01	0.64
NDF	723	719	722	735	4.01	719	728	727	3.10	0.69	<0.01	0.34	0.14	0.34

[a,b]Different letters within a row indicate significant differences among initial BW categories according to Tukey's test at the 0.05 significance level.

[1]CON = control; RUP30 = supplement with 30% of RUP; RUP48 = supplement with 48% of RUP; RUP66 = supplement with 66% of RUP.

[2]Body weight categories: L = light, M = medium, and H = heavy.

[3]Probability of treatment effects: S = effect of supplement versus nonsupplemented animals; L = linear effect of RUP levels; Q = quadratic effect of RUP levels; B = body weight categories effect; S × B = interaction between supplementation level and initial BW categories.

[4]DOM = digestible OM.

[5]Supplement allowance was standardized across treatments at the beginning of the study; however, actual supplement intake differed among RUP treatments.

initial BW categories (light, medium, and heavy), SUP × BW_{ij} is the fixed effect of the interaction between SUP level and initial BW categories, and ε_{ijk} is the random error. The interaction effect of treatment and block was tested for all variables, and because it was not significant, it was removed from the model.

For all models, residuals were tested for normality (Shapiro and Wilk, 1965). For gene expression data, we used a log-normal distribution, and means were back-transformed for presentation in the text. For all analyses, a significance level of 0.05 was used to determine the type I error. The main effects of supplementation were decomposed by preplanned orthogonal contrasts to test (1) CON versus the mean of supplemented animals, (2) the linear effect of increasing RUP levels (30%, 48%, and 66% of CP in the supplement), and (3) the quadratic effect of RUP levels. Contrast coefficients were generated to ensure orthogonality and were tested within the GLIMMIX framework. When appropriate, differences among initial BW category means were compared using Tukey's test at a significance level of 0.05.

The number of experimental units per treatment (n = 7) was determined by a power analysis using ADG as the primary variable, assuming an α level of 0.05, a power of 0.95, a CV of 7% (mean of 480 g/d and SD of 24.5 g/d), and an expected difference of 13.5%. For liver gene expression, the number of 5 experimental units per treatment was determined using CPS1 expression as the primary variable, with an α level of 0.05, a power of 0.90 a coefficient of variation of 8% (mean of 3 and SD of 0.24), and an expected difference of 16% (Ryan, 2013).

RESULTS

Intake and Digestibility

No interactions between treatment and period or treatment and BW were detected for intake variables ($P > 0.05$; Table 4). Intake and apparent digestibility increased over the experimental periods ($P < 0.01$), except for RDP intake ($P = 0.08$). Body weight class affected all intake variables (except RDP) and digestibility (except NDF), with heavy heifers showing greater intake and digestibility compared with medium and light animals (Table 4). An interaction between treatment and BW was observed for OM digestibility ($P < 0.05$), showing a quadratic effect for medium heifers and a linear increase for heavy heifers.

Supplemented animals had greater intake and digestibility than CON animals ($P < 0.01$), except for pasture intake and NDF intake and digestibility (Table 4). Supplement intake increased linearly with dietary RUP content (1.28, 1.42, and 1.41 kg/d for RUP30, RUP48, and RUP66, re-

Table 5. Performance and body measurements of grazing crossbred Holstein × Gyr dairy heifers (n = 7) that were either not supplemented or supplemented with different levels of RUP and with different initial BW

Item[4]	Treatment[1]					BW[2]				Probability[3]				
	CON	RUP30	RUP48	RUP66	SEM	L	M	H	SEM	S	L	Q	B	S × B
Initial BW, kg	290	287	288	288	10.5	238^{b}	306^{a}	344^{a}	10.2	0.87	0.95	0.98	<0.01	0.99
Final BW, kg	304	316	332	320	5.09	261^{b}	345^{a}	373^{a}	4.5	<0.01	0.52	<0.01	0.03	0.34
ADG, kg/d	0.18	0.33	0.52	0.37	0.04	0.26^{b}	0.45^{a}	0.34^{a}	0.045	<0.01	0.52	<0.01	0.03	0.34
BCSi, points	3.12	3.16	3.23	3.16	0.69	3.08	3.12	3.31	0.50	0.93	0.99	0.93	0.95	0.99
BCSf, points	3.00	3.12	3.08	3.22	0.67	2.97	3.09	3.25	0.49	0.85	0.92	0.91	0.94	0.99
Body measurement, cm														
Withers height	125.8	125.1	126.5	125.6	0.74	124.3	126.7	126.3	0.63	0.91	0.71	0.21	0.08	0.89
Thoracic perimeter	158.7	163.7	168.7	165.8	1.82	162.2	165.5	165.0	1.80	<0.01	0.43	0.09	0.56	0.21
Body length	111.5	114.8	112.8	113.9	1.63	110.1	115.1	114.6	1.54	0.23	0.69	0.43	0.13	0.50
Rump height	128.1	129.0	130.5	130.4	0.35	129.4^{ab}	130.6^{a}	128.6^{b}	0.30	<0.01	0.01	0.09	<0.01	0.17

^{a,b}Different letters within a row indicate significant differences among initial BW categories according to Tukey's test at the 0.05 significance level.

[1]CON = control; RUP30 = supplement with 30% of RUP; RUP48 = supplement with 48% of RUP; RUP66 = supplement with 66% of RUP.

[2]Body weight categories: L = light, M = medium, and H = heavy.

[3]Probability of treatment effects: S = effect of supplement versus nonsupplemented animals; L = linear effect of RUP levels; Q = quadratic effect of RUP levels; B = body weight categories effect; S × B = interaction between supplementation level and initial BW categories.

