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EFFECT OF CALCIUM NITRATE SUPPLEMENTATION ON NUTRITION, PERFORMANCE, AND ENTERIC METHANE EMISSIONS IN CATTLE FED LOW-QUALITY TROPICAL FORAGE

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Dissertação apresentada ao Programa de Pós- Graduação em Ciência Animal (Nutrição e Produção de Ruminantes no Trópico Úmido) da Universidade Federal Rural do Rio de Janeiro, como requisito parcial para a obtenção do grau de Mestre em Ciência Animal, Área de concentração: Zootecnia.

Orientadora: Prof.ª Dr.ª Luana Marta de Almeida Rufino.

Coorientador: Dr. João Paulo Pacheco Rodrigues.

Coorientador: Dr. Rafael Gonçalves Tonucci.

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RESUMO

BARBOSA, Mariana Gomes. **Efeito da suplementação com nitrato de cálcio sobre a nutrição, desempenho e emissões entéricas de metano em bovinos alimentados com forragem tropical de baixa qualidade.** 2026, 50p. Mestrado em Ciência Animal. Programa de Pós – Graduação em Ciência Animal, Instituto de Zootecnia, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Seropédica, 2026, Dissertação.

A produção de metano entérico representa importante perda energética na pecuária de corte e constitui um desafio ambiental relevante. O uso de aditivos alimentares surge como alternativa promissora para reduzir a produção de metano (CH_4) no rúmen, sendo o nitrato de cálcio um composto com potencial mitigador por atuar como sumidouro alternativo de hidrogênio. Objetivou-se avaliar o efeito da inclusão do nitrato de cálcio na dieta de bovinos de corte alimentados com forragem de baixa qualidade sobre as emissões de CH_4 entérico, desempenho produtivo e parâmetros metabólicos. O experimento foi conduzido na Embrapa Gado de Leite, com duração de 88 dias, utilizando-se 26 novilhos Nelore castrados, com idade inicial de 14 meses, distribuídos em delineamento inteiramente casualizado, com quatro tratamentos: 0 (CON), 25 (LOW), 50 (INT) e 100 (HIG) g Kg na matéria seca^{-1} (MS) de inclusão do nitrato de cálcio no suplemento. A dieta foi composta por feno de *Cynodon dactylon* cv. Tifton 85., *ad libitum* e suplemento, 0,5% do peso vivo. Foram avaliados o consumo, digestibilidade, fermentação ruminal, produção de CH_4, perfil de consumo do suplemento, parâmetros sanguíneos e excreções urinárias. Não foram observados efeitos dos tratamentos sobre os consumos de MS total, forragem, matéria orgânica, fibra em detergente neutro e energia ($P > 0,05$). Contudo, verificou-se redução no consumo de MS do suplemento ($P = 0,011$), consumo relativo de suplemento (g suplemento/g MS ingerida; $P = 0,005$), consumo de proteína bruta e carboidratos não fibrosos ($P = 0,002$) e digestibilidade da proteína bruta ($P = 0,001$). Houve aumento do peso vivo final ($P = 0,045$) e ganho médio diário ($P = 0,045$). Não houve efeito sobre o pH, nitrogênio amoniacal ruminal e concentrações absolutas de ácidos graxos voláteis ($P > 0,05$), porém observou-se aumento na relação acetato:propionato ($P = 0,008$) e acetato:(propionato + butirato) ($P = 0,033$). Houve redução em 10% da emissão diária de CH_4 (L/dia), na relação CH_4:CO_2, no rendimento (L CH_4 Kg^{-1} MS) em 13% e na intensidade de emissão (L CH_4 Kg^{-1} GMD) em 47%, com maiores reduções nos níveis mais elevados de inclusão. Observou-se interação entre tratamento e tempo pós-fornecimento para o perfil de consumo do suplemento ($P < 0,001$), com menor ingestão imediata e maior dispersão do consumo ao longo do tempo para o HIG. Não houve efeito dos tratamentos sobre o balanço de nitrogênio, ureia, ácido úrico ou parâmetros sanguíneos ($P > 0,05$); entretanto, a excreção urinária de nitrato e nitrito aumentou linearmente. Conclui-se que a suplementação com nitrato de cálcio foi eficaz na mitigação das emissões entéricas de CH_4, promovendo reduções na produção diária, no rendimento e na intensidade de emissão de CH_4, sem comprometer o metabolismo nitrogenado dos animais. O melhor desempenho produtivo ocorreu no INT, indicando que o aditivo constitui estratégia viável para mitigação de metano em novilhos nelore submetidos a sistemas baseados em forragens de baixa qualidade.

