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Performance, nitrogen metabolism, and mammary gland development of grazing Holstein×Gyr crossbred heifers supplemented with different levels of rumen undegradable protein

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Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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ABSTRACT

OLIVEIRA, Gabriella Maria Mota de, M.Sc., Universidade Federal de Viçosa, October, 2024. **Performance, nitrogen metabolism, and mammary gland development of grazing Holstein×Gyr crossbred heifers supplemented with different levels of rumen undegradable protein**. Adviser: Alex Lopes da Silva. Co-advisers: Polyana Pizzi Rotta, Luciana Navajas Renno, Simone Eliza Facioni Guimaraes, Mariana Magalhães Campos and Carlos Augusto de Miranda Gomide.

The objective was to assess the impact of supplementing with increasing levels of RUP on the performance, N metabolism, and mammary gland development of grazing dairy heifers. Twenty-eight crossbred Holstein×Gyr dairy heifers (initial BW of 278 ± 50.4 kg) were grazed intermittently on Megathyrsus maximus cv. BRS Quênia grass pasture for an experimental period of 84 d, divided into four sampling sub-periods of 21 d each. The experiment followed a randomized block design, consisting of three treatments with increasing levels of RUP and a control group (CON). The supplemented groups received 30% (RUP30), 48% (RUP48), or 66% (RUP66) of RUP in their supplement. The supplement was provided at 0.5% of the animals' BW, with a consistent CP content around 24% across all treatments. Sampling of pasture, feces, and urine was performed on four consecutive d in each sub-period. On d 0 and 19 of each sub-period, the animals were weighed, and biometric measurements were recorded. Ultrasonic mammary gland images were taken on d 0, 42, and 84. Blood samples were collected on the same d to measure the concentrations of IGF-1, albumin, total proteins, urea, and glucose. At 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, relative DMI (g/kg of BW), and nutrient intake when compared to CON animals. A quadratic effect of RUP level was observed for supplement DMI, pasture DMI, DMI, and nutrient intake with higher values noted in the RUP48 treatment. The supplemented animals had a significant increase in the digestibility of DM, CP, and OM. There was a linear increase in the digestibility of NDF and OM across the RUP level. Supplemented animals achieved higher BW, ADG, thoracic circumference, and rump height when compared to 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 to 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 to the CON animals and there was no effect of RUP level on this variable. Supplemented animals had higher levels of urea and blood IGF-1 when compared to CON animals. A quadratic effect of RUP level was observed for total serum protein, with higher levels in the RUP48 treatment. Supplementation resulted in higher BCS, greater mean horn diameter, and improved reproductive tract tone and score. An increasing linear effect was observed for uterine tone across the RUP levels, with higher values in the RUP66 treatment. Supplemented 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 and N metabolism.

Keywords: weight gain,; protein metabolism,; protein supplementation.

RESUMO

OLIVEIRA, Gabriella Maria Mota de, M.Sc., Universidade Federal de Viçosa, outubro de 2024. **Desempenho, metabolismo do nitrogênio e desenvolvimento da glândula mamária de novilhas mestiças Holandesa×Gyr em pastejo suplementadas com diferentes níveis de proteína não degradável no rúmen**. Orientador: Alex Lopes da Silva. Coorientadores: Polyana Pizzi Rotta, Luciana Navajas Renno, Simone Eliza Facioni Guimaraes, Mariana Magalhães Campos e Carlos Augusto de Miranda Gomide.

O objetivo foi avaliar o impacto da suplementação com níveis crescentes de PNDR sobre o desempenho, metabolismo do nitrogênio e desenvolvimento da glândula mamária de novilhas leiteiras em pastejo. Vinte e oito novilhas mestiças Holandês × Gir (Peso médio inicial = 278 ± 50,4 kg), foram manejadas de forma intermitente em pastagem de Capim Megathyrsus maximusCv. BRS Quênia, por um período experimental de 84 dias, dividido em quatro subperíodos amostrais de 21 dias cada. O experimento seguiu o delineamento em blocos casualizados, composto por três tratamentos com níveis crescentes de PNDR e um grupo controle (CON). Os grupos suplementados receberam 30% (PNDR30), 48% (PNDR48) e 66% (PNDR66) de PNDR em seu suplemento. O suplemento foi fornecido à 0,5% do PV dos animais, com um teor constante de PB de 24% em todos os tratamentos. A amostragem de pastagem, fezes e urina foi realizada em quatro dias consecutivos no final de cada subperíodo. Nos dias 0 e 19 de cada subperíodo, os animais foram pesados e as medidas biométricas foram registradas. Imagens de ultrassom da glândula mamária foram realizadas nos dias 0, 42 e 84. As amostras de sangue foram coletadas no mesmo dia para medir as concentrações de IGF-1, albumina, proteínas totais, ureia e glicose. Nos dias 0, 42 e 84, o desenvolvimento do trato reprodutivo foi avaliado por palpação transretal com aparelho de ultrassom. O tecido hepático foi amostrado no dia 0 e 84. Os animais suplementados apresentaram maior consumo de CMS, CMS relativo (g/kg de PV) e ingestão de nutrientes quando comparado aos animais CON. Observou-se efeito quadrático do nível de PNDR para consumo de suplemento, CMS da pastagem, CMS e ingestão de nutrientes, com maiores valores observados no tratamento PNDR48. Os animais suplementados tiveram um aumento significativo na digestibilidade da MS, PB e MO. Houve um aumento linear na digestibilidade da FDN e MO com o aumento dos níveis de PNDR. Os animais suplementados obtiveram maior PC, GMD, circunferência torácica e altura da garupa quando comparados aos animais CON. Observou-se efeito quadrático do nível de PNDR para PC e GMD, com valores mais elevados no tratamento PNDR48. Os animais suplementados apresentaram maior consumo de N, excreção urinária e

fecal de N e síntese microbiana de PB quando comparados aos animais CON. Observou-se efeito quadrático do nível de PNDR para ingestão de N e excreção fecal de N, com maiores valores no tratamento PNDR48. Os animais suplementados apresentaram menor contagem de pixels na glândula mamária quando comparados aos animais CON e não houve efeito do nível de PNDR sobre essa variável. Os animais suplementados apresentaram maiores níveis de ureia e IGF-1 no sangue quando comparados aos animais CON. Observou-se efeito quadrático do nível de PNDR para a proteína sérica total, com níveis mais elevados no tratamento PNDR48. A suplementação resultou em maior ECC, maior diâmetro médio do corno uterino, e melhor tônus e pontuação do trato reprodutivo. Um efeito linear crescente foi observado para o tônus uterino em todos os níveis de PNDR, com valores mais altos no tratamento PNDR66. Os animais suplementados apresentaram menor expressão hepática da enzima glutâmico-oxaloacética transaminase 1 (GOT1). Além disso, foi observado um efeito quadrático para a expressão de GOT1 entre os níveis de PNDR, com menor expressão nos tratamentos PNDR48 e PNDR66. Em conclusão, um nível de PNDR de 48% no suplemento é a recomendação ideal para novilhas leiteiras mestiças Holandês × Gir em pastejo, pois de modo geral melhorou o desempenho e o metabolismo do N.

Palavras-chave: ganho de peso, ; metabolismo proteico, ; suplementação proteica.

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LIST OF ACRONYMS AND ABBREVIATIONS

CON	Control
RUP30	Treatment with 30% RUP in the supplement
RUP48	Treatment with 48% RUP in the supplement
RUP66	Treatment with 66% RUP in the supplement
PM	Protein Metabolism
DOM	Digestible organic matter
NDFi	Indigestible neutral detergent fiber
AU	Animal unit
NPK	Nitrogen, Phosphorus, Potassium
N	Nitrogen
K	Potassium
mm^2	Square Millimeters
mg	Milligrams
nM	Nanomolar
MHz	Megahertz
Ct	Thershold cycle
IGFBP3	Included insulin-like growth factor-binding protein 3
CPS1	Carbamoyl phosphate synthetase
GOT1	Glutamic transaminase - oxaloacetic

SUMMARY

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 significant proportion of the costs of dairy production systems and is often not given sufficient attention by farmers (Lowe et al.,2016; Silva, 2024). In this context, it is important to recognize that one of the most effective ways to reduce the production costs of heifers is to ensure that these animals reach their first calving as early as possible (Hutchison et al., 2017; Machado et al., 2020). As reproductive traits are closely correlated with BW, strategies to improve ADG and thus reduce age 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, one of the main crossbreeds used in this type of system is the Holstein×Gyr cattle, as it is more resistant to grazing at high temperatures and also 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). However, tropical pastures usually provide an unbalanced diet, which could lead to lower animal performance and affect the profitability of the system (Detmann et al., 2014a). Therefore, to mitigate the nutritional limitations of pastures, an appropriate concentrate supplementation program should be implemented (Machado et al., 2019).

Tropical pastures are generally energy surplus, indicating the need for protein supplementation, even during the rainy season (Detmann, et al., 2014b; a). 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; Machado et al., 2020; Castro et al., 2023a). Furthermore, protein supplementation can increase the utilization of basal forage resources by improving forage 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, better nitrogen retention and can prevent the deposition of body fat (Tomlinson et al., 1997; Bethard et al., 1997; Bascom et al.,2007). This could lead to higher ADG levels without excessive fat deposition in the carcass 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 CP content of about 24% in the supplement is most suitable for these animals Holstein × Gyr with 350 kg and 19 months 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, the authors found no negative effects on the development of the mammary glands. In addition, this proportion improved the balance of nitrogenous compounds, resulting in lower nitrogen excretion into the environment (Silva et al., 2018a; b).

Although the results presented above are concise, there is still a considerable need for further studies to evaluate the effects of protein supplementation on dairy heifers' performance. In particular, there is an urgent need to evaluate RUP-rich protein supplements to determine the most appropriate levels for grazing animals. Therefore, we hypothesized that the use of a supplement with a CP content of 24% in combination with an increased amount of RUP would improve performance, protein metabolism and mammary gland development and reduce nitrogen excretion in grazing dairy heifers. The aim of this study was to evaluate the intake, performance, mammary gland development, N balance and N metabolism and gene expression of crossbred Holstein×Gyr dairy heifers supplemented with different levels of RUP.

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, MG. The geographical coordinates are 21°33'22" S and 43°6'15" W. Temperature and precipitation data were collected for the experimental period from January 19, 2023 to April 12, 2023 through the meteorological station A557 in Coronel Pacheco, MG, through the INMET system (Figure 1).

Animals, experimental design and treatments

The study was conducted as a randomized block design with 28 crossbred $^{5}/_{8}$ Holstein × Gyr heifers. These heifers were divided into three blocks based on their

initial BW: Block 1 had an average BW of 331.6 ± 6.65 kg, Block 2 had an average BW of 298.3 ± 12.06 kg, and Block 3 had an average BW of 257.22 ± 20.43 kg. At the beginning, all heifers were treated against ectoparasites with Fipronil Base at a dosage of 0.1 mL/kg of BW. The animals were kept intermittently in paddocks with *Megathyrsus maximus* cv. BRS Quênia and subjected to one of four treatments: i) no supplementation or control group (**CON**; n=7); ii) supplementation with 30% of the supplement CP as RUP (**RUP30**; n=7); iii) supplementation with 48% of the supplement CP as RUP (**RUP48**; n=7); and iv) supplementation with 66% of the supplement CP as RUP (**RUP66**; n=7) (Table 1). All animals had *ad libitum* access to the mineral supplement, which was placed in two covered troughs in the resting area. The experimental period lasted 84 d and was divided into four sub-periods of 21 d each.