[4]BCSi = initial BCS; and BCSf = final BCS.

spectively; $P < 0.01$). Pasture DMI showed a quadratic response ($P = 0.03$), with the highest intake in RUP48 (5.80 kg/d) compared with RUP30 (5.43 kg/d) and RUP66 (5.06 kg/d). A similar quadratic pattern was observed for total DMI and several nutrient intakes. Digestibility of NDF increased linearly with RUP level ($P < 0.01$).

Performance and Body Measurements

Initial BW did not differ among treatments ($P > 0.05$; Table 5). Supplemented heifers had greater final BW (316–332 vs. 304 kg; $P < 0.01$) and ADG (0.33–0.52 vs. 0.18 kg/d; $P < 0.01$) than CON animals. Quadratic effects of RUP were observed for final BW and ADG ($P < 0.01$), with the greatest values in RUP48 (332 kg and 0.52 kg/d). Thoracic perimeter and rump height also increased with supplementation ($P < 0.05$). Rump height increased linearly with RUP level ($P = 0.01$), with the highest values in RUP48 and RUP66.

Nitrogen Balance and Microbial Protein Synthesis

All N balance variables were affected by period ($P < 0.05$), except for efficiency of N use (Table 6). Supplemented animals had greater N intake (165–176 vs. 116 g/d; $P < 0.01$), fecal and urinary N excretion, and N retention (26–47 vs. 19 g/d; $P < 0.05$) compared with CON heifers. Efficiency of N use did not differ between supplemented and nonsupplemented groups.

Microbial protein synthesis was greater in supplemented heifers (401–495 vs. 338 g/d; $P < 0.05$). Nitrogen intake and fecal N excretion showed quadratic responses to RUP level ($P < 0.05$), with the highest values in the RUP48 treatment.

Mammary Gland Ultrasound, Blood Analysis, and Reproductive Assessment

Glucose (61.8–68.3 vs. 60.6 mg/dL; $P < 0.05$), serum urea (27.6–44.2 vs. 15.4 mg/dL; $P < 0.01$), and IGF-1 (176–202 vs. 119 ng/mL; $P < 0.01$) were greater in supplemented heifers than in CON heifers (Table 7). Increasing RUP level linearly decreased serum urea ($P < 0.01$) and quadratically affected total protein ($P = 0.05$), with the highest concentration observed in the RUP48 treatment (6.25 g/dL).

Reproductive traits were improved by supplementation, horn diameter (1.24–1.34 vs. 1.17 cm; $P = 0.03$), uterine tone (2.35–2.77 vs. 2.05; $P < 0.01$), and reproductive tract score (1.97–2.15 vs. 1.60; $P = 0.03$). Uterine tone increased linearly with RUP level ($P < 0.01$).

Gene Expression

Nonsupplemented heifers had higher hepatic expression of IGF-1, IGFBP3, and GOT1 compared with supplemented animals (Table 8). Among supplemented heifers, GOT1 expression showed a quadratic response ($P < 0.01$), with the highest value observed in the RUP30 treatment (0.08). A similar pattern was observed for IGFBP3 ($P < 0.01$), with the greatest value in RUP30 (0.40). In contrast, IGF-1 and CPS1 expression decreased linearly with increasing dietary RUP ($P < 0.05$).

DISCUSSION

Supplementary feeding is crucial for optimal heifer production in tropical grazing systems. Previous studies have indicated that under these conditions, a CP level of

Table 6. Nitrogen balance and microbial protein synthesis of grazing crossbred Holstein × Gyr dairy heifers (n = 7) that were either not supplemented or supplemented with different levels of RUP and with different initial BW

Item	Treatment[1]					BW[2]				Probability[3]				
	CON	RUP30	RUP48	RUP66	SEM	L	M	H	SEM	S	L	Q	B	S × B
Nitrogen balance, g/d														
Intake	116	165	176	162	5.3	136[a]	162[b]	166[b]	4.07	<0.01	0.72	0.04	<0.01	0.73
Fecal excretion	35.5	40.9	46.8	41.4	1.66	39.2	43.9	40.4	1.26	<0.01	0.81	0.01	0.08	0.50
Urinary excretion	60.0	88.9	75.2	84.8	6.89	60.0[b]	80.5[a]	91.1[a]	4.53	<0.01	0.67	0.17	<0.01	0.37
Urinary urea, mg/d	176	217	217	201	11.1	193	198	216	8.4	0.02	0.32	0.59	0.27	0.15
Retained	18.5	26.4	47.0	32.8	8.11	34.1	28.4	31.1	5.34	0.03	0.57	0.08	0.82	0.15
Efficiency of nitrogen use, g/g	0.15	0.15	0.28	0.17	0.053	0.23	0.16	0.17	0.034	0.42	0.81	0.06	0.41	0.17
Microbial synthesis														
Microbial protein, g/d	338	401	495	442	33.2	386	411	459	25.3	0.01	0.37	0.09	0.24	0.48
Microbial efficiency, g/kg DOM	108	120	113	134	18.0	156[a]	99[b]	101[ab]	13.7	0.56	0.56	0.55	0.01	0.44
N use for microbial synthesis, g/g	0.47	0.40	0.46	0.46	0.038	0.47	0.43	0.45	0.029	0.62	0.28	0.52	0.67	0.64

[a,b]Different letters within a row indicate significant differences among initial BW categories according to Tukey's test at the 0.05 significance level.

[1]CON = control; RUP30 = supplement with 30% of RUP; RUP48 = supplement with 48% of RUP; RUP66 = supplement with 66% of RUP.

[2]Body weight categories: L = light, M = medium, and H = heavy.

[3]Probability of treatment effects: S = effect of supplement versus nonsupplemented animals; L = linear effect of RUP levels; Q = quadratic effect of RUP levels; B = body weight categories effect; S × B = interaction between supplementation level and initial BW categories.