Palavras-chave: Aditivo alimentar; fermentação ruminal; metabolismo; nutrição de ruminantes.

ABSTRACT

BARBOSA, Mariana Gomes. **Effect of calcium nitrate supplementation on nutrition, performance, and enteric methane emissions in cattle fed low-quality tropical forage.** 2026, 50p. Master's in Animal Science. Postgraduate Program in Animal Science, Institute of Animal Science, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Seropédica, 2026, Dissertation.

Enteric methane production represents a significant energy loss in beef cattle production and constitutes an important environmental challenge. The use of feed additives has emerged as a promising alternative to reduce methane (CH_4) production in the rumen, with calcium nitrate being a compound with mitigation potential due to its role as an alternative hydrogen sink. The objective of this study was to evaluate the effect of including calcium nitrate in the diet of beef cattle fed low-quality forage on enteric CH_4 emissions, productive performance, and metabolic parameters. The experiment was conducted at Embrapa Dairy Cattle and lasted 88 days, using 26 castrated Nellore steers with an initial age of 14 months, distributed in a completely randomized design with four treatments: 0 (CON), 25 (LOW), 50 (INT), and 100 (HIG) $g\ kg^{-1}$ of calcium nitrate inclusion in the dry matter (DM) of the supplement. The diet consisted of *Cynodon dactylon* cv. Tifton 85 hay, offered ad libitum, and supplement at 0.5% of body weight. Intake, digestibility, ruminal fermentation, CH_4 production, supplement intake pattern, blood parameters, and urinary excretions were evaluated. No treatment effects were observed on total DM intake, forage intake, organic matter, neutral detergent fiber, or energy intake ($P > 0.05$). However, there was a reduction in supplement DM intake ($P = 0.011$), relative supplement intake (g supplement/g DM intake; $P = 0.005$), crude protein and non-fiber carbohydrate intake ($P = 0.002$), and crude protein digestibility ($P = 0.001$). Final body weight ($P = 0.045$) and average daily gain ($P = 0.045$) increased. There was no effect on ruminal pH, ammonia nitrogen, or absolute concentrations of volatile fatty acids ($P > 0.05$); however, an increase was observed in the acetate:propionate ratio ($P = 0.008$) and acetate:(propionate + butyrate) ratio ($P = 0.033$). A 10% reduction in daily CH_4 emissions (L/day), CH_4:CO_2 ratio, a 13% reduction in methane yield (L CH_4 kg^{-1} DM), and a 47% reduction in methane emission intensity (L CH_4 kg^{-1} ADG) were observed, with greater reductions at higher inclusion levels. An interaction between treatment and time post-feeding was observed for supplement intake pattern ($P < 0.001$), with lower immediate intake and greater dispersion of intake over time in HIG. There was no effect of treatments on nitrogen balance, urea, uric acid, or blood parameters ($P > 0.05$); however, urinary nitrate and nitrite excretion increased linearly. It is concluded that calcium nitrate supplementation was effective in mitigating enteric CH_4 emissions, promoting reductions in daily production, methane yield, and emission intensity without compromising nitrogen metabolism. The best productive performance was observed in INT, indicating that this additive is a viable strategy for methane mitigation in Nellore steers under low-quality forage-based systems.

Keywords: Feed additive; metabolism; ruminal fermentation; ruminant nutrition.

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1 INTRODUCTION

Brazilian beef cattle production is undergoing a dynamic process of transformation. As the world's largest beef exporter, Brazil also became the leading country in global beef production in 2025. According to the United States Department of Agriculture (USDA), the country produced 12.35 million tons of beef, surpassing, for the first time, the United States, with an estimated production of 11.81 million tons. Despite this prominent position, extensive production systems still predominate, relying largely on tropical forages, with an average stocking rate of 0.93 animal units per hectare (AU/ha) (ABIEC, 2025). Furthermore, only about 20% of Brazilian beef cattle are managed in feedlot systems, with the majority of these cattle undergoing confinement during the finishing phase. This relatively low level of intensification highlights the significant potential for productivity gains through improved pasture management and intensification strategies.

In this context, impact assessments that accurately reflect the Brazilian reality, using approaches that simultaneously account for carbon emissions and removals, are essential for understanding the current carbon footprint of national beef production systems (Oliveira et al., 2022). It is well established that forage-based diets, characterized by a high fiber content and low digestibility, tend to result in greater enteric CH_4 production due to the predominance of acetate and hydrogen production in the rumen (Koscheck et al., 2020). Nevertheless, additional mitigation strategies can be adopted to enable progress toward more sustainable production systems.