General management

The animals were kept in paddocks with *Megathyrsus maximus* cv. BRS Quênia, covering a total area of about 2 ha. This area was divided into 10 paddocks of 2,000 m² each and a resting area of 430 m². The paddocks were fertilized with 42 kg of a NPK mixture (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 that the animals were in a paddock after the animals had left the paddock. In total, 197.4 kg/ha N and 197.4 kg/ha K were applied during the entire trial. The resting area was equipped with drinking fountains and feeders for mineral supplementation.

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 AU/ha, with an occupation period of 2 days until day 49 of the experimental period. In 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).

The experimental concentrate supplements were administered at a level of 0.5% (5 g/kg BW) 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 to monitor the leftovers when there were. Daily, the animals were brought to the management area at 0900 each d, where they received supplementary feed 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 kept in the same way as the others but were not given any supplementation.

At the time of preparation of the feed supplements, samples were taken to determine their chemical composition. The composition and availability of the forage in the grazing layer were estimated in each sub-period by taking samples from three randomly selected paddocks. The forage mass was quantified by measuring the average height at 20 points. Subsequently, two points corresponding to the average height of vegetation in each paddock were sampled at 15 cm from the ground using a 0.5×1.0 m metal frame (Gomide et al., 2007). For chemical composition, the sample was taken using the same method, but at 50% of the average vegetation height (Gomide et al., 2007).

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% maize meal, 47.5% soybean meal, 6% mineral supplement, and 2% urea.

Performance, intake and digestibility

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

Titanium dioxide was used as a marker to estimate fecal excretion. It was administered at a dose of 15 g/d for nine consecutive d, starting on the 9th d of each sub-period (Titgemeyer et al., 2001). The marker was administered directly into the animals' esophagus using an applicator. To determine the digestibility of the feed, four fecal samples were collected on four consecutive d of each sub-period according to

the following schedule: 0600 on d 15, 1000 on d 16, 1400 on d 17, and 1800 on d 18. The titanium dioxide content in the feces was quantified using a sulfur digestion method (INCT-CA M-007/2 method; Detmann et al., 2021).

Supplement, forage, and feces samples were subjected to partial drying at 55°C for 72 h immediately after collection (INCT-CA G-001/2 method). They were then ground with a knife mill, first through a 2 mm sieve and then through a 1 mm sieve. Samples ground to 1 mm were analyzed for DM (INCT-CA G-003/1 method), CP (INCT-CA N-001/2 method) and ash content (INCT-CA M-001/2 method) according to the methods described by Detmann et al. (2021). In addition, the samples were analyzed for the NDF content corrected for ash (INCT-CA F-002/2 and INCT-CA M-002/2 method). Samples ground to 2 mm were used to determine the concentration of indigestible NDF (**NDFi**; INCT-CA method F-009/2; Detmann et al., 2021). In brief, supplement, forage, and fecal samples were incubated in the rumen of three heifers via a rumen cannula for a period of 288 h and the residual NDF was determined. The NDFi was used as an internal indicator for estimating pasture intake by the animals.

Microbial protein synthesis and nitrogen balance

The urine samples were collected on the same d and at the same times as the fecal samples by stimulated urination. After sampling, the urine was filtered through gauze and a 50 mL aliquot of concentrated urine was collected. Another aliquot of 10 mL was also 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.

Concentrated urine samples were used to determine the total N concentration using the INCT-CA N-001/2 method (Detmann et al., 2021). The samples previously diluted in sulfuric acid were used to determine the concentrations of creatinine, urea, uric acid and allantoin. Creatinine was determined using Labtest's colorimetric determination kit (Labtest Creatinine), while urea and uric acid concentrations were measured using Biotek's EON microplate spectrophotometer with Gen5 software and LABTEST determination kits. Finally, allantoin concentrations were determined using the technique described by Chen and Gomes (1992).

The daily urinary excretion volume was estimated by dividing the daily urinary excretion of creatinine by the concentration of creatinine in the urine of each animal.

The daily urinary excretion of creatinine was estimated using the model proposed by Chizzotti et al. (2008) for heifers:

$$\mathrm{CE} = 32.2 - 0.0109 \times \mathrm{BW}$$

where: CE = daily creatinine excretion (mg/kg BW) and BW = body weight (kg).

The total excretion of purine derivatives was calculated from the sum of the amounts of allantoin and uric acid excreted in the urine. Absorbed purines were calculated from the total excretion of purine derivatives, using the recovery values for absorbed purines as purine derivatives and the endogenous contribution to purine excretion, based on the model of Prates et al. (2012):

$$\mathrm{AP} = (\mathrm{PD} - 0.439 \times \mathrm{BW}^{0.75}) / 0.99$$

where: AP = absorbed purines (mmol/d), PD = excretion of purine derivatives (mmol/d), 0.439 × BW0.75 = endogenous contribution to purine excretion and 0.99 = recovery of purines absorbed as purine derivatives in urine.

The ruminal synthesis of microbial compounds was calculated as a function of the absorbed purines according to the method described by Chen and Gomes (1992):

$$\mathrm{Nmic} = 70 \times \mathrm{AP} / 0.93 \times 0.11 \times 1000$$

where: Nmic = synthesis of microbial nitrogenous compounds in the rumen (g/d), AP = absorbed purines (mmol/d), 70 = nitrogen content in microbial purines (mg N/mol), 0.93 = intestinal digestibility of microbial purines (Barbosa et al., 2011), and 0.11 = purine N/total N ratio of bacteria (Prates et al., 2012).

The production of MP of microbial origin was calculated as follows:

$$MPmic = Nmic \times 6.25 \times 0.65$$

where: MPmic = flow of MP of microbial origin (g/d), Nmic = synthesis of microbial nitrogen-containing compounds in the rumen (g/d) and 0.65 = conversion factor from microbial CP to MP of microbial origin (Nasem, 2021).

The daily intake of rumen degradable protein (**RDP**) was considered equal to the microbial CP (Nmic × 6.25), assuming 100% efficiency in N recycling (NASEM, 2021). The daily flow of digestible RUP was calculated by the difference between the daily intake of CP and the estimated intake of RDP.

$$RUPd = (CPi - RDP) \times 0.80$$

where: RUPd = flow of rumen undegradable protein in the digestible rumen (g/d), CPi = crude protein intake (g/d), RDP = rumen degradable protein (g/d), and 0.80 = average digestible of RUP (NASEM, 2021).

The daily flow of MP was determined by summing the flow of MP of microbial origin and digestible RUP. In addition, the N balance was calculated as the difference between the total N intake and the total N excreted via feces and urine.

Mammary gland ultrasound, blood analysis, and reproductive assessment

On d 0 (before the start of the study), 42 and 84 of the trial period, ultrasound images of the mammary gland were taken to assess its development. The images were obtained using a B-mode ultrasound machine with a microconvex transducer at a frequency of 6 MHz (M6Vet, Mindray). One image of each quarter of the mammary gland was acquired in a standardized probe position with a 45° inclination relative to the base of the teat, always in the caudocranial direction (Nishimura et al., 2011; Albino et al., 2017).

Images were processed using ImageJ software (NIH, USA) calibrated to 100 pixels/cm using the Straight Tracer tool. Within each mammary gland image, three squares of equal area (16 mm^2) were randomly selected that were located near the growth of the mammary duct (Nishimura et al., 2011). The average pixel value within each square was evaluated in 8-bit images and numerically represented on a scale of 256 gray levels (0 = black, 255 = white) according to the brightness intensity. Therefore, adipose tissue shows a higher brightness intensity and therefore higher pixel values, while parenchymal tissue shows a lower intensity and consequently lower pixel values (Albino et al., 2015; Silva et al., 2018).

Blood samples were collected on day 0 (prior to the study's initiation) and on the final day of each sub-period, 4 hours after supplementation, to measure serum levels of insulin-like growth factor (**IGF-1**), urea nitrogen, glucose, total protein, and albumin. Blood was collected by puncturing the coccygeal vein into vacuum tubes containing separator gel and a coagulation activator. The tubes were placed in a Styrofoam box with ice and then transported to the laboratory where they were centrifuged at 3,000 rpm for 20 minutes. Ten serum samples were taken from each animal, placed in prepared Eppendorf tubes and stored at -20°C for further analysis.

Quantification of urea-N, glucose, albumin, and total protein levels was conducted using an automated biochemical analyzer (model BS200) with Bioclin kits. The methods used included fixed-time kinetics for urea-N, a colorimetric enzymatic method for glucose, the bromocresol green method for albumin, and the biuret method for total protein. IGF-1 levels were quantified using a chemiluminescence method with

an enzyme-linked immunosorbent assay (ELISA) microplate reader. For this analysis, the Liaison XL-Diasorin device was employed along with the Diasorin kit (LIAISON® IGF-1 [REF 313231]).

At time points d 0, 42 and 84, the development of the reproductive tract was assessed by transrectal palpation with an ultrasonic device by a veterinarian. A B-mode ultrasound-equipped device with a linear 6 MHz transducer (M6Vet, Mindray) was used for this assessment. During the ultrasound examination, the areas of the left and right ovary, the left and right uterine horn, the diameter of the corpus luteum (if present) and the tone of the uterus (scored on a scale of 1 to 5) were recorded (Rosenkrans and Hardin, 2003; Stevenson et al., 2008). Based on the data obtained, the development of the reproductive tract of the animals was classified as follows. Animals with no uterine horn tone and follicles smaller than 8 mm were scored 1 and classified as prepubertal. Animals with a slight tone and follicles between 8 and 10 mm received a score of 2 and were classified as peripubertal. Animals with tight horns, follicles larger than 10 mm and the presence of a corpus luteum were scored 3 and classified as pubertal (Stevenson et al., 2008).

Liver tissue collection and gene expression

Five animals from each treatment group were randomly selected for liver tissue sampling. Samples were collected on the first and last d of the study. Liver tissue was collected using the Tru-Cut needle biopsy technique 4 h prior to supplementation (Mølgaard et al., 2012). An incision was made between the 11th and 12th ribs on the right side of the animal. The needle was inserted approximately five times to collect liver tissue (Miranda et al., 2010). After collection, all samples were stored in cryovials and immediately frozen in liquid nitrogen.

First, RNA was extracted from the liver tissue samples using the organic solvent method with Trizol (InvitrogenTM, Thermo Fisher Scientific) according to the manufacturer's protocol. The total RNA extracted was quantified using a NanoDrop spectrophotometer and its integrity was assessed by agarose gel electrophoresis at a concentration of 1%. The RNA samples were then reverse transcribed into cDNA using the High-Capacity Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). The resulting cDNA was quantified with 1 µL of sample using the

NanoDrop spectrophotometer. The cDNA was then diluted to a concentration of 5 ng/μL and stored at -20°C until PCR analysis.