~24% in the supplement is suitable for Holstein × Gyr heifers grazing on *Megathyrsus maximus* cv. Mombaça pastures. For feedlot heifers, enhancing RUP content in their diet can boost performance, as demonstrated by Silva et al. (2018a). Consequently, this study was conducted to investigate the effects of RUP content in dietary supplements on the performance and nitrogen metabolism of grazing heifers.

Supplementation Effects

As expected from the experimental design, pasture DMI (expressed in kg/d and g/kg BW) was lower in supplemented than in CON heifers, with reductions of 4.7 and 6.6%, respectively. Conversely, total DMI increased by 16.6% (kg/d) and 15.4% (g/kg BW) in supplemented animals compared with the CON group, indicating a substitution effect in which the supplement partially replaced forage intake but increased overall nutrient consumption. This greater total intake likely enhanced ruminal microbial growth, improving DM digestibility and, consequently, animal performance and N retention. Similar substitution effects were reported by Machado et al. (2020) in grazing Holstein × Gyr heifers supplemented with 12% or 24% CP.

Pasture DMI should be interpreted with caution, as values were not directly measured but derived from estimated fecal output and an internal marker (iNDF). Although the double-marker approach combining TiO_2 and iNDF is widely validated and remains one of the most reliable methods for grazing cattle, small deviations in estimated fecal excretion may influence the absolute magnitude of intake.

Nutrient digestibility can be influenced by factors such as intake level, supplement composition, and pasture quality and availability (Castro et al., 2023). In the present study, supplementation improved the digestibility of several nutrients, likely due to the inclusion of readily fermentable ingredients that stimulate the growth of nonfibrolytic bacteria and enhance overall nutrient utilization (Camargo et al., 2022). The combination of these ingredients with nutrients from high-quality pasture also ensured adequate N supply for fibrolytic microorganisms, preventing competition among microbial groups. Consequently, NDF digestibility did not differ between supplemented and nonsupplemented heifers, consistent with the fact that a forage CP concentration above 100 g/kg DM is sufficient to sustain fibrolytic activity (Detmann et al., 2014b; Lazzarini et al., 2016; de Oliveira Franco et al., 2017).

Animal performance in grazing systems depends on feed intake, digestibility, and forage availability and quality (Camargo et al., 2022; Castro et al., 2023). Consistent with the present results, several studies have reported higher growth rates in supplemented grazing heifers. Castro et al. (2023) observed that increasing supplement CP levels (12%–36%) improved ADG and body measurements of Holstein × Gyr heifers grazing *Urochloa decumbens*. Similarly, Machado et al. (2019) found that protein supplementation (24% CP at 0.5% BW) on *Megathyrsus maximus* cv. Mombaça pastures increased ADG compared with both nonsupplemented and energy-supplemented groups.

Overall, protein supplementation consistently improves ADG in grazing heifers. The results from Machado et al. (2019) and the present study emphasize the importance of protein supplementation even when animals graze high-quality forage with relatively high CP content. During the rainy season, the high digestibility of DM and

Table 7. Blood metabolites, mammary gland development, and reproductive traits of grazing crossbred Holstein × Gyr dairy heifers (n = 7) that were either not supplemented or supplemented with different levels of RUP and with different initial BW

	Treatment[1]					BW[2]				Probability[3]				
Item	CON	RUP30	RUP48	RUP66	SEM	L	M	H	SEM	S	L	Q	B	S × B
Mammary gland pixel value														
Log of pixels, mm[2]	39.4	38.5	34.2	38.1	2.59	39.5	39.6	33.4	2.13	0.43	0.91	0.23	0.13	0.96
Blood parameter														
Total protein, g/dL	6.22	5.69	6.25	6.05	0.149	5.90	6.04	6.20	0.134	0.25	0.12	0.05	0.30	0.11
Glucose, mg/dL	60.6	66.5	68.3	61.8	2.07	62.3	65.3	65.3	1.62	0.04	0.13	0.11	0.40	0.55
Albumin, g/dL	2.54	2.48	2.59	2.59	0.06	2.56	2.55	2.55	0.052	0.80	0.21	0.50	0.99	0.29
Urea, mg/dL	15.4	44.2	36.6	27.6	1.53	27.6[b]	34.4[a]	30.9[ab]	1.24	<0.01	<0.01	0.77	<0.01	0.27
IGF-1, ng/mL	119	176	202	191	17.5	154	162	201	14.4	<0.01	0.55	0.41	0.20	0.07
Reproductive evaluation														
Average horn diameter, cm	1.17	1.24	1.28	1.34	0.045	1.16[b]	1.29[ab]	1.32[a]	0.039	0.03	0.13	0.90	0.01	0.58
Tonus[4]	2.05	2.35	2.60	2.77	0.145	1.95[a]	2.33[b]	3.05[c]	0.098	<0.01	0.03	0.81	<0.01	0.69
Reproductive tract score[5]	1.60	1.97	1.91	2.15	0.22	1.51[b]	1.93[ab]	2.29[a]	0.12	0.03	0.54	0.48	0.01	0.90

[a–c]Different letters within a row indicate significant differences among initial BW categories according to Tukey's test at the 0.05 significance level.

[1]CON = control; RUP30 = supplement with 30% of RUP; RUP48 = supplement with 48% of RUP; RUP66 = supplement with 66% of RUP.

[2]Body weight categories: L = light, M = medium, and H = heavy.

[3]Probability of treatment effects: S = effect of supplement versus nonsupplemented animals; L = linear effect of RUP levels; Q = quadratic effect of RUP levels; B = body weight categories effect; S × B = interaction between supplementation level and initial BW categories.

[4]Scored from 1 to 5.

[5]Scored from 1 to 3.

NDF increases energy supply, which can create an imbalance between energy and protein availability. Under these conditions, protein supplementation helps correct this imbalance and supports additional daily gains of ~0.2 kg/d (Detmann et al., 2014b).

The greater urinary and fecal N excretion observed in supplemented heifers resulted from their higher CP intake from both pasture and supplement. In the rumen, dietary protein and urea are hydrolyzed to ammonia, part of which is incorporated into microbial protein, with excess being absorbed, converted to urea in the liver, and excreted via urine or feces (Reynolds and Kristensen, 2008; Hailemariam et al., 2021). Consequently, higher CP intake likely increased ruminal ammoniacal N concentration and N excretion, consistent with reports by Camargo et al. (2022). Despite greater N losses, N retention also increased in supplemented heifers, indicating improved overall N status and utilization efficiency (Detmann et al., 2014b; Lazzarini et al., 2016). The higher serum urea concentration in supplemented animals reflects this elevated N turnover and is directly related to the increased CP intake and ruminal ammonia production (Detmann et al., 2014b; Camargo et al., 2022). The interpretation of N balance and microbial protein synthesis values should be made with caution because these variables were derived from estimated rather than directly measured urinary N excretion. Although the approach based on creatinine excretion and purine derivatives is well validated for grazing cattle, small deviations in predicted urinary volume may influence the final N balance calculations. Therefore, the values presented should be regarded as relative indicators of treatment effects rather than absolute measures of biological efficiency.

The greater MP intake of supplemented heifers likely enhanced IGF-1 activity, leading to higher circulating IGF-1 concentrations (Castro et al., 2023). Protein supplementation reduces myofibrillar protein degradation that would otherwise supply N for recycling, thereby increasing N retention and growth (Batista et al., 2016; de Oliveira Franco et al., 2017). Supplementary feeding also provides additional fermentable substrates that promote rumen microbial development and improve feed degradability and utilization (de Oliveira Franco et al., 2017). The higher microbial protein synthesis observed in supplemented heifers supports this improved rumen environment, in which N compounds and readily available energy act synergistically to enhance nutrient use efficiency (Souza et al., 2010).

The higher protein-to-energy ratio consumed by supplemented heifers likely favored mammary parenchyma development compared with the CON group. The increase in circulating IGF-1 concentrations further supports the existence of a more favorable hormonal environment for mammary growth (Thissen et al., 1994; Albino et al., 2017). Improved nutrient intake, performance, and N retention in supplemented animals also contributed to better reproductive traits. Under nutritional imbalance, reproduction is one of the first functions to be compromised (Valentim et al., 2019). Adequate metabolic homeostasis is required for the synthesis of hormones regulating GnRH release, such as leptin, IGF-1, and ghrelin (D'Occhio et al., 2019). Therefore, protein supplementa-

Table 8. Relative hepatic gene expression in grazing crossbred Holstein × Gyr dairy heifers (n = 5) that were either not supplemented or supplemented with different levels of rumen RUP and with different initial BW

Item[4]	Treatment[1]				SEM	BW[2]			SEM	Probability[3]				
	CON	RUP30	RUP48	RUP66		L	M	H		S	L	Q	B	S × B
IGFBP3	0.33	0.40	0.03	0.04	0.002	0.20	0.20	0.19	0.001	<0.01	<0.01	<0.01	0.85	0.65
IGF1	0.06	0.04	0.02	0.02	0.001	0.05	0.03	0.03	0.001	0.04	0.03	0.41	0.13	0.78
CPS1	2.61	4.22	2.72	1.53	0.451	2.44	3.19	2.35	0.322	0.22	<0.01	0.35	0.06	0.10
GOT1	0.09	0.08	0.02	0.01	0.008	0.05	0.05	0.05	0.005	<0.01	<0.01	<0.01	0.62	0.45

[1]CON = control; RUP30 = supplement with 30% of RUP; RUP48 = supplement with 48% of RUP; RUP66 = supplement with 66% of RUP.

[2]Body weight categories: L = light, M = medium, and H = heavy.

[3]Probability of treatment effects: S = effect of supplement versus nonsupplemented animals; L = linear effect of RUP levels; Q = quadratic effect of RUP levels; B = body weight categories effect; S × B = interaction between supplementation level and initial BW categories.

[4]*IGFBP3* = insulin-like growth factor-binding protein 3; *IGF1* = insulin-like growth factor; *CPS1* = carbamoyl phosphate synthetase; *GOT1* = glutamic transaminase-oxaloacetic (aspartate aminotransferase).

tion likely supported the reproductive development of the heifers; however, these findings should be interpreted with caution given the smaller number of replicates for reproductive measurements.

The lower hepatic expression of GOT1 in supplemented heifers suggests reduced hepatic N turnover, as this enzyme participates in the transamination reactions that channel ammonia into the urea cycle (Kozloski, 2021). Conversely, the higher GOT1 expression in nonsupplemented heifers likely reflects increased hepatic N metabolism associated with less efficient ruminal N utilization and greater urea synthesis (Castro et al., 2023). Although circulating IGF-1 is primarily regulated by hepatic synthesis, the lower hepatic IGF-1 expression observed in supplemented heifers may indicate feedback regulation due to greater nutrient and energy availability. In contrast, the higher IGF-1 expression in CON heifers may represent a compensatory response to lower nutrient intake, as previously described in nutrient-restricted cattle (Radcliff et al., 2004).

Rumen-Undegradable Protein Level Effects

It is important to highlight that the RUP content of the supplements was estimated by a single 16-h in situ incubation, a simplified method that is widely applied in feed evaluation but may lack the absolute accuracy of estimated values, when compared with multiple incubation times and nonlinear modeling of degradation kinetics.

The greater performance of heifers fed RUP48 was associated with their higher intake of supplement, pasture, total DM, CP, and NDF. Similar increases in nutrient intake have been reported when protein sources with higher rumen escape, such as heat-treated soybean meal or corn gluten, were fed to grazing cattle (Camargo et al., 2022).

The DMI is closely related to diet digestibility (Detmann et al., 2014b); thus, improved nutrient availability supports the growth of rumen microorganisms, particularly fibrolytic species, enhancing fiber digestion and feed intake (Rufino et al., 2020). The higher DMI in RUP48 likely reflects a more balanced nutrient supply, combining available energy from the supplement with N from both pasture and supplement to sustain microbial activity. The inclusion of RUP also ensured sufficient amino acid flow to the small intestine while maintaining adequate RDP for ruminal fermentation (Berchielli et al., 2011; Paengkoum et al., 2019). This balanced rumen environment improved nutrient use efficiency and increased digesta passage rate, resulting in greater total nutrient absorption and animal performance.

Camargo et al. (2022) evaluated different RUP sources, including protected soybean meal and corn gluten meal, and reported greater final BW and ADG in supplemented animals compared with those receiving only a mineral supplement. In that study, heifers fed protected soybean meal also showed higher N retention, indicating more efficient MP utilization. Although experimental conditions and RUP sources differed from the present trial, their results similarly highlight the performance benefits of RUP supplementation in grazing cattle.

The greater fecal N excretion observed in the RUP48 treatment compared with RUP30 and RUP66 likely resulted from its higher total CP intake, combining N from both supplement and pasture. This increased N supply may have elevated ruminal ammonia production and urea recycling, as indicated by the positive association between urinary and plasma urea concentrations (Camargo et al., 2022).

The higher CP intake in RUP48 heifers also contributed to their greater serum total protein concentration. Total protein, which reflects both albumin and globulin fractions, is a useful indicator of overall protein status (González, 2000). The mean values observed in RUP48 were within the normal physiological range for cattle (6.6–7.5 g/dL), suggesting that this treatment provided a balanced N supply, avoiding both deficiency and excess.

In contrast, Castro et al. (2023) reported slightly higher total protein concentrations when testing diets with up to 36% CP, reinforcing that matching RUP supplementation to pasture quality helps regulate RDP and NPN availability and prevents excess circulating protein.

The positive reproductive response to RUP supplementation may be related to improved energy efficiency associated with optimal RDP availability. When RDP is supplied in excess or inefficiently utilized, additional energy is required for its excretion, which can impair reproductive function (Tessari et al., 2022). Adequate RUP intake also supports hepatic IGF-1 synthesis, which stimulates GnRH secretion and subsequently the release of gonadotropic hormones, promoting reproductive tract development (D'Occhio et al., 2019).

The reduction in hepatic GOT1 expression with increasing RUP levels suggests a lower need for hepatic ammonia detoxification, as greater RUP supply reduced the availability of excess RDP in the rumen. This indicates a more efficient use of dietary protein, minimizing the conversion of ammonia to urea and, consequently, the energy cost of hepatic N metabolism (Berchielli et al., 2011; Kozloski, 2021). Excess RDP can increase ruminal ammonia production and urea recycling, an energetically demanding process that may limit resources available for growth.

Increasing RUP levels also decreased hepatic expression of IGF-1 and IGFBP3. This response may reflect a metabolic adjustment to higher amino acid availability, leading to reduced hepatic synthesis due to feedback regulation or redistribution of IGF-1 production to extrahepatic tissues, such as the ovaries (Radcliff et al., 2004).

CONCLUSIONS

Supplementation of grazing dairy heifers improved feed intake, nutrient digestibility, and overall performance. Among the evaluated treatments, a supplement containing 48% RUP in total CP provided the most favorable balance between rumen degradable and undegradable protein. Under the conditions of this study (crossbred Holstein × Gyr heifers grazing on *Megathyrsus maximus* cv. BRS Quênia pastures averaging 14% CP), this level optimized intake, growth performance, and nitrogen utilization.

NOTES

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Nonstandard abbreviations used: BCSf = final BCS; BCSi = initial BCS; CON = control; DOM = digestible OM; iBW = initial BW; max. = maximum; min. = minimum; NDFi = indigestible NDF; precip = precipitation; RUP30 = supplementation with 30% of the supplement CP as RUP; RUP48 = supplementation with 48% of the supplement CP as RUP; RUP66 = supplementation with 66% of the supplement CP as RUP.

REFERENCES

Abreu, M. B., C. S. Cunha, J. H. C. Costa, E. K. Miller-Cushon, P. P. Rotta, A. F. Machado, V. C. L. Morais, F. H. M. Chizzotti, and M. I. Marcondes. 2022. Performance and feeding behavior of Holstein and Holstein × Gyr crossbred heifers grazing temperate forages. Trop. Anim. Health Prod. 54:100. https://doi.org/10.1007/s11250-022-03106-w.

Albino, R. L., S. E. F. Guimarães, K. M. Daniels, M. M. S. Fontes, A. F. Machado, G. B. dos Santos, and M. I. Marcondes. 2017a. Technical note: Mammary gland ultrasonography to evaluate mammary parenchymal composition in prepubertal heifers. J. Dairy Sci. 100:1588–1591. https://doi.org/10.3168/jds.2016-11668.

Albino, R. L., M. I. Marcondes, R. M. Akers, E. Detmann, B. C. Carvalho, and T. E. Silva. 2015. Mammary gland development of dairy heifers fed diets containing increasing levels of metabolisable protein:metabolisable energy. J. Dairy Res. 82:113–120. https://doi.org/10.1017/S0022029914000697.

Barbero, R. P., E. B. Malheiros, T. L. R. Araújo, R. L. G. Nave, J. T. Mulliniks, T. T. Berchielli, A. C. Ruggieri, and R. A. Reis. 2015. Combining Marandu grass grazing height and supplementation level to optimize growth and productivity of yearling bulls. Anim. Feed Sci. Technol. 209:110–118. https://doi.org/10.1016/j.anifeedsci.2015.09.010.

Barbosa, A. M., R. F. D. Valadares, S. C. Valadares Filho, D. S. Pina, E. Detmann, and M. I. Leão. 2011. Endogenous fraction and urinary recovery of purine derivatives obtained by different methods in Nellore cattle. J. Anim. Sci. 89:510–519. https://doi.org/10.2527/jas.2009-2366.

Bascom, S. A., R. E. James, M. L. McGilliard, and M. Van Amburgh. 2007. Influence of dietary fat and protein on body composition of jersey bull calves. J. Dairy Sci. 90:5600–5609. https://doi.org/10.3168/jds.2007-0004.

Batista, E. D., E. Detmann, E. C. Titgemeyer, S. C. Valadares Filho, R. F. D. Valadares, L. L. Prates, L. N. Rennó, and M. F. Paulino. 2016. Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage. J. Anim. Sci. 94:201–216. https://doi.org/10.2527/jas.2015-9493.

Berchielli, T. T., A. V. Pires, and S. G. Oliveira. 2011. Nutrição de Ruminantes. 2nd ed. Editora Funep, Jaboticabal, Brazil.

Bethard, G. L., R. E. James, and M. L. Mcgilliard. 1997. Effect of rumen-undegradable protein and energy on growth and feed efficiency of growing Holstein heifers. J. Dairy Sci. 80:2149–2155. https://doi.org/10.3168/jds.S0022-0302(97)76161-7.

Calsamiglia, S., and M. D. Stern. 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. J. Anim. Sci. 73:1459–1465. https://doi.org/10.2527/1995.7351459x.

Camargo, K. D. V., J. D. Messana, L. G. Silva, Y. T. Granja-Salcedo, A. V. L. Dias, K. L. G. C. Alves, P. H. Gonçalves, W. A. Souza, R. A. Reis, and T. T. Berchielli. 2022. Intake, metabolism parameters, and performance of growing beef cattle on pasture supplemented with different rumen undegradable protein with different amino acid profile. Anim. Feed Sci. Technol. 286:115258. https://doi.org/10.1016/j.anifeedsci.2022.115258.

Castro, M. M. D., T. J. DeVries, A. F. Machado, M. M. Ferreira, L. N. Rennó, and M. I. Marcondes. 2023. Metabolic responses and performance of Holstein × Gyr heifers grazing *Brachiaria decumbens* supplemented with varied crude protein levels. PLoS One 18:e0289747. https://doi.org/10.1371/journal.pone.0289747.

Chen, X. B., and M. J. Gomes. 1992. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives: An overview of technical details. International Feed Resources Unit, Rowett Research Institute, Bucksburn Aberdeen, Scotland.

Chizzotti, M. L., S. C. Valadares Filho, R. F. D. Valadares, F. H. M. Chizzotti, and L. O. Tedeschi. 2008. Determination of creatinine excretion and evaluation of spot urine sampling in Holstein cattle. Livest. Sci. 113:218–225. https://doi.org/10.1016/j.livsci.2007.03.013.

de Oliveira Franco, M., E. Detmann, S. de Campos Valadares Filho, E. D. Batista, L. M. de Almeida Rufino, M. Medrado Barbosa, and A. R. Lopes. 2017. Intake, digestibility, and rumen and metabolic characteristics of cattle fed low-quality tropical forage and supplemented with nitrogen and different levels of starch. Asian-Australas. J. Anim. Sci. 30:797–803. https://doi.org/10.5713/ajas.16.0629.

D'Occhio, M. J., P. S. Baruselli, and G. Campanile. 2019. Influence of nutrition, body condition, and metabolic status on reproduction in female beef cattle: A review. Theriogenology 125:277–284. https://doi.org/10.1016/j.theriogenology.2018.11.010.

Detmann, E., M. F. Paulino, S. D. C. Valadares Filh, and P. Huhtanen. 2014a. Nutritional aspects applied to grazing cattle in the tropics: A review based on Brazilian results. Semin. Cienc. Agrar. 35:2829. https://doi.org/10.5433/1679-0359.2014v35n4Suplp2829.

Detmann, E., J. P. P. Rodrigues, T. E. D. Silva, A. S. B. Neto, and M. de O. Franco. 2025. Methods for Feed Analysis. 3rd ed. INCT, Viçosa, Brazil.

Detmann, E., É. E. L. Valente, E. D. Batista, and P. Huhtanen. 2014b. An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. Livest. Sci. 162:141–153. https://doi.org/10.1016/j.livsci.2014.01.029.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77:2695–2703. https://doi.org/10.3168/jds.S0022-0302(94)77212-X.

Gomide, C. A. de M., J. A. Gomide, and E. Alexandrino. 2007. Características estruturais e produção de forragem em pastos de capim-mombaça submetidos a períodos de descanso. Pesqui. Agropecu. Bras. 42:1487–1494. https://doi.org/10.1590/S0100-204X2007001000017.

González, F. H. D. 2000. Indicadores sanguíneos do metabolismo mineral em ruminantes. F. H. D. González, J. O. Barcellos, H. Ospina, and L. A. O. Ribeiro, ed. Gráfica da Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Hailemariam, S., S. Zhao, Y. He, and J. Wang. 2021. Urea transport and hydrolysis in the rumen: A review. Anim. Nutr. 7:989–996. https://doi.org/10.1016/j.aninu.2021.07.002.

Hutchison, J. L., P. M. VanRaden, D. J. Null, J. B. Cole, and D. M. Bickhart. 2017. Genomic evaluation of age at first calving. J. Dairy Sci. 100:6853–6861. https://doi.org/10.3168/jds.2016-12060.

Kozloski, G. V. 2021. Bioquímica Dos Ruminantes. 3rd edição revista e ampliada. UFSM, Santa Maria, Brazil.

Lazzarini, Í., E. Detmann, S. C. V. Filho, M. F. Paulino, E. D. Batista, L. M. A. Rufino, W. L. S. Reis, and M. O. Franco. 2016. Nutritional performance of cattle grazing during rainy season with nitrogen and starch supplementation. Asian-Australas. J. Anim. Sci. 29:1120–1128. https://doi.org/10.5713/ajas.15.0514.

Lowe, J. K. II, C. N. Boyer, A. P. Griffith, J. C. Waller, G. E. Bates, P. D. Keyser, J. A. Larson, and E. Holcomb. 2016. The cost of feeding bred dairy heifers on native warm-season grasses and harvested feedstuffs. J. Dairy Sci. 99:634–643. https://doi.org/10.3168/jds.2015-9475.

Machado, A. F., S. E. F. Guimarães, J. D. Guimarães, G. M. Santos, A. L. Silva, Y. F. R. S. Silva, D. S. Lollobrigida Netto, P. V. F. Correa, and M. I. Marcondes. 2020. Effect of protein supplement level on the productive and reproductive parameters of replacement heifers managed in intensive grazing systems. PLoS One 15:e0239786. https://doi.org/10.1371/journal.pone.0239786.

Machado, W. S., V. L. N. Brandao, V. C. L. Morais, E. Detmann, P. P. Rotta, and M. I. Marcondes. 2019. Supplementation strategies affect the feed intake and performance of grazing replacement heifers. PLoS One 14:e0221651. https://doi.org/10.1371/journal.pone.0221651.

Miranda, M., J. L. Benedito, B. Gutiérrez, M. García-Vaquero, I. Blanco-Penedo, and M. Ló Pez-Alonso. 2010. The interlobular distribution of copper in the liver of beef calves on a high-copper diet. J. Vet. Diagn. Invest. 22:277–281. https://doi.org/10.1177/104063871002200221.

Mølgaard, L., B. M. Damgaard, V. Bjerre-Harpøth, and M. S. Herskin. 2012. Effects of percutaneous needle liver biopsy on dairy cow behaviour. Res. Vet. Sci. 93:1248–1254. https://doi.org/10.1016/j.rvsc.2012.04.001.

NASEM (National Academies of Sciences, Engineering, and Medicine). 2021. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition. The National Academies Press, Washington, DC.

Nishimura, M., T. Yoshida, S. El-Khodery, M. Miyoshi, H. Furuoka, J. Yasuda, and K. Miyahara. 2011. Ultrasound imaging of mammary glands in dairy heifers at different stages of growth. J. Vet. Med. Sci. 73:19–24. https://doi.org/10.1292/jvms.09-0503.

Otto, P. I., S. E. F. Guimarães, L. L. Verardo, A. L. S. Azevedo, J. Vandenplas, A. C. C. Soares, C. A. Sevillano, R. Veroneze, M. de Fatima A. Pires, C. de Freitas, M. C. A. Prata, J. Furlong, R. S. Verneque, M. F. Martins, J. C. C. Panetto, W. A. Carvalho, D. O. R. Gobo, M. V. G. B. da Silva, and M. A. Machado. 2018. Genome-wide association studies for tick resistance in *Bos taurus* × *Bos indicus* crossbred cattle: A deeper look into this intricate mechanism. J. Dairy Sci. 101:11020–11032. https://doi.org/10.3168/jds.2017-14223.

Paz, H. A., T. J. Klopfenstein, D. Hostetler, S. C. Fernando, E. Castillo-Lopez, and P. J. Kononoff. 2014. Ruminal degradation and intestinal digestibility of protein and amino acids in high-protein feedstuffs commonly used in dairy diets. J. Dairy Sci. 97:6485–6498. https://doi.org/10.3168/jds.2014-8108.

Paengkoum, P., S. Chen, and S. Paengkoum. 2019. Effects of crude protein and undegradable intake protein on growth performance, nutrient utilization, and rumen fermentation in growing Thai-indigenous beef cattle. Trop. Anim. Health Prod. 51:1151–1159. https://doi.org/10.1007/s11250-019-01799-0.

Piantoni, P., K. M. Daniels, R. E. Everts, S. L. Rodriguez-Zas, H. A. Lewin, W. L. Hurley, R. M. Akers, and J. J. Loor. 2012. Level of nutrient intake affects mammary gland gene expression profiles in preweaned Holstein heifers. J. Dairy Sci. 95:2550–2561. https://doi.org/10.3168/jds.2011-4539.

Prates, L. L., R. F. D. Valadares, S. C. Valadares Filho, E. Detmann, S. A. Santos, J. M. S. Braga, S. G. Pellizzoni, and K. S. Barbosa. 2012. Endogenous fraction and urinary recovery of purine derivatives in Nellore and Holstein heifers with abomasal purine infusion. Livest. Sci. 150:179–186. https://doi.org/10.1016/j.livsci.2012.08.018.

Quirino, D. F., M. I. Marcondes, L. N. Rennó, P. V. F. Correa, V. C. L. Morais, C. S. Cunha, T. D. A. Silva, A. L. da Silva, E. Miller-Cushon, and P. P. Rotta. 2022. Intake, performance, and feeding behavior of Holstein and Holstein × Gyr heifers grazing intensively managed tropical grasses during the rainy season. Animal 16:100613. https://doi.org/10.1016/j.animal.2022.100613.

Radcliff, R. P., M. J. VandeHaar, Y. Kobayashi, B. K. Sharma, H. A. Tucker, and M. C. Lucy. 2004. Effect of dietary energy and somatotropin on components of the somatotropic axis in Holstein heifers.

J. Dairy Sci. 87:1229–1235. https://doi.org/10.3168/jds.S0022-0302(04)73273-7.
Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim. Sci. 86(suppl_14):E293–E305. https://doi.org/10.2527/jas.2007-0475.
Rosenkrans, K. S., and D. K. Hardin. 2003. Repeatability and accuracy of reproductive tract scoring to determine pubertal status in beef heifers. Theriogenology 59:1087–1092. https://doi.org/10.1016/S0093-691X(02)01171-8.
Rufino, L. M. A., E. D. Batista, J. P. P. Rodrigues, S. C. Valadares Filho, M. F. Paulino, L. F. Costa e Silva, and E. Detmann. 2020. Effects of the amount and frequency of nitrogen supplementation on intake, digestion, and metabolism in cattle fed low-quality tropical grass. Anim. Feed Sci. Technol. 260:114367. https://doi.org/10.1016/j.anifeedsci.2019.114367.
Ryan, T. P. 2013. Sample Size Determination and Power. Wiley Series in Probability and Statistics. John Wiley & Sons Inc.
Sampaio, C. B., E. Detmann, T. N. P. Valente, V. A. C. Costa, S. D. C. Valadares Filho, and A. C. D. Queiroz. 2011. Fecal excretion patterns and short term bias of internal and external markers in a digestion assay with cattle. Rev. Bras. Zootec. 40:657–665. https://doi.org/10.1590/S1516-35982011000300026.
Shapiro, S. S., and M. B. Wilk. 1965. An Analysis of Variance Test for Normality (Complete Samples). Biometrika 52:591–611.
Silva, A. L., E. Detmann, J. Dijkstra, A. M. Pedroso, L. H. P. Silva, A. F. Machado, F. C. Sousa, G. B. dos Santos, and M. I. Marcondes. 2018a. Effects of rumen-undegradable protein on intake, performance, and mammary gland development in prepubertal and pubertal dairy heifers. J. Dairy Sci. 101:5991–6001. https://doi.org/10.3168/jds.2017-13230.
Silva, A. L., E. Detmann, L. N. Rennó, A. M. Pedroso, M. M. S. Fontes, V. C. Morais, A. L. L. Sguizzato, M. B. Abreu, P. P. Rotta, and M. I. Marcondes. 2018b. Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers. Anim. Feed Sci. Technol. 244:1–10. https://doi.org/10.1016/j.anifeedsci.2018.07.019.
Sotelo, D., M. F. Paulino, L. N. Rennó, E. Detmann, R. M. Ortega, D. C. Marquez, L. S. Martins, D. Mageste de Almeida, J. A. da Costa Lima, and F. Henrique de Moura. 2019. Performance and metabolic status of grazing beef heifers receiving increasing protein supplementation pre- and postpartum. Anim. Prod. Sci. 59:1244–1252. https://doi.org/10.1071/AN17485.
Souza, M. A., E. Detmann, M. F. Paulino, C. B. Sampaio, Í. Lazzarini, and S. C. Valadares Filho. 2010. Intake, digestibility and rumen dynamics of neutral detergent fibre in cattle fed low-quality tropical forage and supplemented with nitrogen and/or starch. Trop. Anim. Health Prod. 42:1299–1310. https://doi.org/10.1007/s11250-010-9566-6.
Stevenson, J. L., J. A. Rodrigues, F. A. Braga, S. Bitente, J. C. Dalton, J. E. P. Santos, and R. C. Chebel. 2008. Effect of breeding protocols and reproductive tract score on reproductive performance of dairy heifers and economic outcome of breeding programs. J. Dairy Sci. 91:3424–3438. https://doi.org/10.3168/jds.2007-0804.
Tessari, F. A., R. Pereira da Silva Marques, and M. Valéria de Lima Carvalhal. 2022. Suplementação proteica e nitrogenada vs. reprodução de fêmeas bovinas em pastejo: Revisão. Pubvet 16:1–12. https://doi.org/10.31533/pubvet.v16n08a1193.1-12.
Thissen, J.-P., J.-M. Ketelslegers, and L. E. Underwood. 1994. Nutritional regulation of the insulin-like growth factors. Endocr. Rev. 15:80–101. https://doi.org/10.1210/edrv-15-1-80.
Titgemeyer, E. C., C. K. Armendariz, D. J. Bindel, R. H. Greenwood, and C. A. Löest. 2001. Evaluation of titanium dioxide as a digestibility marker for cattle. J. Anim. Sci. 79:1059. https://doi.org/10.2527/2001.7941059x.
Tomlinson, D. L., R. E. James, G. L. Bethard, and M. L. McGilliard. 1997. Influence of undegradability of protein in the diet on intake, daily gain, feed efficiency, and body composition of Holstein heifers. J. Dairy Sci. 80:943–948. https://doi.org/10.3168/jds.S0022-0302(97)76018-1.
Valentim, J. K., J. P. Mendes, B. B. Przybulinski, F. C. Serpa, D. K. Barbosa, V. A. Rios Castilho, and R. T. R. Pietramale. 2019. Fatores nutricionais aplicados à reprodução de ruminantes. UNICIÊNCIAS 23:77–82.

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