Methane is the main greenhouse gas (GHG) produced by ruminants and accounts for 11.6% of global emissions, representing approximately 43% of total GHG emissions from livestock worldwide. Among these gases, enteric methane generated during ruminal fermentation is particularly important due to its global warming potential (GWP), which is 28 times greater than that of carbon dioxide (CO_2), and its relatively short atmospheric lifetime, averaging 12.4 years (IPCC, 2023). Owing to its significant contribution to the atmospheric carbon balance and its short residence time, reducing methane emissions represents a major opportunity to achieve climate stabilization targets (Reisinger et al., 2021).

Therefore, strategies aimed at reducing CH_4 production are necessary, not only because of their environmental benefits but also because methane formation represents an energetic inefficiency for ruminants. It is estimated that between 6 and 12% of the gross energy intake is lost as methane, depending on diet composition and level of intake (Min et al., 2022). In this regard, the combination of pasture management and strategic supplementation is an alternative that enhances dietary nutritive value, improving not only productive performance but also reducing CH_4 emission intensity per unit of weight gain (Koscheck et al., 2020).

Within this framework, research on nutritional additives has emerged as a promising strategy to mitigate methane emissions, particularly in intensified systems or those amenable to controlled supplementation. A wide range of compounds has been investigated for their ability to modulate ruminal fermentation, including oils, purified bromoform, 3-nitrooxypropanol, organic acids, and nitrates (Almeida et al., 2021). Among these alternatives, nitrate stands out due to its effective potential for enteric methane mitigation, in addition to serving as a source of non-protein nitrogen (NPN) in ruminant diets.

Its mode of action involves functioning as an electron acceptor, acting as an alternative hydrogen (H_2) sink in the rumen and thereby reducing H_2 availability for ruminal methanogenesis (Lee and Beauchemin, 2014). Furthermore, nitrate serves as a source of NPN by providing ammonia in the rumen as a result of complete nitrate reduction. However, issues related to metabolic safety and potential side effects still require further investigation to better elucidate the relationship between calcium nitrate and animal metabolism and to consolidate its use as a feed additive in ruminant nutrition (Lee and Beauchemin, 2014).

The objective of the present study was to evaluate the effects of including the calcium nitrate in the supplementation of Nellore beef cattle fed low quality tropical forage on enteric methane mitigation, productive performance and metabolic parameters.

2 LITERATURE REVIEW

2.1. Supplementation of Grazing Cattle

In Brazil, the seasons of the year are well defined and promote marked variations in both the quantity and quality of available forage as a result of changes in edaphoclimatic conditions. However, pasture as the sole feed source is not always sufficient to meet the nutritional requirements necessary to sustain uniform weight gain throughout the year, particularly during the dry season, when a pronounced reduction in forage nutritive value and biomass occurs (Melo et al., 2022).

In this context, supplementation emerges as a strategy to mitigate the nutritional limitations of tropical pastures, enabling animals to achieve the expected productive performance. In addition, supplementation may increase the digestibility of the fibrous fraction, favoring more efficient use of structural carbohydrates for body tissue synthesis (Medeiros, 2005). The changes promoted in the ruminal environment directly influence the utilization of these carbohydrates by rumen microorganisms, resulting in greater efficiency of fiber use in the diet (Reis et al., 2012).

Beyond effects on animal performance, as a consequence of these nutritional and fermentative modifications, diets with a higher proportion of concentrate tend to result in lower methane emissions when compared with diets composed exclusively of grasses (Hoffmann et al., 2021). In this regard, reductions in methane production (g/day/animal), methane yield (g/kg of dry matter intake), emission intensity (g/kg of average daily gain), and methane conversion factor (Ym%; relative to gross energy intake) have been reported in Nellore cattle grazing high-quality tropical pastures and receiving supplementation during the rainy season (Barbero et al., 2015; Koscheck et al., 2020).

To achieve these positive effects, prior characterization of both the chemical composition and the herbage allowance of the pasture is essential, allowing the supplement to effectively complement nutritional deficiencies. This adjustment is critical to meet crude protein and digestible organic matter requirements, since the efficiency of nitrogen utilization is directly associated with energy availability in the rumen (Reis et al., 2009; Barbero et al., 2015). The capacity to convert feed into animal product can be expressed by the fraction of potentially degradable neutral detergent fiber (pdNDF) in the forage, reinforcing the importance of nutritional strategies that enhance the utilization of this dietary fraction (Allen, 1996).

In addition to dietary composition, supplementation in grazing systems may also alter forage intake as a consequence of changes in ruminal function. In this context, three primary types of responses to supplementation are described: additive, associative (combined), and substitutive effects. Under the additive effect, total digestible energy intake increases, with or without changes in forage intake. The associative (combined) effect is characterized by a reduction in pasture intake accompanied by increased supplement intake, also resulting in greater energy intake. In contrast, under the substitutive effect, total energy intake remains unchanged, indicating partial replacement of forage by the supplement. These responses depend on several factors, including pasture availability and quality, level and composition of the supplement, and management-related aspects such as timing of supplementation and feeder space availability (Moore et al., 1999).

As a result of these pasture–supplement interactions, supplementation has a high potential to increase individual weight gain of beef cattle grazing tropical pastures, particularly during the dry season, when forage is characterized by low crude protein content and digestibility. However, the outcomes achieved are directly related to the level of supplementation, pasture quality, and animal category, requiring continuous adjustments in nutritional management (Tambara et al., 2021). Although the impact of supplementation is more pronounced during the dry season, its use during the rainy season also contributes to improved animal performance, reduced slaughter age, and increased productivity per unit area (Resende et al., 2013).

Considering seasonal variation, during the dry season there is often a decline in the nutritional quality of tropical pastures, mainly characterized by reduced crude protein content and increased lignin concentration in the cell wall (Detmann et al., 2014). Conversely, during the rainy season, when well managed, grasses exhibit a higher leaf proportion and greater rumen-degradable protein content (Delevatti et al., 2019). Under these conditions, energy supplementation can increase energy supply for microbial growth, thereby reducing ruminal ammonia nitrogen concentrations (Costa et al., 2011).

Thus, supplementation in grazing systems represents an efficient strategy to balance animal nutrient requirements through the integration of pasture and supplements. Strategic supplementation of high-quality tropical pastures, providing approximately 1 kg of crude protein intake per day, has resulted in weight gains ranging from 0.800 to 1.200 kg/day (Barbero et al., 2015; Koscheck et al., 2020). Nevertheless, the success of this practice depends on a careful assessment of pasture characteristics, production system, and the types and levels of supplementation adopted, aiming not only at greater productivity per unit area but also at enhanced long-term sustainability and profitability of the system.

2.2. Enteric Fermentation and Methane Production

Ruminal fermentation is an anaerobic process carried out by the microbial population, which converts carbohydrates into short-chain fatty acids (SCFAs), mainly acetic, propionic, and butyric acids. From a metabolic standpoint, the formation of acetate and butyrate releases hydrogen, whereas propionate production creates a competitive pathway for the utilization of

H^+ in the ruminal environment. As a result, during the fermentative process, carbon dioxide (CO_2) and methane (CH_4) are generated, with their amounts varying according to the relative proportions of the acids produced (Owens and Goetsch, 1988). In the rumen, methane is produced primarily through hydrogenotrophic methanogenesis, which uses CO_2 and H_2 as the main substrates for CH_4 formation.

The intensity of this process is directly related to ruminal fermentative conditions, which are strongly modulated by the composition of the diet offered to ruminants. Diets with a high grain content favor propionic acid production, whereas high-forage diets promote acetate production (Kozloski, 2011). In this context, the formation of SCFAs, except propionate, is accompanied by H_2 production, which is subsequently used as an energy source by methanogenic archaea, reducing CO_2 and producing CH_4. The main ruminal methanogenic archaea belong to the genera *Methanobrevibacter*, *Methanosphaera*, *Methanimicrococcus*, and *Methanobacterium*. Consequently, greater acetate production is associated with increased H_2 generation. Hydrogen readily reacts with carbon dioxide molecules to form CH_4 and water through hydrogenotrophic methanogenesis, as described by the following equation:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

From a quantitative perspective, the different fermentative pathways exhibit distinct hydrogen production and consumption balances, which determine substrate availability for methanogenesis. During acetate formation, the fermentation of one mole of glucose results in the production of two moles of CO_2 and four moles of H_2, with this hydrogen being potentially used to reduce one mole of CO_2 to one mole of CH_4. In the butyrate synthesis pathway, the fermentation of one mole of glucose generates approximately 1.5 moles of CO_2 and 0.5 mole of CH_4. In contrast, the propionate formation pathway does not result in net CO_2 production, thereby limiting substrate availability for methanogenesis (Ungerfeld and Kohn, 2006).

In addition to its direct relationship with methane production, hydrogen plays a central role in maintaining the biochemical balance of the ruminal environment. The accumulation of H^+ interferes with enzymatic systems ($NADH + H^+ \leftrightarrow NAD^+$) and ruminal pH regulation. Therefore, the removal of H_2 molecules produced during fermentation is required, with methane formation being the primary pathway (Kozloski, 2011). Methanogenesis is thus a natural and intrinsic process in ruminants and is fundamental to ruminal metabolism. However, it is directly associated with fermentation efficiency and represents an energy loss ranging from 6 to 12% of gross energy intake. Consequently, methane generated through methanogenesis and manure fermentation represents not only an environmental concern but is also associated with reduced animal productive efficiency (Min et al., 2022).

In production systems, these energetic losses may vary, particularly under grazing conditions. In general, as forage maturity advances, methane yield (emissions per unit of feed intake) and methane intensity (emissions per unit of product) tend to increase (Nunes et al., 2023). This increase is associated with higher fiber and lignin contents, as well as lower levels of non-fibrous carbohydrates in tropical forages (Van Soest, 1994).

Given these factors, nutritional strategies aimed at optimizing ruminal fermentation are essential for mitigating CH_4 emissions. Improvements in feed quality and modulation of the ruminal microbial population favor greater energy retention, thereby reducing CH_4 losses, which results in improved animal performance and lower methane production per unit of product (Berndt, 2010).

2.3. Nutritional Feed Additives for Methane Mitigation

Enteric CH_4 emissions arising from ruminal fermentation are influenced by several factors, including animal characteristics, level of feed intake, grain inclusion in the diet, types of carbohydrates present, particle size, feed digestibility, and the use of dietary additives (Cottle et al., 2011). In this context, modulation of ruminal fermentation represents the primary strategy for reducing enteric methane emissions. Accordingly, a wide range of methane-mitigating nutritional additives has been extensively evaluated, including dietary lipid supplementation, chemical inhibitors of methanogenesis, dietary inclusion of algae containing antimethanogenic compounds, alternative electron sinks, phytochemicals, and defaunation through the elimination of ruminal protozoa, among others (Ungerfeld, 2022).

Based on their mechanisms of action, feed additives can be classified into four main categories: those that modulate ruminal microbial fermentation to reduce hydrogen (H_2) production; direct inhibitors of methanogenic archaea; additives that promote redirection of H_2 toward alternative electron incorporation pathways; and compounds that result in CH_4 oxidation (Belanche et al., 2025).

Among additives classified as ruminal fermentation modulators, supplementation with high levels of dietary fat has the potential to reduce CH_4 emissions through multiple mechanisms. These include acting as a hydrogen sink via fatty acid biohydrogenation, increasing ruminal propionate concentrations, and reducing protozoal populations (Almeida et al., 2021). Reductions of up to 20% in methane emissions have been reported with lipid supplementation, particularly when medium-chain or polyunsaturated fatty acids are used (Patra, 2013).

Another example within this same classification is ionophores, which are highly effective against Gram-positive bacteria but exhibit little or no activity against Gram-negative bacteria. Gram-positive bacteria play a key role in the production of acetate, lactate, butyrate, formate, and H_2. Therefore, their inhibition leads to reduced production of these short-chain fatty acids and lower availability of H_2 for methane production by methanogenic archaea (Nagaraja et al., 1997).

Within the group of additives classified as direct methanogenesis inhibitors, those that specifically inhibit methanogenic archaea or microorganisms directly associated with methane production, compounds such as bromochloromethane, 2-bromoethanesulfonate, chloroform, and 3-nitrooxypropanol (3-NOP) are included (Belanche et al., 2025). Among these, 3-NOP has gained prominence due to its high efficacy, acting through inhibition of the enzyme methyl-coenzyme M reductase, thereby blocking methane synthesis at the final step of methanogenesis and promoting CH_4 emission reductions ranging from 20 to 40% (Hristov et al., 2013; Dijkstra et al., 2018).

Inhibition of methyl-coenzyme M reductase by this additive leads to direct metabolic consequences within the ruminal environment. With methane formation, the primary hydrogen sink, being blocked, ruminal H_2 concentrations increase. This excess hydrogen is redirected toward alternative metabolic pathways, most notably propionate production, which represents the most relevant and beneficial route. As a result, changes occur in ruminal SCFA proportions, along with improvements in overall energetic efficiency (Kebreab et al., 2023).

Among naturally derived direct inhibitors, tannins stand out as a viable feed additive alternative, given their well-documented bactericidal and bacteriostatic effects on methanogenic microorganisms (Haque, 2018). Studies evaluating Merino sheep supplemented with tannins have reported reductions in methane emissions, which have been attributed to the toxic effects of plant extracts on methanogenic archaea (Vieira et al., 2020).

In the group of additives that promote hydrogen redirection by reducing its availability to methanogens, nitrates and nitrites directly compete for H_2. Nitrate has shown benefits in methane mitigation while also serving as a source of non-protein nitrogen for the ruminal ecosystem. Compounds such as sulfates, organic acids, fumarate, and malate exhibit similar mechanisms of action by functioning as alternative electron sinks (Belanche et al., 2025).

Overall, considering the different mechanisms discussed, it is important to highlight that many of these strategies do not impair animal productive performance and, in several cases, contribute to its improvement—a key factor for their economic feasibility. Therefore, in addition to absolute CH_4 emissions, performance-related variables should also be evaluated to assess the impact of additive use, such as emission intensity (g CH_4 per kg of product).

2.4. Use of Calcium Nitrate for Enteric Methane Mitigation

Nitrates are a group of compounds that contain the nitrate anion (NO_3^-) in their chemical structure. They are characterized by high water solubility and play an important role in the nitrogen cycle. Nitrate occurs naturally in food, water, soil, and plants and is widely used in industrial and agricultural processes (Oliveira, 2001). In the context of ruminant nutrition, nitrate represents a promising feed additive because, by acting as an electron acceptor, it inhibits ruminal methanogenesis by functioning as an alternative hydrogen sink in the rumen. In this way, nitrate competes with methanogenic archaea for available H_2 in the ruminal environment, thereby contributing to reduced methane production.

After nitrate ingestion and in the presence of suitable substrates, ruminal bacteria express the enzymes nitrate reductase and nitrite reductase. Nitrate present in the rumen is first reduced to nitrite and subsequently to ammonia. Microorganisms such as *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* are involved in this reduction process. Excess ammonia that is not utilized in the rumen enters the bloodstream and is transported to the liver, where it is converted to urea and excreted in the urine (Kozloski, 2009).

In addition to its methane mitigation benefits, nitrate serves as a source of non-protein nitrogen in ruminant diets. Supplementation with NO_3^- does not impair dry matter intake or fiber digestibility and has shown favorable results in grazing systems (Almeida et al., 2021). In that study, overall reductions in methane emission intensity (g CH_4 per kg of animal product) ranged from 10.7% to 18.7%.

Despite the benefits associated with nitrate use, caution is required due to its potential toxic effects. Nitrite formed during nitrate reduction can be toxic when supplied in excessive amounts or when animals are not properly adapted to the diet, potentially leading to methemoglobinemia (Lewis, 1951). Under normal conditions, the activities of nitrate reductase and nitrite reductase are balanced, preventing nitrite accumulation in the rumen. However, excessive dietary nitrate intake may result in increased ruminal nitrite concentrations (Puniya et al., 2015).

Under these conditions, accumulated nitrite may be absorbed through the ruminal wall into the bloodstream, where it reacts with hemoglobin in its ferrous form (Fe^{2+}), leading to the formation of methemoglobin in the ferric form (Fe^{3+}). This compound is incapable of transporting oxygen to tissues and can result in tissue hypoxia (Puniya et al., 2015). Methemoglobin concentrations in blood typically reach their peak within one to two hours after feeding (Guerink et al., 1982). Therefore, despite the demonstrated benefits, further studies are required to consolidate the use of nitrate as an enteric methane–mitigating feed additive in ruminants (Puniya et al., 2015).

3 MATERIALS AND METHODS

The experiment was conducted at the Multi User Laboratory of Bioefficiency and Livestock Sustainability, located at José Henrique Bruschi Experimental Field, belonging to Embrapa Dairy Cattle, in the municipality of Coronel Pacheco, Minas Gerais, Brazil (21°33′22″ S, 43°06′15″ W), from December 28, 2024 to March 25, 2025. The regional climate is classified as Cwa (mesothermal) according to the Köppen classification, characterized by hot and rainy summers and cold, dry winters. All procedures involving animals were approved by the Animal Use Ethics Committee (CEUA) of Embrapa Dairy Cattle (Protocol nº. 3795129424).

3.1. Animals and Experimental Area

A total of 26 steers castrated male Nellore cattle were used, with an initial body weight of 363 ± 28.1 kg (mean ± SD) and an average age of 14 months. The steers were produced through *in vitro* fertilization and were all sired by the same bull, Rem Rardrock (purebred Nellore). The steers were initially allocated to three pens in a free-stall barn using body weight as the allocation criterion. The facility was equipped with rubber mats covered with sawdust bedding, electronic feed bunks (AF-1000 Master Gate, Intergado®, Contagem, MG, Brazil), and a ventilation system (DeLaval®, Jaguariúna, SP, Brazil).

The steers were previously adapted to halter handling and to the free-stall, tie-stall, and respiration chamber environments prior to the data collection periods (Figure 1A, B, and C). This adaptation period facilitated steers acclimation and allowed safe movement between facilities, avoiding abrupt management practices and potential behavioral changes, particularly those affecting dry matter intake (Figure 2).

Figure 1. Steers adaptation to the free-stall (A), tie-stall (B), and respiration chambers (C).

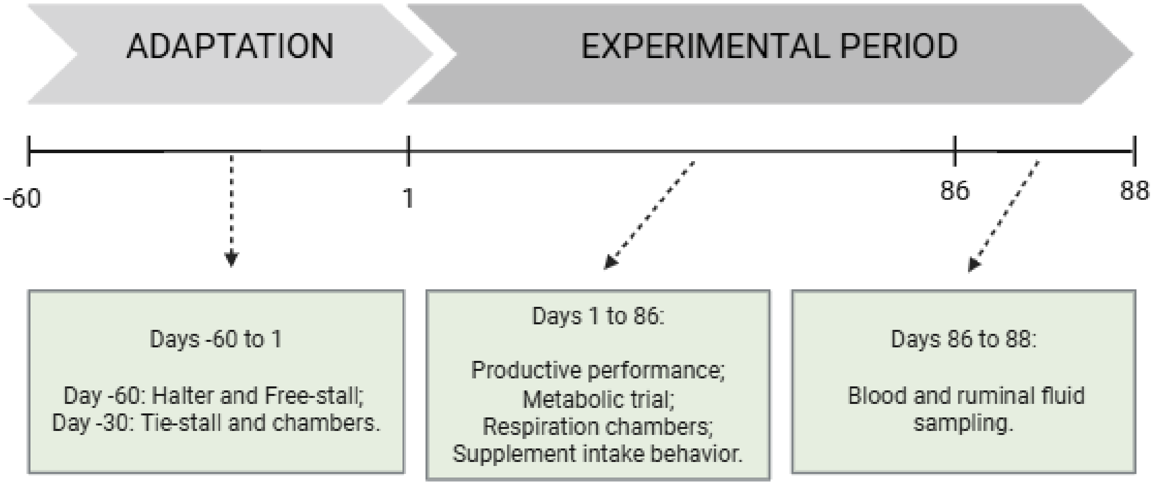

Figure 2. Summary of the experimental period and main sampling procedures.

3.2. Experimental Design and Experimental Diets

Steers were assigned to a completely randomized design with four treatments. The control treatment had eight replicates, while the remaining treatments had six replicates each. The forage source consisted of Cynodon dactylon cv. Tifton 85 hay, offered *ad libitum*. The concentrate was supplied by Nutron Cargill and packaged in 20 Kg bags. Four supplements containing increasing levels of the calcium nitrate were evaluated: 0 g (CON; Probeef® Nutripec; Nutron Cargill), 25 g (LOW), 50 g (INT), and 100 g (HIG) kg^{-1} of supplement dry matter (DM). The additive was incorporated into the supplement as a partial replacement for the non-protein nitrogen (NPN) source used. The experimental diet consisting of low-quality Tifton hay (Table 1) and a protein energy supplement supplied at 0.5% of body weight (BW). The steers had free access to water throughout the experiment.

The additive containing 75.7% nitrate (NO_3^-) in its composition. The supplements were formulated to provide 0, 21.5, 43.1, and 86.1 g of NO_3^- Kg^{-1}, respectively, on DM. All supplements were formulated to be isonitrogenous, achieving a concentration of 90 g/kg of NPN, and contained identical calcium levels.

The forage was offered twice a day, at 08:00 and 13:00, whereas the supplement was supplied at 11:00 in a separate, adapted feeder. The feeding schedule was designed to simulate grazing system behavior, in which cattle typically exhibit longer grazing activity in the early morning, followed by supplement intake during the warmer hours of the day, and a second grazing period in the afternoon. Hay was weighed daily prior to feeding to allow for 5 to 10% orts, ensuring *ad libitum* intake. Supplement allowance was adjusted weekly according to body weight.

Table 1. Composition of Tifton hay and supplements

Item[1]	Hay	Supplement			
		CON	LOW	INT	HIG
DM, g Kg^{-1}	908	936	932	928	917
Composition, g kg DM^{-1}					
OM	929	798	763	777	786
CP	85.3	220.3	221.2	219.4	197.0
NDF	789	155	155	152	156
ADF	427	-	-	-	-
Lignin	74.7	-	-	-	-
EE	14.5	24.5	15.4	18.8	21.0
NFC	40.6	492	453	461	454
GE, Mcal/Kg	4.265	3.593	3.397	3.397	2.853
iFDN	278	51.1	49.2	37.5	37.1
NO_3^-	-	0	21.5	43.1	86.1
Composition, g kg NDF^{-1}					
NDIP	30.1	11.5	13.0	13.5	13.1
NDIA	5.2	23.1	24.9	31.5	27.1
Composition, g kg ADF^{-1}					
ADIP	11.1	-	-	-	-

[1] DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; GE = gross energy; iFDN = Indigestible neutral detergent fiber; NO_3^- = Nitrate; NDIP = neutral detergent insoluble protein; NDIA = neutral detergent insoluble ash; ADOP = acid detergent insoluble protein.

3.3. Supplement Intake Behavior

During the experimental period, three sampling sessions were conducted to evaluate the amount of supplement refusals over time, on days 15, 17, and 30 of the experimental period. To characterize the individual supplement intake pattern, the supplement was offered at 11:00 h, and consecutive measurements of the remaining supplement in the feeder were performed at 60 min intervals, resulting in a total of six post-feeding sampling time points.

3.4. Productive performance

Productive performance was monitored from day 1 to day 86 of the experimental period. The amount of hay and supplement offered and the orts were recorded daily throughout the experiment to determine feed intake, in addition to once a week weighing of the steers (Figure 3) on an automatic weighing platform (Intergado®, Contagem, MG, Brazil). Average daily weight gain was calculated as the difference between the steers' final body weight and initial body weight. Weighings were mandatorily performed before forage offering. During the same handling, steers were led through a footbath to prevent hoof problems.

Figure 3. Body weight recording using an automatic weighing platform.

3.5. Metabolic trial

The metabolic trial was conducted from day 34 to day 52 of the experimental period. During this phase, steers were housed in a tie-stall facility and allocated into three groups evaluated in different periods, in order to facilitate sample collection and to minimize the interval between respiratory exchange measurements. Groups were balanced by body weight and comprised, mandatorily, two steers from each treatment per group.

During the metabolic trial, total fecal collection was performed for five consecutive days. Urine samples were obtained using *spot* sampling at eight time points throughout the day (02:00, 06:00, 10:00, 12:00, 14:00, 16:00, 18:00, and 22:00), with three subsamples collected per animal at each time point. Urine was collected by spontaneous micturition and, when necessary, auditory stimulation using water and manual stimulation of the preputial region were applied to induce urination.

Individual daily fecal output was weighed and homogenized, and a subsample was collected for moisture reduction in a forced-air circulation oven for 72 h and subsequently stored for analysis. Urine samples were immediately filtered through two layers of gauze. A 40 mL aliquot was collected using a syringe and stored in a Falcon tube, while a second tube received a solution containing 32 mL of urine and 8 mL of sulfuric acid (0.36 N). Immediately after processing, all samples were frozen at −20 °C until further analyses.

3.6. Respirometry

Methane production measurements were conducted from day 39 to day 74 of the experimental period using four climate-controlled respirometric chambers, arranged in pairs and configured as an open-circuit system (Aguilera and Prieto, 1986). Each chamber measured 3.68 m in length × 2.56 m in width × 2.24 m in height and was constructed of stainless steel, featuring double walls and lateral glass windows to allow visual contact among steers. These high-precision facilities enable the evaluation of interactions among dry matter intake, climatic conditions, methane emissions, and energy metabolism (Machado et al., 2016). This system is widely recognized as the “gold standard” method for assessing respiratory exchanges and energy metabolism in ruminants (Troy et al., 2016).

Chamber temperature and relative humidity were continuously measured, controlled, and recorded using Metasys software (version 5.1.3.0400; Johnson Controls Inc., Milwaukee, WI). