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Effect of increased levels of non-protein nitrogen in the diet on productive performance, mammary gland development, and nitrogen metabolism of dairy heifers

Ana Carolina Oliveira Ribeiro
Magister Scientiae

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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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Assent:

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This work is dedicated to my parents.

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ABSTRACT

RIBEIRO, Ana Carolina Oliveira, M.Sc., Universidade Federal de Viçosa, July, 2025. **Effect of increased levels of non-protein nitrogen in the diet on productive performance, mammary gland development, and nitrogen metabolism of dairy heifers**. Adviser: Alex Lopes da Silva. Co-advisers: Polyana Pizzi Rotta, Simone Eliza Facioni Guimaraes and Luciana Navajas Renno.

The objective was to evaluate the effects of increasing dietary non-protein nitrogen (NPN) on performance, mammary gland development, and nitrogen metabolism in dairy heifers. Fifteen heifers (7 Holstein and 8 Holstein × Gyr; 259.9 ± 7.5 kg) were assigned to a randomized complete block design with breed as a blocking factor and allocated to diets containing 26%, 42%, or 58% of crude protein (CP) as NPN. The trial lasted 84 days. Intake, digestibility, microbial protein synthesis, blood metabolites, water intake, growth performance, and hepatic gene expression were evaluated. Dry matter (DM) and CP digestibility showed a quadratic response, with greater values at 26% and 58% NPN. Neutral detergent fiber (NDF) and organic matter (OM) digestibility decreased linearly as NPN increased. The 42% NPN treatment improved microbial protein synthesis efficiency and increased mammary parenchymal area. Serum total protein exhibited a quadratic response, with the lowest value at 42% NPN, whereas serum urea concentration was highest at this level. Alanine aminotransferase (ALT) decreased linearly with increasing NPN inclusion. Hepatic carbamoyl phosphate synthetase I (CPS1) expression showed a quadratic response, with lower expression at 42% NPN. Moderate dietary NPN inclusion improved nitrogen metabolism and mammary development without impairing overall growth performance.

Keywords: intake; growth; nitrogen

RESUMO

RIBEIRO, Ana Carolina Oliveira, M.Sc., Universidade Federal de Viçosa, julho de 2025. **Efeito de níveis crescentes de nitrogênio não proteico na dieta de novilhas leiteiras sobre os desempenhos produtivo, o desenvolvimento da glândula mamária e a excreção de nitrogênio**. Orientador: Alex Lopes da Silva. Coorientadores: Polyana Pizzi Rotta, Simone Eliza Facioni Guimaraes e Luciana Navajas Renno.

O objetivo foi avaliar os efeitos do aumento do NNP na dieta sobre o desempenho, o desenvolvimento da glândula mamária e o metabolismo do N em novilhas leiteiras. Quinze novilhas (7 Holandesas e 8 Holandês × Gir; 259,9 ± 7,5 kg) foram distribuídas em delineamento de blocos casualizados, com raça como fator de bloqueio, e alocadas em dietas contendo 26%, 42% ou 58% da PB na forma de NNP. O experimento durou 84 dias. Foram avaliados consumo, digestibilidade, síntese de proteína microbiana, metabólitos sanguíneos e a expressão hepática de genes associados ao metabolismo proteico. A digestibilidade da MS e da PB apresentou resposta quadrática, com maiores valores nos níveis de 26% e 58% de NNP. A digestibilidade da FDN e da MO diminuiu linearmente com o aumento do NNP, assim como o consumo de água. O tratamento com 42% de NNP melhorou a eficiência de síntese de proteína microbiana e aumentou a área do parênquima mamário. A proteína total sérica e a expressão gênica de CPS-1 apresentou resposta quadrática, com menor valor no tratamento 42% NNP, enquanto a concentração de ureia sérica foi maior nesse nível. A ALT diminuiu linearmente. A inclusão moderada de NNP favoreceu o metabolismo do N e o desenvolvimento mamário sem comprometer o desempenho.

Palavras-chave: crescimento; consumo; nitrogênio

Summary

The text is formatted according to the Animal Science Journal guidelines

INTRODUCTION

The efficiency of dairy heifer rearing is one of the main determinants of profitability in milk production systems. Reducing age at first calving, thereby decreasing non-productive days, allows for a faster financial return to the producer and improves overall system efficiency (Silva et al., 2025; Van Niekerk et al., 2021). However, the rearing phase represents a substantial proportion of total dairy production costs and often does not receive the same strategic attention as lactating cows (Silva et al., 2025). Within this context, nutrition constitutes the primary cost component during the growth phase, making it a key target for interventions aimed at improving both biological and economic efficiency. Therefore, identifying nutritional strategies that reduce feeding costs without compromising performance, body development, and future productive potential is essential for the sustainability of dairy operations.

According to Brown et al. (2005), the protein requirement of dairy heifers can be met relatively simply, as microbial protein can supply a large proportion of the metabolizable protein required for growth. This characteristic provides flexibility in diet formulation and allows the inclusion of lower-cost ingredients. In this scenario, NPN sources, such as urea, may be incorporated into diets as a strategy to optimize the cost of the protein fraction.

Urea is highly soluble and is rapidly hydrolyzed in the rumen, releasing ammonia that can be incorporated into microbial protein. However, the efficient utilization of NPN depends on adequate synchrony between ruminal ammonia availability and fermentable energy supply. When provided at elevated levels, the rapid conversion of urea to ammonia may exceed microbial assimilation capacity, resulting in ammonia accumulation in the bloodstream and potential toxic effects (Emmanuel et al., 2015). Furthermore, because the rate of ammonia release from urea often surpasses its rate of microbial utilization, excess nitrogen may be converted into hepatic urea and excreted via urine and feces, thereby reducing nitrogen use efficiency and increasing both economic losses and environmental impact (Highstreet et al., 2010).

Although NPN utilization has been extensively studied in beef cattle and lactating dairy cows, information regarding growing dairy heifers remains limited. Chizzotti et al. (2008) reported that inclusion of up to 46.5% of NPN did not affect performance or microbial protein synthesis in beef steers. Similarly, Zhang et al. (2022) demonstrated that reducing dietary crude protein levels while increasing soluble protein proportions did not impair sheep performance and improved

nitrogen utilization efficiency, reducing environmental impact. However, extrapolation of these findings to growing dairy heifers should be made cautiously, as physiological and metabolic differences may alter responses to NPN inclusion.

Therefore, this study was conducted to evaluate the effects of increasing dietary NPN levels on intake, performance, mammary gland development, nitrogen metabolism, and hepatic expression of genes related to protein metabolism in dairy heifers.

MATERIAL AND METHODS

The experiment was conducted in the Teaching, Research, and Extension Dairy Farm of the Department of Animal Science at the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil). All animal procedures were approved by the university Ethics Committee for the Use of Farm Animals (protocol 027/2023).

Animals, Experimental Design, and Treatments

Fifteen dairy heifers (7 Holstein and 8 Holstein × Gyr crossbred) with an average initial BW of 259.9 ± 7.50 kg and 10 ± 1 months of age were used in this trial. The study used an unbalanced randomized block design with genetic groups as the blocking factor. Animals were randomly assigned to one of 3 dietary treatments (n = 5 per treatment): 1) control diet with 26% CP as NPN (26NPN), 2) diet with 42% CP as NPN (42NPN), and 3) diet with 58% CP as NPN (58NPN). The increasing proportions of NPN among treatments were achieved by progressively increasing urea inclusion in the diets. Diets were formulated to meet the nutritional requirements (NASEM,2021), targeting average daily gain (ADG) of 0.85 kg/d, while maintaining a metabolizable protein to metabolizable energy ratio of 42 ± 1 g/Mcal for all treatments (Table 1).

The experiment lasted 84 days and was divided into four 21-day periods. Prior to the initiation of the trial, animals underwent a 15-day acclimation period to the experimental facilities and handling procedures. Animals were housed in covered individual pens (10 m² each), each equipped with a feeder and an automatic waterer.

Intake and Performance

The total mixed ration (TMR) was offered once daily at 0700 h from the acclimation period through the end of the experimental period. Dry matter intake (DMI) was quantified as the

difference between feed offered and orts from the previous day**.** Feed allowance was adjusted to allow for approximately 5% orts on an as-fed basis. Daily water intake was measured individually using flow meters (Unijato 1/2 in. Dn15 Qn 0.75; Hidrometer, São Paulo, Brazil).

Between days 15 and 18 of each experimental period, samples of corn silage offered and orts from each animal were collected to determine DM and nutrient intake. Daily ort samples were homogenized, quartered, and a 500g subsample was collected from each animal. These subsamples were packaged in plastic bags, properly labeled, and stored at −20°C on each sampling day. At the end of each period, frozen daily subsamples were thawed, thoroughly mixed, and composed by animals for subsequent analyses. Experimental concentrates were prepared in two separate batches, and representative samples were collected from each batch during concentration preparation. Between days 16 and 19 of periods 2 and 4, eight spot fecal samples were collected at 12-h intervals according to the following schedule: d 16 at 0300 and 1500 h; d 17 at 0600 and 1800 h; d 18 at 0900 and 2100 h; and d 19 at 1200 and 0000 h. All fecal samples were collected directly from the rectum of each animal.

Silage, orts, and fecal samples were subjected to partial drying for 72 h at 55°C (INCT-CA method G-001/2). Dried samples were first ground in a knife mill through a 2-mm sieve, after which an aliquot was taken. The remaining material was subsequently reground through a 1-mm sieve for laboratory analyses. Samples ground through 1-mm sieves were analyzed for DM (INCT-CA method G-003/2), CP (INCT-CA method N-001/3), ash (INCT-CA method M-001/3), ash-corrected NDF (INCT-CA methods F-002/3 and M-002/2), starch (INCT-CA method G-007/1) and NPN (INCT-CA method N-002/3) according to Detmann et al. (2025). Samples ground through 2-mm sieves were analyzed for indigestible NDF (iNDF; INCT-CA method F-009/3) according to Detmann et al. (2025). Fecal output was estimated using iNDF as an internal marker.

To evaluate animal performance, heifers were weighed individually after a fasting period using a livestock scale (Balanças Brasil LTDA, model M1, Brazil) and measured for heart girth, withers height, and rump width at the beginning of the experiment and at the end of each period. Measurements were taken on 3 consecutive days, and the mean of the 3 values was used for statistical analysis.

Microbial Protein Synthesis and Nitrogen Excretion

On the same days and times as fecal collections, spot urine samples were obtained by stimulated urination. Urine was filtered through gauze, and a 50-mL aliquot of concentrated urine was

collected. Additionally, a 10-mL aliquot was immediately diluted in 40 mL of 0.036 N sulfuric acid. Both samples were stored at −20°C for subsequent analysis.

In concentrated urine samples, total N concentration was determined by the Kjeldahl method described by Detmann et al. (2025; INCT-CA method N-001/3). In samples diluted in sulfuric acid, concentrations of allantoin, creatinine, and uric acid were analyzed. Creatinine and uric acid were measured using an automated biochemistry analyzer (Mindray BS200E; Mindray, Shenzhen, China) by kinetic colorimetric and enzymatic colorimetric methods, respectively. Allantoin concentrations were determined using the method described by Chen and Gomes (1992). Daily urine output for each animal was estimated by dividing daily urinary creatinine excretion by urinary creatinine concentration. Daily urinary creatinine excretion was estimated using the equation of Chizzotti et al. (2008b) for dairy heifers:

$$CE = 32.2 - 0.0109 \times BW$$

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

Daily excretion of purine derivatives was calculated as the sum of urinary allantoin and uric acid excretion. Absorbed purines were estimated from purine derivatives excretion using the recovery rate of absorbed purines as purine derivatives and the endogenous purine excretion values reported by Prates et al. (2012) for heifers:

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

where AP=absorbed purines (mmol/day); DP=excretion of purine derivatives (mmol/day); 0.439× $BW^{0.75}$=endogenous contribution to purine excretion and 0.99 = recovery of absorbed purines as urinary purine derivatives.

Ruminal microbial N synthesis (Nmic, g/day) was calculated based on absorbed purines (mmol/day) according to the equation of Chen and Gomes (1992):

$$N_{mic} = \frac{70 \times AP}{0.93 \times 0.11 \times 1000}$$

where Nmic = ruminal microbial N synthesis (g/day); AP = absorbed purines (mmol/day); 70 = N content in microbial purines (mg of N/mol); 0.93 = intestinal digestibility of microbial purines according to Barbosa et al. (2011); and 0.11 = purine N to total N ratio of bacteria according to Prates et al. (2012).

Metabolizable protein from microbial origin (MPmic) was calculated using the following equation:

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

where MPmic = metabolizable protein from microbial origin (g/day); Nmic = ruminal microbial N synthesis (g/day); 6.25 = conversion factor from N to crude protein; and 0.65 = conversion factor from microbial crude protein to metabolizable microbial protein (NASEM, 2021).

Nitrogen balance was calculated as the difference between total nitrogen intake and total nitrogen excretion in feces and urine.

Mammary Gland Development and Blood Samples

At the beginning of the experiment and on the last day of each period, ultrasound images of the mammary gland were obtained to assess mammary development. Scans were performed using a B-mode ultrasound device equipped with a micro-convex transducer operating at 6 MHz (DP-20Vet; Mindray, Shenzhen, China). One image was captured from each mammary quarter, with the probe positioned at a standardized 45° angle relative to the teat insertion, always in the caudocranial direction (Nishimura et al., 2011; Albino et al., 2017).

Ultrasound images were processed using ImageJ® software (NIH, Bethesda, MD, USA), calibrated at 100 pixels/cm. For each mammary gland image, three 16-mm² squares were randomly selected adjacent to the developing mammary ducts, following the methodology described by Nishimura et al. (2011). The mean pixel value within each square was determined from 8-bit grayscale images (0 = black, 255 = white), representing brightness intensity (Albino et al., 2015). Using the same calibration, the area of the hypoechoic region, indicative of mammary parenchyma, were quantified (Vang et al., 2024).

Blood samples were collected on the first day of the experiment and on the final day of each experimental subperiod to determine serum concentrations of plasma insulin-like growth factor 1 (IGF-I), blood urea nitrogen (BUN), glucose, total protein, albumin, aspartate aminotransferase (AST), ALT and gamma-glutamyl transferase (GGT). Samples for IGF-I, BUN, total protein, albumin, AST, ALT and GGT analyses were obtained via jugular venipuncture using vacuum tubes containing clot activator and gel separator. For glucose analysis, blood was collected in disposable tubes containing sodium fluoride as an anticoagulant and stabilizer to preserve glucose integrity. Immediately after collection, tubes were placed in insulated boxes containing ice and transported

to the laboratory. Samples were centrifuged at 3,500 × *g* for 15 minutes to separate the serum. A total of five serum samples were collected from each animal, transferred into Eppendorf® tubes, and stored at -20°C until analysis.

Serum concentrations of BUN, glucose, total protein and albumin were measured using an automated biochemical analyzer (Mindray BS200E, Shenzhen, China) with commercial reagent kits (Bioclin®, Belo Horizonte, Minas Gerais, Brazil). Urea-N was determined by a fixed-time kinetic method, glucose by a colorimetric enzymatic method, albumin by the bromocresol green method, and total protein by the biuret method. Serum IGF-I concentrations were determined using a sandwich chemiluminescent immunoassay with a LIAISON® XL analyzer (DiaSorin, Italy) and the commercial LIAISON® IGF-1 kit. AST and ALT activities were determined using the kinetic colorimetric method, whereas GGT activity was measured using the modified Szasz method.

Liver Tissue Biopsy and Gene Expression Analysis

Liver tissue samples were collected on the first and final days of the experimental period for gene expression analysis of insulin-like growth factor binding protein 3 (IGFBP3), IGF-1, CPS1, and glutamic-oxaloacetic transaminase 1 (GOT1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control (Table 2). Liver tissue was sampled using the Tru-Cut needle biopsy technique. Approximately five insertions were performed per animal to obtain sufficient tissue (Pearson and Craig, 1980). Collected samples were immediately placed in cryovials and flash-frozen in liquid nitrogen. Liver tissue was sampled using the Tru-Cut needle biopsy technique. Approximately five insertions were performed per animal to obtain sufficient tissue (Pearson and Craig, 1980). Collected samples were immediately placed in cryovials and flash-frozen in liquid nitrogen.

Total RNA was extracted from liver samples using the Trizol reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. RNA concentration was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was confirmed by 1% agarose gel electrophoresis. Samples were treated with DNase I (Promega Corporation, Madison, WI, USA) and reverse-transcribed into cDNA using the GoScript™ Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA). The resulting cDNA was quantified using the NanoDrop spectrophotometer, diluted to a final concentration of 10 ng/µL, and stored at -20°C until use.

Quantitative real-time PCR (qPCR) was performed in duplicate using a CFX Opus™ 96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) and the GoTaq® qPCR Master Mix Kit (Promega Corporation, Madison, WI, USA). The cycling conditions included an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Melting curve analysis was conducted to verify amplification specificity. Negative controls were included by replacing cDNA with nuclease-free water.

Gene expression levels were normalized to GAPDH using the $2^{-\Delta Ct}$ method, where ΔCt=(Ct_target gene−Ct_endogenous gene), as described by Livak and Schmittgen (2001).

Statistical Analysis

The experiment was conducted using an unbalanced randomized block design. Statistical analyses were performed using the GLIMMIX procedure in SAS (SAS Institute Inc., 2024). For variables measured across periods, such as intake and microbial protein synthesis, the effect of collection period was included in the model as a repeated measure. The selection of the (co)variance structure was based on the corrected Akaike Information Criterion (AIC), using the following model:

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

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 also used to model the covariance structure for repeated measures within animals, B_k = random effect of block, $T \times P_{ijkT}$ = fixed effect of the interaction between treatment and sampling period, ε_{ijklm} = residual error.

Performance and gene expression variables were analyzed using an unbalanced randomized block design, with baseline measurements included as covariates in the statistical model:

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

onde: Y_{ij} = dependent variable, μ = overall mean, $\square_k$ = covariate for baseline measurement, T_i = fixed effect of block, B_j = random effect of block, ε_{ijk} = random error.

The interaction effect between treatment and block was tested for all variables; as it was not significant, it was excluded from the final model. For all models, residuals were tested for

normality using the Shapiro–Wilk test (Shapiro and Wilk, 1965). Gene expression data were analyzed assuming a log-normal distribution, and means were back-transformed for presentation. Observations with studentized internal residuals greater than |2.5| were considered "outliers" and removed from the model. Least squares means were further evaluated using orthogonal polynomial contrasts to assess linear and quadratic effects of dietary NPN levels. Statistical significance was declared at P<0.05, and P-values between 0.05 and 0.10 were considered as marginal significance.

RESULTS

Intake and Digestibility

Starch intake (kg/day and g/kg BW) and NPN intake increased linearly with increasing dietary NPN levels ($P < 0.01$). Starch intake averaged 0.796, 1.05, and 1.46 kg/day, which corresponded to 2.37, 3.29, and 4.24 g/kg BW for the 26NPN, 42NPN, and 58NPN treatments, respectively. Similarly, NPN intake increased from 0.053 to 0.080 and 0.122 kg/day for the 26NPN, 42NPN, and 58NPN treatments, respectively. In addition, a marginally significant quadratic effect was observed for RUP intake ($P = 0.07$) and MP intake ($P = 0.10$), with animals fed the 42NPN diet exhibiting the lowest intake among treatments (Table 3).

A linear decrease was observed in water intake relative to body weight (mL/kg BW; $P = 0.03$) and relative to dry matter intake (L/kg DM; $P < 0.01$) as dietary NPN levels increased. Water intake averaged 33.6, 26.1, and 24.2 mL/kg BW, and 1.58, 1.15, and 1.07 L/kg DM for the 26NPN, 42NPN, and 58NPN treatments, respectively. Moreover, water intake expressed as liters per day showed a marginally significant linear decrease with increasing dietary NPN levels ($P = 0.07$), averaging 10.3, 7.64, and 7.97 L/day for the 26NPN, 42NPN, and 58NPN treatments, respectively (Table 3).

A quadratic treatment effect was detected for the apparent digestibility of DM ($P < 0.01$) and CP ($P = 0.04$), with higher values observed in the 26NPN (699 g/kg) and 58NPN (680 g/kg) treatments compared with the 42NPN (660 g/kg) treatment. In contrast, the apparent digestibility of OM ($P = 0.04$) and NDF ($P < 0.01$) decreased linearly across treatments. Apparent OM digestibility averaged 731, 712, and 711 g/kg, whereas apparent NDF digestibility averaged 678, 642, and 611 g/kg for the 26NPN, 42NPN, and 58NPN treatments, respectively (Table 3).

Performance, Nitrogen Balance, and Microbial Protein Synthesis

No treatment effect was observed on animal performance or body measurements ($P > 0.05$; Table 4). The animals had an average initial BW of 260 kg, a final BW of 365 kg, and an ADG of 1.26 kg/day.

Nitrogen intake ($P = 0.09$) and urinary nitrogen excretion ($P = 0.06$) were marginally affected, both quadratically by NPN levels (Table 5). Nitrogen intake averaged 208, 190, and 209g/day for the 26NPN, 42NPN, and 58NPN treatments, respectively. A similar pattern was observed for urinary nitrogen excretion, with values of 129, 105, and 120 g/day for the 26NPN, 42NPN, and 58NPN treatments, respectively.

A marginal significance for interaction between treatment and collection period was observed for nitrogen retained ($P = 0.08$) and nitrogen use efficiency ($P = 0.06$). In period 2, retained nitrogen showed a linear increasing marginal significance ($P = 0.06$; Figure 1A), with the 26NPN group exhibiting lower nitrogen retention (14.57 g/day) compared with the 42NPN (40.53 g/day) and 58NPN (41.84 g/day) treatments. Nitrogen use efficiency also displayed linear behavior ($P = 0.05$; Figure 1B), with the 26NPN group having the lowest efficiency (0.07 g/g) compared with the 42NPN (0.22 g/g) and 58NPN (0.21 g/g) treatments.

A marginal significance was observed for treatment and collection period interaction for microbial protein synthesis (g/day) and microbial protein synthesis efficiency (g/kg digestible organic matter [DOM]; $P = 0.06$; Table 5). Microbial protein synthesis decreased linearly in period 2 ($P = 0.03$; Figure 2A), with the 26NPN treatment showing the highest production (481.45 g/day), followed by 42NPN (405.73 g/day) and 58NPN (340.36 g/day). Microbial protein synthesis efficiency exhibited a linear effect in period 2 ($P = 0.04$; Figure 2B) and a quadratic effect in period 4 ($P = 0.06$). In period 2, the 26NPN group had the highest efficiency (98.05 g/kg DOM), followed by 42NPN (97.52 g/kg DOM) and 58NPN (76.39 g/kg DOM). In period 4, the 26NPN treatment showed lower efficiency (71.36 g/kg DOM) compared with 42NPN (93.95 g/kg DOM) and 58NPN (84.54 g/kg DOM).

A quadratic treatment effect was observed for microbial efficiency (g/kg DOM; $P = 0.04$), with the 42NPN treatment exhibiting the highest efficiency (95.8 g/kg DOM), followed by the 26NPN (84.7 g/kg DOM) and 58NPN (80.5 g/kg DOM) treatments. A similar quadratic effect was observed for nitrogen use efficiency in microbial protein synthesis ($P = 0.05$), with the 42NPN

treatment showing the highest efficiency (0.359 g/g), followed by the 26NPN (0.319 g/g) and 58NPN (0.303 g/g) treatments (Table 5).

Mammary Gland Development, Blood Parameters, and Liver Gene Expression

No treatment effect was observed on mammary gland pixel count ($P = 0.19$). However, a quadratic effect was detected for parenchyma area ($P < 0.01$), with animals in the 42NPN treatment exhibiting the largest area (1.59 cm²), followed by 58NPN (1.51 cm²) and 26NPN (1.30 cm²). A marginal significance of linear increase was observed for parenchyma perimeter ($P = 0.09$), when dietary NPN level increased (Table 6).

Serum total protein levels exhibited a quadratic effect ($P < 0.01$), with the 26NPN (6.26 g/dL) and 58NPN (6.14 g/dL) treatments showing higher concentrations than the 42NPN treatment (6.02 g/dL). Serum urea levels also displayed a quadratic effect ($P = 0.05$), with the 42NPN group having the highest concentration (32.7 mg/dL), followed by 58NPN (31.8 mg/dL) and 26NPN (29.4 mg/dL). Serum IGF-1 levels increased linearly with increasing NPN levels ($P = 0.02$), averaging 306, 315, and 330 ng/mL for the 26NPN, 42NPN, and 58NPN groups, respectively. Serum ALT levels exhibited a linear effect ($P < 0.01$), decreasing as dietary NPN levels increased. The 26NPN treatment showed the highest ALT concentration (19.78 U/L), followed by 42NPN (19.44 U/L), while the 58NPN treatment presented the lowest value (16.90 U/L).

A quadratic effect of treatment was observed on CPS1 gene expression ($P < 0.01$; Table 7). The 42NPN treatment showed lower expression (1.47) compared with the 26NPN (2.67) and 58NPN (2.52) treatments. No treatment effect was observed on the expression of IGFBP-3, IGF-1, or GOT-1 genes ($P > 0.05$).

DISCUSSION

Although the diets were formulated to be isonitrogenous, the progressive increase in NPN inclusion necessarily required a greater proportion of rapidly fermentable energy sources to maintain dietary protein balance. This adjustment is inherent to formulations with high urea proportions, since NPN partially replaces true protein ingredients, demanding a higher inclusion of carbohydrates to sustain ruminal fermentation (Huber & Poore,1990; Arias et al., 2020).

A linear reduction in NDF digestibility was observed as NPN levels increased. Considering that urea was the main NPN source used, this effect may be attributed to the rapid ruminal hydrolysis of urea and the consequent abrupt release of NH_3. Komatsu and Sakaki (1971) demonstrated that up to 90% of urea-derived NPN can be converted to NH_3 in the rumen within

two hours after ingestion. Such rapid ammoniacal nitrogen release promotes a kinetic mismatch relative to the slower fermentation of structural carbohydrates from forage (Bezerra et al., 2026). Fibrolytic microorganisms exhibit relatively slow growth and require a continuous nitrogen supply at moderate concentrations, whereas transient NH_3 peaks may temporarily exceed microbial incorporation capacity, reducing fiber digestion efficiency (Bezerra et al., 2026; Firkins et al., 2007). Concurrently, increased starch intake favors non-fibrolytic microbial populations, competitively displacing fibrolytic bacteria and reducing plant cell wall digestion efficiency (Guo et al., 2021).

In high-forage diets, such as in the present study, a reduction in fiber digestibility generally leads to lower OM digestibility, since a substantial proportion of dietary OM originates from the fibrous fraction. Furthermore, the quadratic decline observed in crude protein digestibility, particularly in the 42NPN treatment, although not statistically significant, may be attributed to the lower protein intake recorded in this group. This pattern is consistent with the concept that apparent digestibility is positively associated with intake, as higher consumption dilutes the metabolic fecal fraction, thereby increasing apparent digestibility (Oliveira et al., 2020).

It is important to emphasize that studies evaluating NPN sources in diets for dairy heifers are limited. Zanton et al. (2007) assessed diets containing low or high levels of SP and RUP in post-pubertal heifers and found no effects on DM, OM, or NDF digestibility. However, crude protein levels and SP proportions were lower than in the present study, which may have resulted in fewer alterations in the ruminal environment. Similarly, Chizzotti et al. (2008), evaluating increasing NPN levels in diets for Holstein × Nellore steers, did not observe effects of NPN inclusion on apparent digestibility of DM, OM, or NDF. Nevertheless, the NPN proportions were also lower than those used herein. Amos (1987) evaluated different levels of RDP and RUP in Holstein heifers and reported that increasing dietary protein solubility improved DM, NDF, and energy digestibility. However, soybean meals were the main ingredient in the highly degradable treatments, and when compared to urea used in the present study the degradation rates are markedly different.

With the lower RUP intake observed in the 42NPN treatment, there was a reduction in the direct supply of amino acids to the small intestine, increasing reliance on microbial protein as the main source of metabolizable amino acids. Nevertheless, the greater availability of fermentable carbohydrates, particularly starch, may have provided adequate energetic substrate to sustain

microbial growth, favoring ruminal nitrogen incorporation into microbial protein, as described by Dann et al. (2014) and Zebeli et al.(2010). This synchrony between fermentable energy availability and NPN explains the higher microbial protein synthesis efficiency and greater nitrogen utilization efficiency observed in the 42NPN treatment, despite the lower supply of true protein.

Concomitantly, the higher plasma urea concentration associated with lower urinary excretion suggests a metabolic redistribution of nitrogen, with greater body retention. According to Reynolds and Kristensen (2008), at higher nitrogen intake levels there is a proportional increase in the contribution of non-ammoniacal sources to hepatic urea formation, reflecting greater participation of systemic amino acid catabolism rather than direct oxidation of ruminal NH_3. This interpretation is supported by the elevated ALT observed in the 42NPN treatment, indicating increased amino acid flux through transamination pathways and greater integration between peripheral protein metabolism and hepatic gluconeogenesis.

Simultaneously, lower CPS1 expression suggests an adaptive limitation of maximal urea cycle capacity, favoring conservation of body nitrogen and its redistribution to peripheral tissues rather than urinary excretion. Taken together, elevated ALT, reduced CPS1 expression, and increased plasma urea indicate a metabolic reorganization characterized by greater systemic amino acid contribution to ureagenesis, combined with downregulation of hepatic nitrogen excretion capacity.

The higher serum urea concentration concomitant with lower total serum protein observed in the 42NPN treatment may be explained by the combination of lower RUP intake and greater partitioning of absorbed amino acids toward growing tissues rather than plasma protein synthesis. Total serum protein reflects the chronic balance among intestinal amino acid supply, hepatic synthesis, and peripheral utilization (González & Scheffer, 2018; Eremenko and Eremenko, 2024). Thus, while the 26NPN and 58NPN treatments, which showed greater crude protein digestibility, presented higher serum total protein concentrations, the 42NPN treatment appears to have prioritized structural amino acid incorporation.

This hypothesis is reinforced by the linear increase in IGF-1 concentrations and the greater mammary parenchyma observed in this treatment. IGF-1 is a central mediator of somatic growth and mammary development and is strongly regulated by the animal's energetic status and metabolic efficiency (McGuire et al., 1992). Therefore, despite lower overall digestibility and

lower RUP intake, the 42NPN treatment promoted a metabolic environment favorable to tissue growth, supported by greater microbial efficiency and systemic nitrogen redistribution.

Additionally, water intake exhibited a linear decreasing effect as dietary urea inclusion increased. Thirst regulation in mammals is primarily mediated by central osmotic mechanisms. Urea, being a highly permeable solute, rapidly diffuses between intra- and extracellular compartments, preventing the establishment of effective osmotic gradients capable of inducing cellular dehydration. Consequently, even under elevated plasma urea concentrations, hypothalamic osmoreceptors are not strongly activated, reducing the stimulus for water intake (Steiger Burgos et al., 2001; McKinley & Johnson, 2004; Dijkstra et al., 2013). Thus, the higher serum urea observed in treatments with greater NPN provides a plausible physiological mechanism for the concomitant reduction in water consumption.

CONCLUSION

Increasing dietary non-protein nitrogen levels altered nitrogen metabolism and nutrient digestibility without affecting growth performance of dairy heifers. Although higher NPN inclusion reduced fiber digestibility, moderate inclusion improved microbial protein synthesis efficiency, enhanced nitrogen use efficiency, and promoted greater mammary parenchymal development. These responses were accompanied by metabolic adjustments in hepatic nitrogen metabolism, suggesting improved systemic nitrogen redistribution rather than increased nitrogen excretion.

Therefore, moderate dietary inclusion of NPN can be considered a viable nutritional strategy to optimize nitrogen utilization and support mammary development in growing dairy heifers without compromising overall performance.

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TABLES AND FIGURES

Table 1. Ingredient and chemical composition of diets containing increasing concentrations of non-protein nitrogen fed to dairy heifers.

Item[1]	Treatments[2]		
	26NPN	42NPN	58NPN
Diet formulation, g/kg DM			
Corn silage	731	730	734
Corn meal	16.1	83.2	149.0
Soybean meal	237.7	159.7	77.6
Urea	0.0	11.3	22.3
Mineral premix[2]	12.6	13.0	13.4
Sodium chloride	1.58	1.60	1.58
Ammonium sulfate	0.63	1.12	1.58
Chemical composition, g/kg DM[3]			
DM, as fed	397	398	397
CP	173	174	172
Starch	17.04	53.43	88.93
CF	29	29	29
NDF	382	368	355
NDFi	117	117	118
NPN, g/kg N	258	418	580
ME, Mcal/kg	2.64	2.58	2.46
MP/ME, g/Mcal	41.9	42.5	42.9

[1]DM = dry matter; CP = crude protein; CF = crude fat; NDF = neutral detergent fiber; NDFi = indigestible neutral detergent fiber; NPN = non-protein nitrogen; ME = metabolizable energy; MP:ME = metabolizable protein to metabolizable energy ratio.

[2] Calcium (min): 170 g/kg; Calcium (max): 200 g/kg; Phosphorus: 60 g/kg; Sodium: 70 g/kg; Potassium: 6 g/kg; Magnesium: 15.6 g/kg; Sulfur: 45 g/kg; Iron: 1,200 mg/kg; Zinc: 2,000 mg/kg; Copper: 540 mg/kg; Manganese: 2,000 mg/kg; Iodine: 50 mg/kg; Cobalt: 10 mg/kg; Selenium: 30 mg/kg; Vitamin A: 314,000 IU/kg; Vitamin D: 47,100 IU/kg; Vitamin E: 2,000 IU/kg; Vitamin B1: 64.8 mg/kg; Vitamin B2: 64.8 mg/kg; Vitamin B6: 64.8 mg/kg; Vitamin B12: 420 µg/kg; Niacin: 204 mg/kg; Pantothenic acid: 130.2 mg/kg; Monensin: 1,200 mg/kg.

Table 2. Gene names, primer sequences, and GenBank accession numbers for liver tissue gene expression analysis.

Genetic symbol[1]	Sequence	NCBI
IGFBP3	Forward TTACAAGAAAAAGCAGTGCCGC Reverse TGCCCGTACTTATCCACACAC	NM_174556.1
IGF-1	Forward TGCACTTCAGAAGCAATGGG Reverse GCATCTTCACCTGCTTCAAGA	NM_001077828.1
CPS1	Forward GGCTTAACCAATGTGACGGC Reverse TGTGTGCTGTTTGTGCCTTG	NM_001192258.1
GOT1	Forward AAGACAATGGCAGACCGCAT Reverse TACTCAACCTGCTTGGGGTTC	BT020856.2
GAPDH	Forward AAGGTCGGAGTGAACGGATTC Reverse ATGGCGACGATGTCCACTTT	NM_001034034.2

[1]IGFBP3 = insulin-like growth factor-binding protein 3; IGF-1 = insulin-like growth factor 1; CPS1 = carbamoyl phosphate synthetase I; GOT1 = glutamate-oxaloacetate transaminase (aspartate aminotransferase); GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Nutrient intake and apparent digestibility in dairy heifers fed diets containing increasing concentrations of non-protein nitrogen.

Item[1]	Treatments[2]			SEM	P-value [3]			
	26NPN	42NPN	58NPN		P	T×P	L	Q
Intake								
DM, kg/day	7.15	6.83	7.42	0.246	0.01	0.99	0.45	0.12
DM, g/kg BW	22.1	22.1	22.4	0.45	0.01	0.98	0.68	0.82
OM, kg/day	6.55	6.28	6.87	0.227	0.01	0.99	0.34	0.12
CP, kg/day	1.28	1.21	1.31	0.04	0.04	0.99	0.55	0.11
Starch, kg/day	0.796	1.05	1.46	0.047	0.22	0.98	<0.01	0.19
Starch, g/kg BW	2.37	3.29	4.24	0.089	0.01	0.36	<0.01	0.19
NPN, g/day	53	80	122	3.5	<0.01	0.47	<0.01	0.10
RDP, g/day	428	424	393	52.32	0.73	0.06	0.44	0.73
RUP, g/day	792	767	921	47.92	0.02	0.61	0.08	0.13
MP, g/day	959	889	992	41.82	<0.01	0.72	0.58	0.10
CP: DOM, g/kg	261	265	265	4.13	0.05	0.40	0.33	0.38
NDF, kg/day	2.70	2.48	2.61	0.088	<0.01	0.99	0.50	0.11
Water, L/day	10.3	7.64	7.97	1.333	0.75	0.95	0.07	0.17
Water, mL/kg BW	33.6	26.1	24.2	3.675	0.08	1.00	0.03	0.45
Water, L/kg DM	1.58	1.15	1.07	0.129	0.03	0.91	0.01	0.23
Apparent digestibility, g/kg								
DM	699	660	680	4.982	0.27	0.34	0.02	<0.01
OM	731	712	711	7.124	0.14	0.29	0.04	0.25
CP	776	753	771	7.867	0.87	0.66	0.66	0.05
NDF	678	642	611	8.939	0.10	0.17	<0.01	0.83

[1]DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber.

[2]26NPN = diet with 26% of CP as non-protein nitrogen; 42NPN = diet with 42% of CP as non-protein nitrogen; 58NPN = diet with 58% of CP as non-protein nitrogen.

[3]Probability of treatment effects: P = collection period effect; T×P = interaction between treatment and collection period; L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentration

Table 4. Body weight and growth performance in dairy heifers fed diets containing increasing concentrations of non-protein nitrogen.

Item[1]	Treatments[2]			SEM	Probability[3]	
	26NPN	42NPN	58NPN		L	Q
Initial BW, kg	260	253	268	25.015	0.83	0.72
Final BW, kg	364	364	367	3.385	0.54	0.85
ADG, kg/day	1.25	1.25	1.28	0.036	0.54	0.85
Body measurements, cm						
Withers height	128	127	127	0.864	0.58	0.85
Thoracic perimeter	168	168	168	0.58	1.00	0.86
Rump width	37.9	37.2	37.9	0.607	0.65	0.56

[1]BW = body weight; ADG = average daily gain.

[2]26NPN = diet with 26% of CP as non-protein nitrogen; 42NPN = diet with 42% of CP as non-protein nitrogen; 58NPN = diet with 58% of CP as non-protein nitrogen.

[3]Probability of treatment effects: L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations.

Table 5. Microbial protein synthesis and nitrogen balance in dairy heifers fed diets containing increasing concentrations of non-protein nitrogen.

Item	Treatments[1]			SEM	P-value [2]			
	26NPN	42NPN	58NPN		P	T×P	L	Q
Nitrogen balance. g/day								
Intake	208	190	209	8.559	0.02	0.76	0.91	0.09
Fecal excretion	46.5	47.2	47.9	2.971	0.10	0.90	0.75	1.00
Urinary excretion	129	105	120	8.018	0.28	0.16	0.44	0.06
Retained[3]	32.7	37.3	41.6	7.102	0.22	0.08	0.37	0.98
Efficiency of nitrogen use. g/g[3]	0.155	0.200	0.198	0.036	0.54	0.06	0.37	0.55
Microbial synthesis								
Microbial protein. g/day	428	424	394	52.321	0.73	0.06	0.44	0.73
Microbial efficiency. g/kg DOM[3]	84.7	95.8	80.5	13.006	0.20	0.06	0.56	0.04
N use for microbial synthesis. g/g[3]	0.319	0.359	0.303	0.044	0.45	0.17	0.58	0.05

[1]26NPN = diet with 26% of CP as non-protein nitrogen; 42NPN = diet with 42% of CP as non-protein nitrogen; 58NPN = diet with 58% of CP as non-protein nitrogen.

[2]Probability of treatment effects: P = collection period effect; T×P = interaction between treatment and sampling period; L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations.

[3]P-values are not shown due to significant treatment × sampling period interactions. Interaction decompositions are presented in Figures 1 and 2.

Table 6. Mammary gland ultrasonographic measurements and blood parameters dairy heifers fed diets containing increasing concentrations of non-protein nitrogen.

Item	Treatments[1]			SEM	Probability[2]			
	26NPN	42NPN	58NPN		P	T×P	L	Q
Mammary gland parenchyma measurements								
Pixels count. pixel/mm²	90.9	89.5	95.2	3.061	<0.01	0.75	0.22	0.19
Area. cm^2	1.30	1.59	1.52	0.098	0.01	0.11	0.01	0.01
Blood characteristics								
Total Protein. g/dL	6.26	6.02	6.14	0.138	0.62	0.48	0.05	<0.01
Glucose. mg/dL	83.8	83.2	85.8	2.125	<0.01	0.89	0.25	0.27
Albumin. g/dL	3.43	3.48	3.40	0.036	0.07	0.94	0.59	0.15
Urea. mg/dL	29.4	32.7	31.8	0.849	0.12	0.88	0.05	0.05
IGF-1. ng/mL	306	315	330	7.540	0.63	0.49	0.02	0.74
AST. U/L	54.5	52.8	54.4	1.319	0.05	0.12	1.00	0.32
ALT. U/L	19.8	19.4	16.9	0.698	0.48	0.40	<0.01	0.08
GGT. U/L	17.5	16.7	16.8	0.517	0.04	0.72	0.35	0.50

[1]26NPN = diet with 26% of CP as non-protein nitrogen; 42NPN = diet with 42% of CP as non-protein nitrogen; 58NPN = diet with 58% of CP as non-protein nitrogen.

[2]Probability of treatment effects: P = collection period effect; T×P = interaction between treatment and collection period; L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations

Table 7. Hepatic gene expression in dairy heifers fed diets containing increasing concentrations of non-protein nitrogen.

Item[1]	Treatments[2]			SEM	Probability[3]	
	26NPN	42NPN	58NPN		L	Q
IGFBP3	0.319	0.471	0.498	0.122	0.32	0.71
IGF-1	0.082	0.071	0.086	0.020	0.87	0.55
CPS1	2.67	1.47	2.52	0.227	0.65	<0.01
GOT1	0.319	0.400	0.371	0.079	0.69	0.60

[1]IGFBP3 = insulin-like growth factor-binding protein 3. IGF-1 = insulin-like growth factor. CPS1 = carbamoyl phosphate synthetase. and GOT1= glutamic transaminase-oxaloacetic (Aspartate aminotransferase).

[2]26NPN = diet with 26% of CP as non-protein nitrogen; 42NPN = diet with 42% of CP as non-protein nitrogen; 58NPN = diet with 58% of CP as non-protein nitrogen.

[3]Probability of treatment effects: L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations.

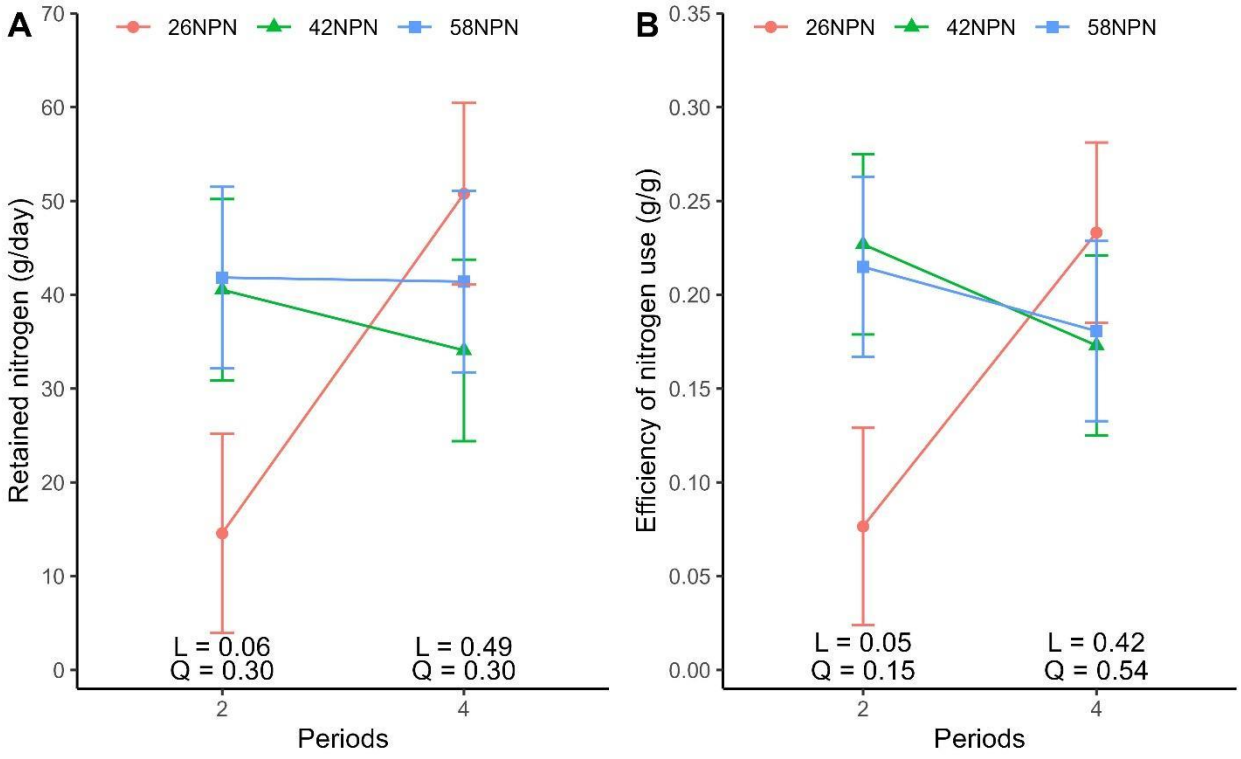

Figure 1. Interaction effects of sampling period and treatment on retained nitrogen (g/day; A) and nitrogen utilization efficiency (g/g; B). L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations

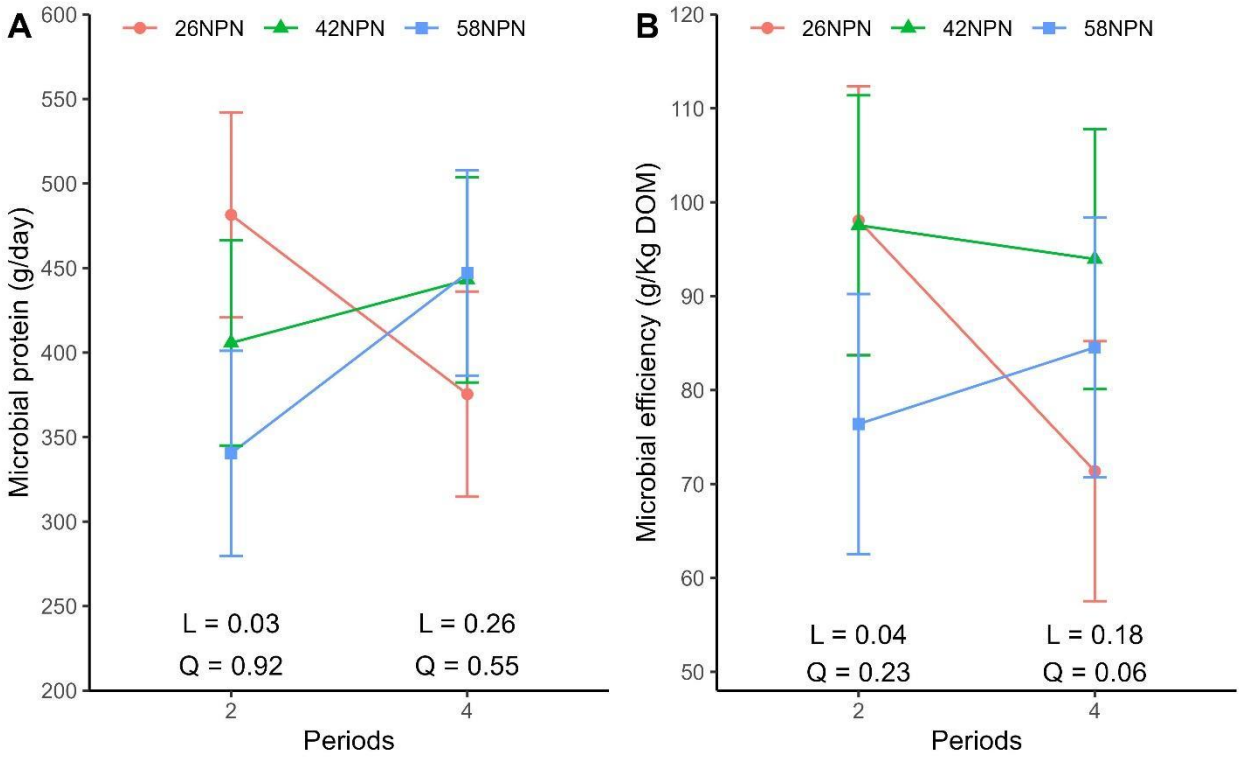

Figure 2. Interaction effects of sampling period and treatment on microbial protein synthesis (g/day; A) and microbial efficiency (g/g; B). L = linear effect of NPN concentrations; Q = quadratic effect of NPN concentrations