Real-time PCR analysis was performed with duplicate samples using a CFX96 Real-Time System (Bio-Rad) thermal cycler. SyBR Green GoTaq qPCR Master Mix Reagent (Promega Corporation) was used according to the manufacturer's protocol. The efficiency was tested for each gene with serial dilutions of cDNA at concentrations of 5, 15 and 45 ng/μL and different concentrations of master mix for the reactions of 100, 200 and 400 nM. The efficiency was considered acceptable when the amplification of the target gene was comparable to that of the control gene, resulting in an efficiency between 80-100%. This value was determined based on calculations using the Ct values of the PCR instruments. The amplification protocol for all reactions started with a first step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and ended with a final step at 60°C for 60 seconds.

The expression of each target gene for each animal was calculated by subtracting the cycle threshold (Ct) of the control gene (endogenous gene) from that of the target gene (Ct of endogenous gene – Ct of target gene). The results were expressed relative to GAPDH using the formula $2^{-\Delta Ct}$, where ΔCt= (Ct target gene–Ct endogenous gene). Target genes evaluated in liver tissue included insulin-like growth factor-binding protein 3 (IGFBP3), insulin-like growth factor (IGF-1), carbamoyl phosphate synthetase (CPS1), glutamate transaminase - oxaloacetic acid (GOT1) and endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 3).

In Situ Degradability Trial

To estimate the RDP and RUP of the diets, a rumen-cannulated Holstein cow (BW 680 kg) was used to perform the *in situ* rumen experiment. The animal was fed twice daily a diet containing corn silage, hay, concentrate, cottonseed, and urea. Nylon bags (15 × 8 cm, with a porosity of 50 μm) filled with approximately 5 g of feed samples (corn meal, soybean meal and rumen-protected soybean meal), and ground to 2 mm were used for incubation. The samples were incubated in triplicate for 16 h (Calsamiglia and Stern, 1995; Paz et al., 2014). The bags were then washed under running water and dried in an oven at 55° C. The DM and CP content was analyzed in the residues according to INCT methods – CA G-003/1 and N-001/2 (Detmann et al., 2021). The amount of CP (%DM) that had disappeared after incubation was used to

estimate the RDP content (%DM) of the ingredients. The RUP content (%DM) was calculated based on the difference between the CP and RDP content.

Statistical analysis

The experiment was conducted using a completely randomized block design and analyses were performed using the GLIMMIX procedure in SAS (SAS Institute, 2024). For intake, digestibility, serum hormones/metabolites, and mammary gland development, the effect of sampling sub-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 criterion (AICC) using the following model:

$$Y_{ijk} = \mu + T_i + P_j + \delta_{ij} + B_k + (T \times P)_{ijl} + \varepsilon_{ijklm},$$

where: Y_{ijk} = dependent variable, μ= overall mean, T_i = fixed effect of treatment, P_j = fixed effect of sampling period, δ_{ij} = random error representing the variance between animals within treatments, which is equal to the covariance between repeated measurements within animals, B_k = random effect of block, $T \times P_{ijkT}$ = fixed effect of the interaction between treatment and collection period, and ϵ_{ijklm} = random error.

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

$$Y_{ij} = \mu + T_i + B_j + \varepsilon_{ijk},$$

where: Yij = dependent variable, μ = overall mean; Ti = fixed effect of treatment, B_k = random effect of block, ε_{ij} = random error. The interaction effect of treatment and block was tested for all variables, and as it was not significant, it was removed from the model.

For all models, residuals were tested for normality (Shapiro and Wilk, 1965). Gene expression data were analyzed assuming a lognormal 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 and a significance level between 0.05 and 0.10 was used as trend. Means were decomposed using orthogonal contrasts to compare the control group with the supplemented groups and to assess the linear and quadratic effects of RUP levels in the supplement.

The number of replicates of seven was determined by a power analysis, taking into account an alpha of 0.05, a power of 0.95 and a coefficient of variation of 7%. The

number of five replicates for gene expression in the liver was determined with an alpha of 0.05, a power of 0.90 and a coefficient of variation of 8% (Ryan, 2013).

RESULTS

Intake and Digestibility

No interaction effect between treatment and sampling period was observed for any of the variables related to intake and apparent digestibility ($P > 0.05$; Table 4). A significant effect of time was observed for all variables analyzed in relation to intake and apparent digestibility ($P < 0.001$), except for RDP intake ($P = 0.107$; Table 4). In general, intake increased over the course of the study. For example, CP intake increased from period 1 to periods 2, 3 and 4 as the animals grew. CP digestibility showed a continuous increase until the third period, followed by a decrease in the fourth period (data not shown).

The DMI of the pasture in relation to BW was higher ($P = 0.008$) in the CON treatment than in the average of the supplemented animals, with values of 20.09 and 18.10 g/kg BW, respectively. On the other hand, the supplemented animals had a higher average DMI compared to the CON treatment ($P < 0.001$), with values of 6.45 and 5.53 kg/d, respectively. Similar patterns were observed for DMI in relation to BW (supplemented = average 22.37 g/kg BW and CON = 20.07 g/kg BW), CP (supplemented = average 0.98 kg/d and CON = 0.71 kg/d), RDP (supplemented = average 438 g/d and CON = 328 g/d), RUP (supplemented = average 569 g/d and CON = 378 g/d), MP (supplemented = average 731 g/d and CON = 515 g/d; $P < 0.05$; Table 4).

With respect to dietary RUP content, a quadratic effect on supplement intake was observed ($P = 0.009$; Table 4), with higher consumption in treatments RUP48 (1.31 kg/d) and RUP66 (1.30 kg/d) than in treatment RUP30 (1.19 kg/d). In addition, a quadratic effect ($P = 0.028$) of RUP content on DMI of pasture was observed, with higher intake in the RUP48 treatment (5.54 kg/d) than in the RUP30 (5.17 kg/d) and RUP66 (4.93 kg/d) treatments. A similar pattern was observed for DM, CP, NDF, and PM intake ($P < 0.05$; Table 4). A linear effect was observed for the CP:DOM ratio ($P = 0.028$), which was higher in the RUP30 treatment.

The supplemented animals had higher DM digestibility compared to the CON treatment ($P < 0.001$), with average values of 645 and 615 g/kg, respectively. A similar

pattern was observed for the digestibility of CP (supplemented = average 728 g/kg and CON = 692 g/kg) and OM (supplemented = average 664 g/kg and CON = 638 g/kg) ($P < 0.05$; Table 4). In the supplemented animals, the digestibility of NDF and OM increased linearly with increasing RUP content in the supplement ($P < 0.05$; Table 4).

Performance and body measurements

There was no difference in the initial body weight of the animals between treatments ($P > 0.05$; Table 5). The supplemented animals had a higher final body weight compared to the CON treatment, with an average weight of 321 kg and 303 kg, respectively ($P < 0.001$; Table 5). In addition, a similar pattern was observed for ADG (supplemented = average of 0.393 kg/d and CON = 0.170 kg/d), BCS (supplemented = average 3.17 and CON = 3.02), thoracic perimeters (supplemented = average of 166 cm and CON = 157 cm) and rump height (supplemented = average of 129 cm and CON = 127 cm) ($P < 0.05$). We also observed a trend towards greater body length ($P = 0.068$) in the supplemented animals compared to the CON treatment, with averages of 112 cm and 109 cm, respectively (Table 5).

A quadratic effect of RUP content ($P < 0.05$) on final BW and ADG was observed in the supplemented animals, with the highest values observed in the RUP48 treatment (330 kg and 0.49 kg/d, respectively; Table 5). In addition, a trend towards a quadratic response in chest circumference was observed in the supplemented animals ($P = 0.055$), with the highest value measured in the RUP48 treatment. A linear growth trend was also observed for rump height ($P = 0.087$), with the highest values recorded in the RUP48 and RUP66 treatments (Table 5).

Nitrogen balance and microbial protein synthesis

The variables related to N balance showed no interaction effect between treatment and sampling period ($P > 0.05$; Table 6), except for, a significant interaction for urinary urea excretion ($P < 0.001$; Figure 3A).

Regardless of the period studied, the CON animals had lower urinary urea excretion compared to the supplemented animals, with average values of 175 and 205 mg/d, respectively. In the supplemented animals, a quadratic effect of RUP levels ($P < 0.05$) was observed in periods 1 and 2, with the highest urinary urea excretion recorded in the RUP30 treatment (167 mg/d) in the 1° period and in the RUP48

treatment (253 mg/d) in the 2° period. In the 3° period, a linear decrease in urinary urea excretion was observed with increasing RUP in the supplement (P = 0.006), while in the fourth period there was no significant difference between the RUP contents (P > 0.05).

Similarly, a significant interaction was observed for the efficiency of microbial protein synthesis (P = 0.022; Figure 2A) and for the efficiency of N utilization for microbial protein synthesis (P = 0.013; Figure 2B). The supplemented animals had a higher efficiency of microbial protein synthesis (P < 0.05) in periods 1 and 4, with values of 138 g/kg and 78 g/kg compared to the CON treatment, which had values of 57.73 and 80 g/kg, respectively. A quadratic effect was observed for RUP values in periods 1 and 3, with treatments RUP48 and RUP66 showing higher efficiency. In contrast, a decreasing linear effect was observed in period 2 (Figure 2A).

In terms of the efficiency of N utilization for microbial protein synthesis, the supplemented animals achieved higher values (P < 0.05) in the 1° period with an average of 0.613 g/kg than the CON group, which achieved an average of 0.318 g/kg (Figure 2B). In periods three and four, the CON group achieved higher values (P < 0.05) with average values of 0.609 and 0.366 g/kg, respectively, compared to the supplemented group, which had average values of 0.494 and 0.298 g/kg, respectively. Regarding the RUP content, a quadratic effect was observed in period 3 and a linear effect in period 4, with the highest efficiency recorded in the RUP48 treatment.

All variables related to N balance showed significant effects in relation to the sampling period (P < 0.05; Table 6). An increasing trend was observed for urea-N, N uptake, urinary N excretion, N retention and N utilization efficiency over each assessment period. Microbial protein production and microbial efficiency increased until the 3° period, followed by a decrease in the 4° period. Nitrogen utilization efficiency for microbial synthesis was higher in periods 1 and 3 and lower in periods 2 and 4.

The supplemented animals showed a trend (P = 0.051) towards higher microbial protein production compared to the CON group, with average values of 438 and 328 g/d, respectively. N intake (supplemented = average 155 g/d and CON = 114 g/d), fecal N excretion (supplemented = average 42 g/d and CON = 33.9 g/d) and urinary N excretion (supplemented = average 79.7 g/d and CON = 61.9 g/d) were also higher in the supplemented animals than in the CON treatment (P < 0.05; Table 6). In addition, a trend (P = 0.082) towards higher N retention was observed in the supplemented

animals with an average of 32.14 g/d than in the CON treatment with a mean value of 18.87 g/d (Table 6).

Supplemented quadratic effect ($P < 0.05$; Table 6) was observed for N uptake and fecal N excretion in the RUP animals, with the highest values recorded in the RUP48 treatment (167 and 44.86 g/d, respectively). Urinary N excretion ($P = 0.089$), N retention ($P = 0.081$) and N utilization efficiency ($P = 0.051$) showed a trend towards a quadratic effect, indicating lower urinary N excretion and higher N retention and N utilization efficiency in the RUP48 treatment (Table 6).

Mammary gland ultrasound, blood analysis, and reproductive assessment

There was no interaction effect between treatment and sampling period for the number of pixels in the mammary gland images or for serum levels of total protein, glucose, urea, IGF-1, average horn diameter, tone and reproductive tract score ($P > 0.05$; Table 7).

In contrast, an interaction effect between treatment and time was observed for serum albumin levels ($P = 0.041$, Table 7). The results showed no difference ($P > 0.05$) between the supplemented and CON groups in periods 2and 4. In the supplemented animals, a quadratic effect was observed in the 2°period ($P = 0.029$), with the highest value recorded in the RUP48 treatment (2.61 g/dL; Figure 3B).

A significant effect of period ($P < 0.05$) was observed for glucose, urea and uterine tone. These blood parameters showed a progressive increase in glucose and urea levels from the 2° to the 4° sampling period. In contrast, uterine tone showed a steady decline over the same period (data not shown).

The supplemented animals had a lower pixel count ($P < 0.05$) in the mammary gland images compared to the CON treatment, with an average of 36.62 and 41.54 pixels/mm², respectively. For blood parameters, the supplemented animals had higher levels ($P < 0.05$) of urea (average 32.87 mg/dL) and IGF-1 (186 ng/mL) than the CON treatment, which had average levels of 21.63 and 139 ng/mL, respectively. When assessing reproductive capacity, supplemented animals had a higher uterine tone (supplemented = mean of 2.38 and CON = 1.88) and reproductive tract score (supplemented = mean of 1.88 and CON = 1.48; Table 7).

There was a quadratic effect ($P = 0.018$) for total protein in blood serum, with the RUP48 treatment having the highest value of 6.31 g/dL (Table 7). In addition, there

was a trend towards a quadratic effect for serum glucose and urea levels, with higher values observed for the RUP30 and RUP48 treatments than for the RUP66 treatment (Table 7). In addition, there was a linear increase ($P < 0.001$) in uterine tone, with the RUP66 treatment having the highest value of 2.57.

Gene expression

There was no effect of supplementation ($P = 0.134$; see Table 8) on the expression of the enzyme CPS1 in the liver. The supplemented animals showed lower expression levels of GOT1, IGF-1 and IGFBP3 compared to the CON treatment.

A quadratic effect was observed for the enzyme GOT1 in the supplemented animals ($P < 0.001$; see Table 8), with the highest expression recorded in the RUP30 treatment. A similar pattern was observed for IGFBP-3 ($P < 0.001$), with the highest value recorded in the RUP30 treatment group (0.40). In contrast, IGF-1 levels showed a linear decrease ($P = 0.015$) with increasing dietary RUP content.

DISCUSSION

Supplementation effects

DMI of pasture, measured in kg/d and g/kg BW, was lower in the supplemented animals compared to the CON group, with reduction by 6.6 % and 9.9 % respectively. Conversely, the supplemented animals showed a 16% increase in DMI (kg/d) and an 11.4% increase in relative DMI (g/kg BW) compared to the control group. These results indicate that the supplementation had a substitutive effect by increasing the total DMI of the animals. As a result, the supplemented animals had a higher total nutrient intake, which led to increased microbial growth in the rumen, improved DM digestibility and consequently better N performance and N retention. Machado et al (2020) found a similar response when evaluating different protein levels (12 and 24%) in the supplement of Holstein heifers × Gyr in grazing. In this study, the supplemented animals obtained higher dry matter intake and lower pasture intake when compared to the control group that consumed only pasture.

The digestibility of nutrients can be influenced by several factors, including the level of intake, the chemical composition of the concentrate and the quality and availability of pasture (Castro et al., 2023a). In the present study, supplementation

increased the digestibility of several of the variables analyzed. This is supported by the presence of highly available ingredients in the supplemented diet that promote the growth of non-fibrolytic bacteria and improve nutrient utilization and digestibility (Camargo et al., 2022). In addition, the provision of these readily available ingredients was combined by supplementation with nutrients from high quality pasture. This approach also provided the fibrolytic bacteria access to their primary and limiting substrate, N (Lazzarini et al., 2015; Franco et al., 2016). Thus, there was no competition for nutrients between the microbial groups, resulting in similar NDF digestibility between the supplemented and non-supplemented animals. A CP content of 100 g/kg of forage is considered sufficient to support fibrolytic microorganisms, which explains the lack of effect on forage digestibility (Detmann et al., 2014b; Camargo et al., 2022).

Animal performance in grazing systems is related to feed intake, digestibility, availability and quality of forage (Camargo et al., 2022; Castro et al., 2023a). Several studies have shown positive effects of supplementation on the performance of grazing animals, supporting the results observed in this study. In this context, Castro et al. (2023a) investigated supplementation with different CP levels (12, 24, and 36%) in animals grazing on *Urochloa* decumbens. The supplement was provided at 0.5% of BW (5g/kg BW) in the rainy season (February – March), rain-drought transition (April - June), drought (June - September) and rainy season (October - January). The supplemented animals had a higher ADG compared to the control group, with average values of 0.45 kg/d and 0.39 kg/d, respectively. In addition, the supplemented animals also had a larger chest circumference, body length, and withers and rump heights. Machado et al. (2019) tested two supplementation strategies for animals grazing on high quality pasture (*Megathyrsus maximus* cv. Mombaça). The animals received a protein supplement with 24 % CP and an energy supplement with 8.19 % CP, both administered at 0.5 % of BW. The animals receiving the protein supplement achieved an ADG of 0.570 kg/d, while the control group (without supplement) achieved an ADG of 0.308 kg/d and the group with the energy supplement an ADG of 0.346 kg/d.

It is therefore clear that protein supplementation has a positive effect on ADG in grazing animals. The results of Machado et al. (2019) as well as those of our study underline the importance of protein supplementation, even when high-quality forage with a high CP content is used. Although the focus on forage quality is often on CP content, it is important highlight that high digestibility of DM and NDF during the rainy

season also contributes to improved energy availability. As a result, there is an imbalance in the ratio between energy and protein that does not meet the nutritional requirements of the animals (Detmann & Valadares Filho, 2010). In this context, the administration of protein supplements can lead to additional gains of up to 0.200 kg/d (Detmann & Valadares Filho, 2010; Detmann et al., 2014b).

The higher N excretion in the urine and feces of supplemented animals compared to the non-suplemented animals is due to the increased CP intake from both the supplement and the roughage. In the rumen the true protein and urea undergo hydrolysis to release the N from the compounds to make the N available in the medium. Then, the assimilatory and biosynthetic pathways produce amino acids and peptides used by the cell. Part of the ammonia released is used for microbial protein synthesis, and what is not used is absorbed in the portal vein, in which part is recycled in the liver and returns to the animal and the other part is excreted in urine and feces (Hailemariam et al., 2021; Reynolds & Kristensen, 2008).

This higher intake may have triggered an increase in amoniacal-N in the rumen, which can be transported into the blood by diffusion, leading to increased N excretion in urine and feces (Van Soest, 1997; Camargo et al., 2022). Increased N retention indicates that an improvement in N status through protein supplementation is necessary to improve N retention (Detmann et al., 2014; Lazzarini et al., 2015).

Due to the higher daily CP intake, the supplemented animals showed increased higher urea concentration in the serum. These higher urea values correlate with the increased CP intake, which directly influences the increase in amoniacal-N in the rumen. This variable shows a positive and proportional relationship with the amount of CP in the diet (Detmann et al., 2014b; Camargo et al., 2022). In addition, a significant interaction between time and treatment was observed for urinary urea excretion. In periods 1, 2 and 3, the supplemented animals had a higher urinary urea excretion than the non-supplemented animals. In these periods, protein supplementation combined with good forage availability due to fertilization and higher rainfall resulted in increased protein intake by the supplemented animals, which explains the higher urea excretion observed. In the fourth period, however, the non-supplemented animals excreted more urea than the supplemented animals. During this period, the availability and quality of pasture decreased. Without supplementation, the animals had to meet their nutrient requirements from the limited biomass available to maintain rumen activity and survival. In the absence of readily available ingredients, available N was diverted to

urea recycling to maintain rumen function. This process may have led to increased urea losses during recycling (Franco et al., 2016).

The higher MP intake of the supplemented animals led to increased activity of IGF-1, which subsequently increased its level (Castro et al., 2023a). Protein supplementation thus reduces the degradation of myofibrillar protein, which would otherwise be required to mobilize N for recycling and survival. This leads to greater N retention and improved performance, as observed in this study (Batista et al., 2016; Franco et al., 2016; Castro et al., 2023a).

Supplementary feeding creates a better rumen environment by providing an increased supply of substrates (carbohydrates and proteins) that are important for the development of rumen microorganisms. This increases the degradability and utilization of the feed, leading to improved performance and intake of animals in a grazing system (Franco et al., 2016). Our results of microbial protein synthesis support the idea of an improved rumen environment and show higher levels in supplemented animals (Table 6). In this way, nitrogenous compounds and a more readily available energy source work together to improve nutrient utilization by the animals' metabolism (Souza et al., 2010).

The efficiency of microbial protein synthesis (g/kg DOM) and N use showed a significant interaction between treatment and time. In the 1° period, the supplemented animals showed higher microbial efficiency, which was due to the increased availability of energy from the supplement and the greater quantity and quality of biomass in the paddocks. In the 4° period, the non-supplemented animals had a higher microbial efficiency (80.34 g/kg DOM) than the supplemented animals (78.13 g/kg DOM). This result can be attributed to the animals' efforts to maximize efficiency and ensure the supply of available pasture. In addition, the lower biomass production in the paddocks during this period resulted in a lower supply of non-protein nitrogen (NPN) and pasture energy. Consequently, the animals had to manage the limited available resources more efficiently (Castro et al., 2023a).

In the 1° period, the highest observed values for N utilization efficiency in the supplemented animals are directly related to the greater availability of nutrients from the supplement. Although pasture was available, its nutritional value gradually improved as management measures were implemented throughout the experimental period. This explains why the animals that did not receive supplementation utilized N less efficiently for microbial synthesis. On the other hand, the non-supplemented

animals achieved higher N utilization values than the supplemented animals in periods 3 and 4. This can be explained by the fact that these animals had fewer nutrients available and were dependent on energy and protein from the pasture and N from recycling. In addition, the lower pasture production during these periods led these animals to increase N use efficiency to support the development of rumen microorganisms (Detmann et al., 2014b; Castro et al., 2023a).

The higher protein/energy ratio (g BW/kg DOM, Table 4) consumed by the supplemented animals had a positive effect on the development of the mammary parenchyma compared to the control group . In addition, as previously mentioned, the supplemented animals had higher circulating IGF-1 levels, indicating that supplementation provided a favorable hormonal environment for mammary gland development (Thissen et al.,1994; Albino et al.,2017).

The higher intake, performance and N retention of the supplemented animals also had a positive effect on reproductive variables. In case of nutritional imbalance, reproduction is the first function to be affected (Valentim et al., 2019). Maintaining metabolic homeostasis is crucial for the production of hormones that regulate the release of GnRH, such as leptin, IGF-1 and ghrelin (D'Occhio et al., 2019). Therefore, the results suggest that supplementation was crucial for the reproductive development of the animals.

The lower gene expression of the enzyme GOT1 in the liver of the supplemented animals indicates a reduced hepatic N metabolism, as GOT1 is involved in one of the pathways for the entry of free ammonia into the liver. Free ammonia can be absorbed from the digestive tract or generated from other amino acids by transamination reactions with α-ketoglutarate in the cytoplasm to form glutamate (Kozloski, 2021). However, the higher expression of GOT1 in non-supplemented animals indicates a higher N intake from the pasture. This correlates with lower rumen utilization due to lower nutrient intake from the supplement, which leads to increased urea production in the liver (Castro et al., 2023b).

Although the literature indicates that circulating serum levels of IGF-1 are dependent on hepatic synthesis of this hormone, our results suggest the opposite. It is hypothesized that the lower expression of IGF-1 observed in the supplemented animals is a response to the higher availability of energy and protein. These animals, which had an excess of nutrients, showed a lower expression of IGF-1. In the CON group, on the other hand, IGF-1 expression was higher due to the lower nutrient intake,

which meant that the animals were more efficient and only used pasture, resulting in higher IGF-1 expression in the liver (Radcliff et al., 2004).

RUP levels effects

The higher performance of the RUP48 animals is related to the higher intake of supplement, pasture, DM, CP, NDF and MP observed in this treatment. Camargo et al (2022) observed higher intakes of supplement, NDF and CP in animals fed protected soybean meal and corn gluten compared to the control group fed pasture and a mineral supplement when testing intakes of different sources of replenishment.

The DMI is strongly influenced by diet digestibility (Detmann et al., 2014b). Therefore, increasing the availability of nutrients promote their use for the growth of rumen microorganisms, especially fibrolytic bacteria, which significantly improve fiber digestibility and consequently increase DMI (Detmann et al., 2014b; Rufino et al., 2020). In this study, the higher intake observed in the RUP48 treatment could be related to a better nutrient balance in the diet. This treatment provided the necessary nutrients to maintain a favorable rumen environment, including high available energy from the supplement, N from the pasture and supplement, which is essential for microbial growth, and RDP, which provides the carbon skeleton for the microorganisms (Berchielli et al., 2011). In addition, the inclusion of RUP was crucial to direct protein utilization into the small intestine while ensuring that the amount of RDP available in the rumen was neither limited nor excessive (Paengkoum et al., 2019). This more favorable rumen environment led to improved utilization of the diet and increased the passage rate of ingested material. As a result, intake and nutrient absorption increased, which improved the overall nutrient supply (Berchielli et al., 2011).

Among the supplemented animals, the animals in the RUP66 treatment had higher values for NDF and OM digestibility. To maximize bacterial growth in animals in a grazing system, a minimum CP dietary level of 70-80 g/kg DM is required (Sampaio et al., 2009). In this study, the minimum CP forage content was 95.7 g/kg DM and the maximum was 151.9 g/kg DM. Although the RUP66 treatment did not receive RDP supplementation to ensure a good rumen environment and improved digestibility, the high CP level of the forage was sufficient to meet the animals' requirements.

The results of this study indicate that supplementation with RUP sources leads to a higher MP intake. This is attributed to a more favorable rumen environment that

promotes microbial protein production (the main source of MP in ruminants) and an increase in RUP, both of which contribute to the increase in MP content (Souza et al., 2020). In this sense, animals treated with RUP48 achieved higher levels of MP, which improves the flow of amino acids (AA) into the small intestine and increases final BW and ADG compared to animals receiving other levels of RUP (Tomlinson et al., 1997; Silva et al., 2018a).

Camargo et al. (2022), who studied different sources of RUP (protected soybean meal and corn gluten meal), observed higher final BW (average 346.5 kg) and ADG (average 0.95 kg/d) in animals supplemented with these sources of RUP compared to the group receiving only a mineral supplement and feed (final BW = 323 kg and ADG = 0.75 kg/d). In the same study, the animals receiving protected soybean meal had higher N retention, indicating a more efficient utilization of MP. Although the cited study had different experimental conditions and used different sources of RUP, it emphasizes the performance benefits of RUP sources when feeding grazing animals.

The higher fecal N excretion in the RUP48 treatment compared to RUP30 and RUP66 is due to the increased intake of supplemental feed in combination with CP intake from pasture, which likely resulted in higher NH3-N production. Ammonia is a key indicator of N flux to the liver, where urea recycling takes place. There is therefore a correlation between increased urea excretion and plasma urea levels (Camargo et al., 2022).

Regarding the interaction between treatment and time for urea excretion, the first evaluation period showed that a higher RUP inclusion and a lower CP content in the pasture resulted in lower urea excretion. This was due to the lower availability of NPN and RDP in the rumen, together with a greater amount of available energy, which probably reduced the level of free ammonia in the rumen and consequently the need to recycle excess free N (Berchielli et al., 2011; Castro, et al., 2023a). In the second period, the RUP48 treatment resulted in higher urea excretion compared to the other treatments. This increase was due to a higher intake and CP content of the pasture, resulting in a higher protein intake by the animals. In the third evaluation period, the RUP48 treatment maintained its urea excretion from the second period, while the other levels showed an increase in urea excretion. The increase in urea excretion in the third period compared to the first period could be due to an improvement in the structure and quality of the pasture, which increased the protein content and provided a greater

amount of more digestible nutrients. This could have led to an excess of ammonia in the rumen, which required increased recycling (Machado et al., 2020).

For efficient microbial protein synthesis, it is important that NPN, true protein and energy reach the rumen (Berchielli et al., 2011). These substrates enable the production of carbohydrates, which are converted into short-chain fatty acids, and the carbon skeletons of the true proteins are used to form microbial cells (Berchielli et al., 2011). The higher value of microbial efficiency (g/kg DOM) observed in the RUP66 treatment during the first period can be attributed to the higher energy supply from the finely ground corn and pasture protein, which provided the necessary substrates for microbial growth and synthesis of microbial protein. Paengkoum et al (2019), investigating different levels of RUP (15%, 25% and 35%) for growing cattle, observed a linear increase in proteolytic and amylolytic bacteria. These bacteria produced microbial protein by decreasing RDP uptake and increasing RUP. In the third period, the RUP48 treatment achieved higher microbial efficiency due to improved forage quality characterized by higher CP content and higher digestibility associated with more easily digestible nutrients. This contributed to a more efficient utilization of nutrients for microbial N synthesis.

The higher efficiency of N utilization for microbial synthesis observed in the RUP48 treatment during the third period is attributed to the improved nutritional balance in this treatment. This balance provided the required amounts of nutrients to the rumen, which improved N utilization. In addition to improved nutrient availability, the higher DMI in this treatment may have resulted in a higher passage rate, making more substrate available to the microorganisms (Berchielli et al., 2011). This decrease in efficiency in the fourth period may be attributed to the lower production and protein content of the pasture, which limited the available nutrients and decreased microbial efficiency despite the overall benefits seen in the RUP48 treatment. In general, it can be concluded that the RUP48 treatment achieved a balanced intake of energy, RDP and RUP, resulting in improved responses related to the rumen environment.

The already mentioned higher CP intake and the higher protein status of the animals in the RUP48 treatment also contributed to the increased total protein and albumin levels in the blood. Blood protein levels are used to monitor protein intake in animals. Total protein, which includes both albumin and globulins, directly reflects protein excess or deficiency (González et al., 2000). The total protein level considered normal for an adult bovine is 6.6 - 7.5 g/dL (González et al., 2000; González and Silva,

2017). Thus, the average results of the RUP48 treatment indicate that the animals received a balanced protein intake and avoided both deficiency and excess. This balance in the overall diet contributed to better N metabolism and overall efficiency. The values obtained in the present study were lower than those of Castro et al. (2023), who found values closer to the recommended limit (7.5 g/dL) when testing different protein levels (12%, 24% and 36%) in crossbred Holstein×Gyr heifers on pasture. These results suggest that the inclusion of RUP in the animal's diet matched to pasture quality helps to regulate the amount of NNP and RDP available to the animal. This approach prevents the animal from reaching a state of excess circulating protein (hyperproteinemia).

Another parameter used to assess protein status is the aforementioned albumin. In the present study, there was an interaction between treatment and time, with animals in the RUP48 treatment having higher levels in the second period (42 d after the start of the experiment). Albumin makes up about 50% of total protein and is important as a protein reserve for the animals, as well as transporting free fatty acids, amino acids and other substances (González and Scheffer, 2018). Its deficit or excess is directly related to the long-term availability of protein in the diet. The values observed in the RUP48 treatment are within the reference range of 2.5 - 3.8 g/dL (González et al., 2000; González and Silva, 2017). This increase in albumin in the RUP48 treatment during the second period is closely related to the increase in the CP content of the pasture. This increase in pasture CP content was significant from the first to the second period and was attributed to improved management and fertilization. Despite the above-mentioned increase in pasture CP content, the addition of RUP helped to regulate albumin synthesis.

The enchanced absorption, digestibility, and performance due to the better amino acid supply also led to greater uterine tone, which improved with increasing RUP levels. The positive effect of RUP on reproduction is attributed to the lower energy loss associated with higher RDP availability. In cases where RDP is not used efficiently or is present in excess, energy is expended to excrete it, which can negatively affect reproductive performance (Tessari and Tessari, 2022). In addition, the intake of proteins, especially RUP, increases IGF-1 synthesis in the liver. Increased IGF-1 levels stimulate the release of GnRH, which in turn triggers the release of reproductive hormones. These hormones significantly influence the development of the reproductive tract (D'Occhio et al., 2019).

The lower expression of the enzyme GOT1 with increasing RUP levels suggests that the additional RUP provided a lower amount of free RDP that could not otherwise be adequately utilized. This suggests that the added RUP was used more effectively, minimizing the need to convert excess RDP to ammonia and thus reducing the burden on the liver for N metabolism. To avoid intoxication, this RDP would be absorbed through the rumen wall and recycled in the liver. The process of urea recycling is highly variable and is influenced by several factors, including the type of diet (such as the ratio of roughage to concentrate and the level of rumen degradable proteins and carbohydrates) and the level of intake (Kozloski, 2021). Therefore, an excess of RDP in the diet leads to an increased amount of rumen ammonia that is recycled. This process is also very energy consuming, as the formation of a single molecule of urea from 2 molecules of ammonia in the liver requires the use of 2 moles of ATP. However, high levels of RDP can lead to both N and energy losses that could otherwise be used for the maintenance and growth process of the animal (Berchielli et al., 2011).

An increase in RUP levels impaired IGF-1 synthesis and led to its decrease. This decrease could be related to liver overload caused by an imbalance between energy and protein, as the amino acids reaching the liver were larger. This may have led to a higher energy expenditure to control this excess, which impaired the synthesis of IGF-1 and the IGFBP-3 binding protein. Additionally, this pattern may be a result of a synthesis of IGF-1 in other tissues, like ovarium (Lucy, 2011).

CONCLUSIONS

Supplementing dairy heifers in a grazing system resulted in improved intake, digestibility and overall performance. In addition, protein supplementation provided a greater development of parenchymal tissue in the mammary gland, due to the higher protein-energy ratio. Among the levels tested in this study, a concentrate with 48% RUP of total CP was found to be the optimal recommendation for crossbred Holstein×Gyr dairy heifers in grazing systems with the BRS Quênia cultivar with an average protein level of 14%, as it resulted in improved intake, performance and N utilization. The higher MP intake from the 48% RUP treatment resulted in greater N retention, improving utilization and reducing excretion into the environment.

In addition to the performance improvements, the higher N retention provided to the group with 48% of RUP, will have a lower environmental impact by generating less N excretion to the soil, which will reduce the pollution of the water table, rivers and lakes.

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, & M. I. Marcondes. 2022. Performance and feeding behavior of Holstein and Holstein × Gyr crossbred heifers grazing temperate forages. Trop Anim Health Prod, *54*(2), 100. https://doi.org/10.1007/s11250-022-03106-w

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

Albino, R. L., S. E. F. Guimarães, K. M. Daniels, M. M. S. Fontes, A. F. Machado, G. B. Santos, and M. I. Marcondes. 2017. 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

Alves, C. P., B.J. Cirino, A. K. P. Rocha, D. S. M. Vieira, D. S. Eugênio, e M. L. M. V. Leite .2021. Respostas morfofisiológicas das plantas forrageiras sob manejo de cultivo e pastejo: Uma revisão. Res Soc Dev, 10, e10610615405. https://doi.org/10.33448/rsd-v10i6.15405

Barbero, R. P., E. B. Malheiro, 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. V. 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 cattle1. J. Anim Sci, *89*:510–519. https://doi.org/10.2527/jas.2009-2366

Bascom, S. A., R. E. James, M. L. McGilliard, & M. Van Amburgh. 2007. Influence of Dietary Fat and Protein on Body Composition of Jersey Bull Calves. J Dairy Sci, *90*(12), 5600–5609. https://doi.org/10.3168/jds.2007-0004

Batista, E. D., E. Detmann, E. C. Titgemeyer, S. C. V. 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 forage1. J. Anim Sci, 94: 201–216. https://doi.org/10.2527/jas.2015-9493

Berchielli, T. T., A. V. Pires, e S. G. Oliveira. 2011. Metabolismo de proteínas. Páginas 265-297 em Nutrição de ruminantes.2 ed. T. Berchielli, A. Pires, & S. Oliveira. Editora Funep.

Bethard, G. L., R. E. James, & M. L. McGilliard .1997. Effect of Rumen-Undegradable Protein and Energy on Growth and Feed Efficiency of Growing Holstein Heifers. J Dairy Sci, *80*(9), 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 ruminants2. J. Anim Sci, 73(5), 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. 2023a. Metabolic responses and performance of Holstein × Gyr heifers grazing *Brachiaria* decumbens supplemented with varied crude protein levels. PLoS one, 18(8), e0289747. https://doi.org/10.1371/journal.pone.0289747

Castro, M. M. D., T. J. DeVries, A. F. Machado, P. V. F. Correa, and M. I. Marcondes. 2023b. Expression of enzymes involved in the urea cycle and muscle and mammary gland development of Holstein × Gyr heifers in a rotational grazing system supplemented with increasing protein levels. J. Dairy Sci., 106(10), 6951–6960. https://doi.org/10.3168/jds.2022-22969

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. https://www.researchgate.net/publication/265323654

Chizzotti, M. L., S. C. V. 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

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., and S. C. V. Filho. 2010. On the estimation of non-fibrous carbohydrates in feeds and diets. Arq Bras Med Vet Zootec, 62: 980–984. https://doi.org/10.1590/S0102-09352010000400030

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

Detmann, E., L. Silva, G. Rocha, M. Palma, and J. Rodrigues .2021. Métodos para análise de alimentos .2° ed.Suprema

Detmann, E., M. F. Paulino, S. C. V. Filho, and P. Huhtanen. 2014a. Nutritional aspects applied to grazing cattle in the tropics: a review based on Brazilian results. Semin Cienc Agrar, 35(4Supl), 2829. https://doi.org/10.5433/1679-0359.2014v35n4Suplp2829

Franco, M. O., E. Detmann, S. C. V. Filho, E. D. Batista, L. M. A. Rufino, M. M. Barbosa, and A. R. Lopes. 2016. Intake, digestibility, and rumen and metabolic characteristics of cattle fed low-quality tropical forage and supplemented with nitrogen and different levels of starch. Asian-Australasian J. Anim Scis, 30: 797–803. https://doi.org/10.5713/ajas.16.0629

Gomide, C. A. M., J. A. Gomide, e 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. e S. C. Silva. 2017. Perfil bioquímico sanguíneo. Páginas 463-515 em Introdução à bioquímica clínica veterinária. 3 ed. Editora UFRGS.http://hdl.handle.net/10183/237269

González, F. H. D., e J. F. Scheffer. 2018. Perfil sanguíneo: ferramenta de análise clínica, metabólica e nutricional. Páginas 30-45 em Doze leituras em bioquímica clínica veterinária. 1 ed. Editora LACvet. Félix H.D. González.

González, F. H. D., J. Barcellos, H. Ospina, P. Luiz, e A. Ribeiro. 2000. Indicadores do metabolismo proteico utilizados nos perfis metabólicos de rebanhos. Páginas 23-30 em Perfil metabólico em ruminantes seu uso em nutrição e doenças nutricionais. Félix H. D. González, Julio Barcellos, Harold Ospina Patiño e Luiz Alberto Ribeiro.Editora UFRGS.

Hailemariam, S., S. Zhao, Y. He, & J. Wang. 2021. Urea transport and hydrolysis in the rumen: A review. Animal Nutrition, *7*(4), 989–996. https://doi.org/10.1016/j.aninu.2021.07.002

Hutchison J.L., P.M. VanRaden, D.J. Null , J.B. Cole , D.M. Bickhart. 2017. Genomic evaluation of age at first calving. J Dairy Sci. 100: 1–9. https://doi.org/10.3168/jds.2016-12060.

Jank, L., C. M. S. Andrade, R. B. Amorim, M. Claudio, M. Macedo, J. R. Valério, J. Verzignassi, A. H. Zimmer, C. D. Fernandes, M. F. Santos, e R. M. Simeão .2017. O capim-BRS Quênia (*Panicum maximum* Jacq.) na diversificação e intensificação das pastagens Técnico Comunicado. 1–20.

Kozloski, G. V.2021. Digestão, absorção e matabolismo visceral. Páginas 113-177 em Bioquímica dos ruminantes. 3 ed. Editora da UFSM.

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 2015. Nutritional Performance of Cattle Grazing during Rainy Season with Nitrogen and Starch Supplementation. Asian-Australasian J. Anim Scis, 29:1120–1128. https://doi.org/10.5713/ajas.15.0514

Lowe, J. K., 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

Lucy Matthew C. (2011) Growth hormone regulation of follicular growth. Reproduction, Fertility and Development 24, 19-28. https://doi.org/10.1071/RD11903

Machado, A. F., S. E. F. Guimarães, J. D. Guimarães, G. M. Santos, A. L. Silva, Y. F. R. S. Silva, D. S. L. 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(10), 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(9), e0221651. https://doi.org/10.1371/journal.pone.0221651

Miranda, M., J. L. Benedito, B. Gutiérrez, M. García-Vaquero, I. Blanco-Penedo, and M. L. Pez- Alonso.2010. The interlobular distribution of copper in the liver of beef calves on a high-copper diet. J Vet Diagn Invest (Vol. 22).

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

Nasem. 2021. National Academies of Sciences, Engineering, and Medicine. https://doi.org/https://doi.org/10.17226/25806

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. In J. Vet. Med. Sci (Vol. 73, Issue 1).

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, A. P., M. de Fatima, 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, & 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*(12), 11020–11032. https://doi.org/10.3168/jds.2017-14223

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

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

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. V. 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, P. P. Rotta. 2022. Intake, performance, and feeding behavior of Holstein and Holstein × Gyr heifers grazing intensively managed tropical grasses during the rainy season. Anim Feed Sci Technol, *16*(9), 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., & N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis1. 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. V. Filho, M. F. Paulino, L. F. C. 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, Thomas P. Sample size determination and power. John Wiley & Sons, 2013.

Sampaio, C. B., E. Detmann, I. Lazzarini, M. A. Souza, M. F. Paulino, and S. C. V. Filho. 2009. Rumen dynamics of neutral detergent fiber in cattle fed low-quality tropical forage and supplemented with nitrogenous compounds. Revista Brasileira de Zootecnia, 38: 560–569. https://doi.org/10.1590/S1516-35982009000300023

SAS Institute. 2024. SAS OnDemand for Academics. Available from https://www.sas.com/en_us/software/on-demand-for-academics.html

Sguizzato, A. L. L., S. E. F. Guimarães, G. M. Santos, E. F. Santos, and M. I. Marcondes. 2024. Is bovine somatotropin an alternative strategy to overcome the detrimental effects of high-gain diets on prepubertal Holstein × Gyr heifers? PLoS one, 19(4), e0300728. https://doi.org/10.1371/journal.pone.0300728

Shapiro, S. S., and M. B. Wilk .1965. An analysis of variance test for normality (complete samples). Biometrika, 52(3/4), 591–611.

Silva, A. L., E. Detmann, J. Dijkstra, A. M. Pedroso, L. H. P. Silva, A. F. Machado, F. C. Sousa, G. B. 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.

Silva, L. H. R. 2024. Technology-driven improvements in Dairy calf and heifer management: performance and ecnomic impacts. 104 p. Dissertação (Mestrado em Ciências Agrárias) – Universidade Federal de Viçosa.

Sotelo, D., M. F. Paulino, L. N. Rennó, E. Detmann, R. M. Ortega, D. C. Marquez, L. S. Martins, D. M. Almeida, J. A. C. Lima, and F. H. Moura. 2019. Performance and metabolic status of grazing beef heifers receiving increasing protein supplementation pre- and postpartum. Animal Production Science, 59: 1244. https://doi.org/10.1071/AN17485

Souza, M. A., E. Detmann, M. F. Paulino, C. B. Sampaio, Í. Lazzarini, and S. C. V. 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(6), 1299–1310. https://doi.org/10.1007/s11250-010-9566-6

Souza, V. C., J. D. Messana, E. D. Batista, K. L. G. C. Alves, Lino Dias, A. V., L. M. Campos, L. O. Lima, Y. T. Granja-Salcedo, L. R. Faria, G. M. Carvalho, A. V. Pires, and T. T. Berchielli . 2020. Assessing amino acid utilization in young Nellore steers fed high-concentrate diets with different sources and levels of nitrogen. Anim Feed Sci Technol, 269, 114642. https://doi.org/10.1016/j.anifeedsci.2020.114642

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., e F. A. Tessari 2022. Suplementação proteica e nitrogenada vs. reprodução de fêmeas bovinas em pastejo: Revisão. Pubvet, 16(08). https://doi.org/10.31533/pubvet.v16n08a1193.1-12

Thissen, J.P., J.M. Ketelsegers, & L. E. Underwood.1994. Nutritional Regulation of the Insulin-Like Growth Factors*. Endocr Rev, *15*(1), 80–101. https://doi.org/10.1210/edrv-15-1-80

Titgemeyer, E. C., C. K. Armendariz, D. J. Bindel, , R. H. Greenwood, & C. A. Löest. 2001. Evaluation of titanium dioxide as a digestibility marker for cattle. J Anim Sci, *79*(4), 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

Van Soest PJ. 1994. Nitrogen metabolism. Page 290-311 in Nutritional Ecology of the Ruminant . 2 ed. Ithaca: Cornell University Press.

TABLES

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

Item	Supplemented treatments		
	RUP30	RUP48	RUP66
Supplement composition, g/kg of DM			
Corn grain fine ground	85.9	73.7	62.5
Bypass soybean meal	-	17.9	37.5
Soybean meal	10.6	6.7	-
Urea	3.5	1.7	-
Chemical composition, g/kg of DM			
Dry matter, as fed	919.6	913.9	915.5
Organic matter	948.3	939.8	942.0
Crude protein	232.9	247.3	251.3
Neutral detergent fiber	122.6	138.2	131.9
Indigestible neutral detergent fiber	19.5	16.5	17.6
Ether extract	45.7	39.6	36.6
Non fiber carbohydrate	610.3	577.9	513.2
RUP/CP[1]	286	479	668
Starch[2]	606.4	532.1	446.6
Digestible energy[2], Mcal/kg	3.45	3.55	3.64
MP/ME[2], g/Mcal	27.9	38.6	51.9

[1]Amount of RUP in total CP (calculated using values obtained in the *in situ* trial).
[2]Estimated by NASEM (2021)
Mineral composition: 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 (150,00 UI/kg), Vitamin D (40,000 UI/kg) and Vitamin E (1,000 UI/kg).

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/paddock	190	175	181	163
DM pasture allowance, kg/animal/d	6.78	6.26	5.68	5.27
Chemical composition, g/kg of DM[2]				
Dry matter, as fed	254.9	205.2	244.8	251.6
Organic matter	908.8	908.6	912.8	911.5
Crude protein	95.7	144.2	146.6	135.8
RUP/CP[3]	405.3	357.5	345.3	384.0
Neutral detergent fiber	715.9	708.2	702.8	697.6
Indigestible neutral detergent fiber	166.0	132.4	153.5	122.2
Ether extract	13.5	14.2	11.1	12.2
Non fiber carbohydrate	89.9	54.6	51.8	63.6

[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]Amount of RUP in total CP (calculated using values obtained in the *in situ* trial).

Table 3. Genes, primers sequence and access number used for liver tissue samples

Genetic symbol	Sequence	NCBI
IGFBP3[1]	Forward TTACAAGAAAAAGCAGTGCCGC Reverse TGCCCGTACTTATCCACACAC	NM_174556.1
IGF1[2]	Forward TGCACTTCAGAAGCAATGGGA Reverse GGCATCTTCACCTGCTTCAA	NM_001077828.1
CPS1[3]	Forward GGCTTAACCAATGTGACGGC Reverse TGTGTGCTGTTTGTGCCTTG	NM_001192258.1
GOT1[4]	Forward GGCTTAACCAATGTGACGGC Reverse TACTCAACCTGCTTGGGGTTC	NM_177502.2
GAPDH[5]	Forward AAGGTCGGAGTGAACGGATTC Reverse ATGGCGACGATGTCCACTTT	NM_001034034.2

[1]insulin-like growth factor-binding protein 3; [2]Insulin-like growth factor; [3]carbamoyl phosphate synthetase; [4]glutamic transaminase - oxaloacetic (Aspartate aminotransferase); [5]glyceraldehyde-3-phosphate dehydrogenase.

Table 4. Intake and apparent digestibility of nutrients of grazing crossbred Holstein×Gyr dairy heifers, either not supplemented or supplemented with different levels of rumen undegradable protein

Item[1]	Treatments[2]				SEM[3]	P-value[4]				
	CON	RUP30	RUP48	RUP66		P	T×P	S	L	Q
Intake										
Supplement DM, kg/d	-	1.19	1.31	1.30	0.16	<0.01	0.30	-	<0.01	0.09
Pasture DM, kg/d	5.53	5.17	5.54	4.93	0.26	<0.01	0.36	0.07	0.34	0.02
DM, kg/d	5.53	6.24	6.86	6.25	0.35	<0.01	0.75	<0.01	0.98	0.06
Pasture, g/kg BW	20.0	18.6	18.8	16.8	1.59	<0.01	0.18	0.08	0.05	0.15
DM, g/kg BW	20.0	22.6	23.2	21.2	1.56	<0.01	0.25	0.02	0.11	0.10
OM, kg/d	4.92	5.17	6.46	5.76	0.64	<0.01	0.69	0.81	0.33	0.58
CP, kg/d	0.71	0.95	1.04	0.97	0.05	<0.01	0.94	<0.01	0.57	0.01
NDF, kg/d	3.93	3.79	4.08	3.65	0.19	<0.01	0.36	0.53	0.43	0.02
RDP, g/d	328	396	476	442	35.2	0.10	0.05	0.08	0.31	0.16
RUP, g/d	378	583	585	540	57.1	<0.01	0.05	<0.01	0.42	0.62
MP, g/d	515	710	765	718	49.7	<0.01	0.60	<0.01	0.76	0.04
CP:DOM, g CP/kg DOM	227	290	254	259	22.3	<0.01	0.21	<0.01	0.02	0.07
Apparent digestibility, g/kg										
DM	615	640	647	648	7.63	<0.01	0.17	<0.01	0.24	0.58
NDF	725	718	726	734	4.31	<0.01	0.05	0.73	0.00	0.99
CP	692	737	719	729	11.5	<0.01	0.14	<0.01	0.45	0.12
OM	638	640	675	679	25.6	<0.01	0.15	0.01	0.00	0.15

[1]DM= Dry matter; OM= Organic matter; CP= Crude protein; NDF=Neutral detergent fiber; RDP= Rumen degradable protein; RUP= Rumen undegradable protein; MP= Metabolizable protein; DOM= Digestible organic matter.
[2]CON=Control; RUP30= supplement with for 30% of RUP, RUP48= supplement with for 48% of RUP, RUP66= supplement with for 66% of RUP;
[3]SEM= Standard error of the mean;
[3]P= Sampling period effect; T×P= Interaction between treatment and sampling period; S= Effect of supplement versus non-supplemented animals; L= Linear effect among RUP levels; Q=Quadratic effect among RUP levels.

Table 5. Body weight and performance of grazing crossbred Holstein×Gyr dairy heifers, not supplemented and supplemented with different levels of rumen undegradable protein

Item[1]	Treatments[2]				SEM[3]	P-value[4]		
	CON	RUP30	RUP48	RUP66		S	L	Q
Initial BW, kg	277	274	276	275	33.8	0.77	0.91	0.92
Final BW, kg	303	315	330	320	5.09	<0.01	0.43	0.01
ADG, kg/d	0.17	0.32	0.49	0.37	0.06	<0.01	0.43	0.01
BCSi	3.05	3.07	3.10	3.12	0.08	0.75	0.58	0.68
BCS	3.02	3.16	3.12	3.22	0.09	0.01	0.28	0.11
Body measurements, cm								
Withers height	125	124	126	125	0.82	0.87	0.55	0.21
Thoracic perimeter	157	163	169	166	2.02	<0.01	0.29	0.05
Body length	109	113	111	113	2.89	0.06	0.97	0.36
Rump height	127	129	130	130	0.67	0.04	0.08	0.55

[1]BW=Body weight; ADG=Average daily gain, BCSi=Initial body condition score; BCS= body condition score;
[2]CON=Control; RUP30= supplement with for 30% of RUP; RUP48= supplement with for 48% of RUP; RUP66= supplement with for 66% of RUP;
[3]SEM= Standard error of the mean;
[4]S= Effect of supplement versus non-supplemented animals; [3]L= Linear effect among RUP levels; [4]Q=Quadratic effect among RUP levels.

Table 6. Microbial protein synthesis and nitrogen balance of grazing crossbred Holstein×Gyr dairy heifers, either not supplemented or supplemented with different levels of rumen undegradable protein

Item	Treatments[1]				SEM[2]	P-value[3]				
	CON	RUP30	RUP48	RUP66		P	T×P	S	L	Q
Nitrogen balance, g/d										
Intake	114	153	167	146	9.4	<0.01	0.94	<0.01	0.57	0.01
Fecal excretion	33.9	41.0	44.9	40.4	1.48	<0.01	0.41	<0.01	0.77	0.01
Urinary excretion	61.9	82.7	71.1	85.4	0.16	0.02	0.14	0.01	0.76	0.08
Urinary Urea, mg/d[4]	175	209	213	193	8.69	<0.01	<0.01	-	-	-
Retained	18.9	28.0	41.7	26.7	7.96	<0.01	0.73	0.08	0.88	0.08
Efficiency of nitrogen use, g/g	0.12	0.16	0.25	0.13	0.05	0.01	0.64	0.27	0.57	0.05
Microbial synthesis										
Microbial protein, g/d	328	396	476	442	35.2	0.10	0.05	0.08	0.31	0.16
Microbial efficiency, g/Kg DOM[4]	109	120	120	118	15.4	<0.01	0.02	-	-	-
N use for microbial synthesis, g/g[4]	0.45	0.41	0.46	0.47	0.03	<0.01	0.01	-	-	-

[1]CON=Control; RUP30= supplement with for 30% of RUP, RUP48= supplement with for 48% of RUP, RUP66= supplement with for 66% of RUP;
[2]SEM= Standard error of the mean;
[3]P= Sampling period effect; T×P= Interaction between treatment and sampling period; S= Effect of supplement versus non-supplemented animals; L= Linear effect among RUP levels; Q=Quadratic effect among RUP levels.
[4]P-values were not shown due to the interaction effect between treatment and sampling period. The interaction decompositions are shown in Figs. 2 and 3.

Table 7. Mammary gland pixel count, blood parameters, and reproductive evaluation of grazing crossbred Holstein×Gyr dairy heifers, either not supplemented or supplemented with different levels of rumen undegradable protein

Item[1]	Treatments[1]				SEM[2]	P-value[3]				
	CON	RUP30	RUP48	RUP66		P	T×P	S	L	Q
Mammary gland pixel value										
Log of pixels, mm²	41.5	36.6	34.5	38.6	2.44	0.15	0.89	0.04	0.49	0.23
Blood parameters										
Total Protein, g/dL	6.18	5.72	6.31	6.00	0.15	0.44	0.09	0.33	0.20	0.01
Glucose, mg/dL	64.1	65.3	67.0	63.1	1.42	0.02	0.53	0.49	0.24	0.07
Albumin, g/dL[5]	2.53	2.49	2.61	2.59	0.06	0.35	0.04	-	-	-
Urea, mg/dL	21.6	34.2	35.1	29.1	2.48	<0.01	0.61	<0.01	0.20	0.07
IGF-1, ng/mL	139	175	202	183	21.4	0.28	0.61	0.03	0.74	0.25
Reproductive Evaluation										
Average horn diameter, cm	1.15	1.21	1.26	1.31	0.06	0.09	0.73	0.03	0.18	0.57
Tonus	1.88	2.16	2.43	2.57	0.32	<0.01	0.68	0.00	0.03	0.67
Reproductive tract score	1.48	1.84	1.82	2.02	0.24	0.34	0.90	0.03	0.54	0.63

[1]CON=Control; RUP30= supplement with for 30% of RUP, RUP48= supplement with for 48% of RUP, RUP66= supplement with for 66% of RUP;
[2]SEM= Standard error of the mean;
[3]P= Sampling period effect; T×P= Interaction between treatment and sampling period; S= Effect of supplement versus non-supplemented animals; L= Linear effect among RUP levels; Q=Quadratic effect among RUP levels.
[3]P-values were not shown due to the interaction effect between treatment and sampling period. The interaction decompositions is shown in Fig. 3.

Table 8. Liver gene expression of grazing crossbred Holstein×Gyr dairy heifers, either not supplemented or supplemented with different levels of rumen undegradable protein

Item[1]	Treatments[2]				SEM[3]	P-value[4]		
	CON	RUP30	RUP48	RUP66		S	L	Q
CPS1	2.71	3.23	3.36	2.91	0.27	0.13	0.55	0.37
GOT1	0.09	0.08	0.01	0.01	0.00	<0.01	<0.01	<0.01
IGF-1	0.06	0.04	0.02	0.02	0.00	0.01	0.01	0.53
IGFBP3	0.35	0.40	0.02	0.03	0.01	<0.01	<0.01	<0.01

[1]Carbamoyl-phosphate synthase 1; Glutamic-oxaloacetic transaminase 1; Insulin-like Growth Factor; Insulin-like Growth Factor Binding Protein 3;
[2]CON=Control; RUP30= supplement with for 30% of RUP, RUP48= supplement with for 48% of RUP, RUP66= supplement with for 66% of RUP;
[3]SEM= Standard error of the mean;
[4]S= Effect of supplement versus non-supplemented animals ; L= Linear effect among RUP levels; Q=Quadratic effect among RUP levels.

FIGURES

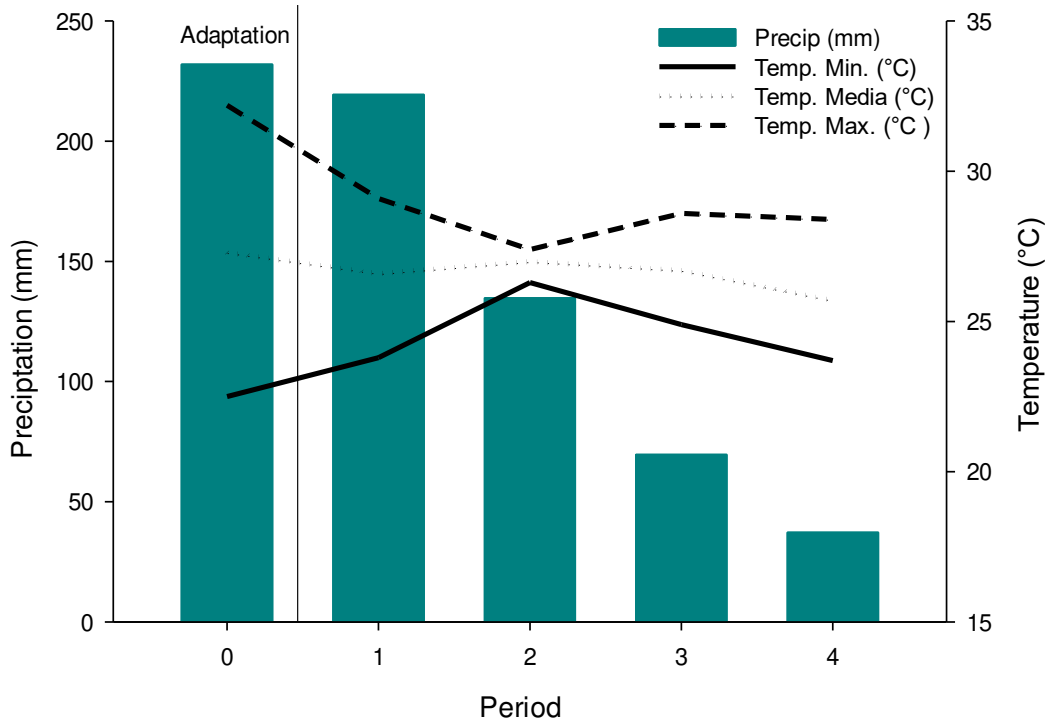

Figure 1. Precipitation data and minimum, average and maximum temperatures were recorded during the adaptation period (January 4 to 18) and the experimental sub-periods: one (January 19 to February 8), two (February 9 to March 1), three (March 2 to 22), and four (March 23 to April 12). Source: INMET. Retrieved on: https://tempo.inmet.gov.br/TabelaEstacoes/A001.

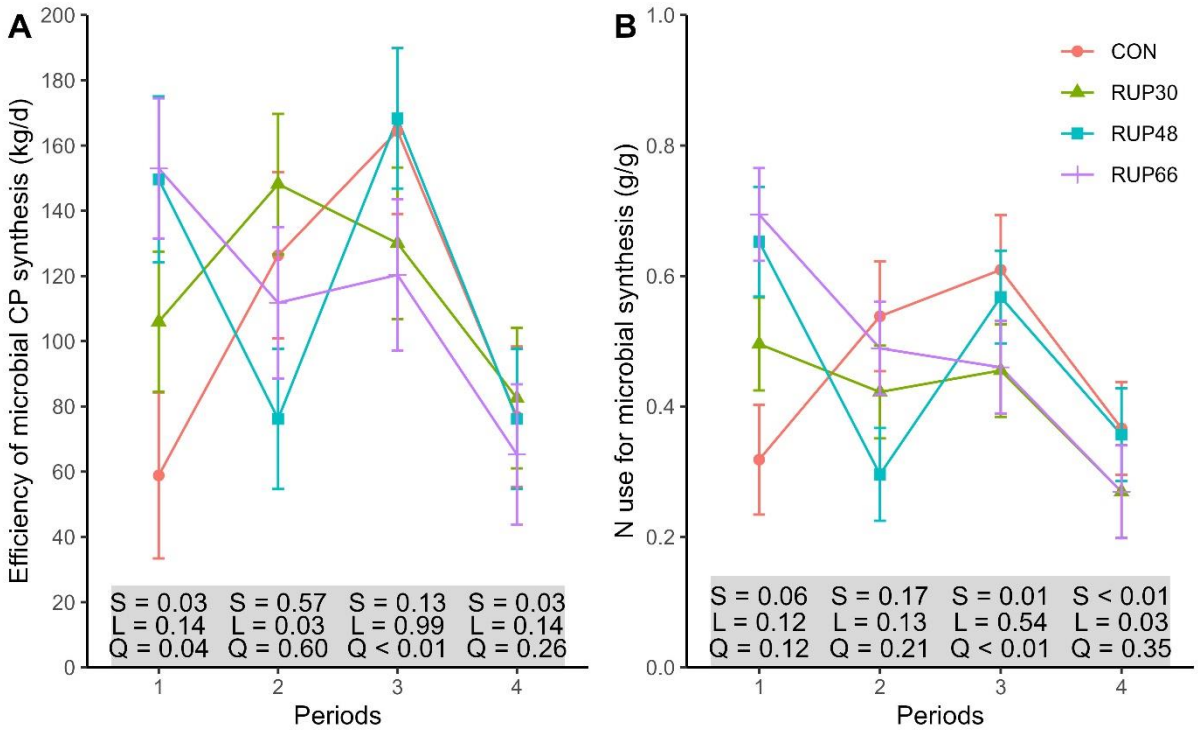

Figure 2. Interaction effects of sampling period (time) and treatment for the efficiency of microbial CP synthesis (**A**) and for N utilization for microbial synthesis (**B**). Within each period, "S" represents the effect of supplement versus non-supplemented animals, "L" represents the linear effect of RUP, and "Q" represents the quadratic effect of RUP.

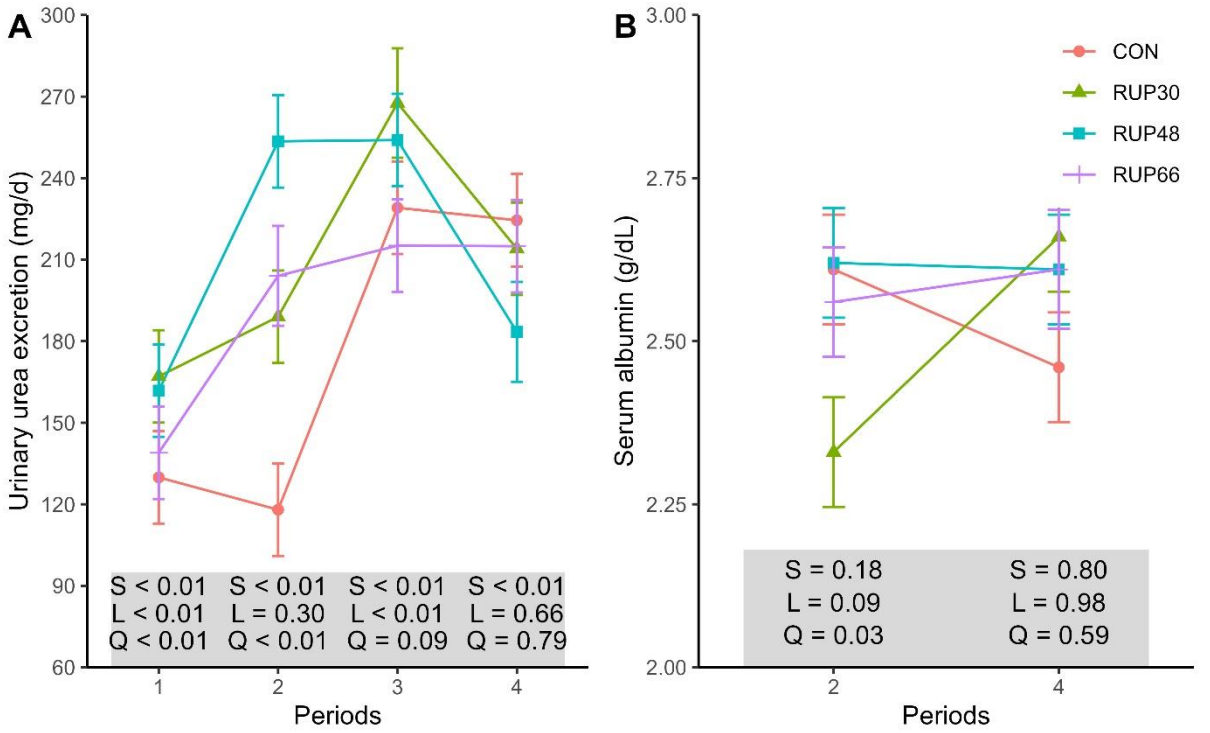

Figure 3. Interaction effects of sampling period (time) and treatment for the urinary urea excretion (**A**) and serum albumin concentration (**B**). Within each period, "S" represents the effect of supplement versus non-supplemented animals, "L" represents the linear effect of RUP, and "Q" represents the quadratic effect of RUP.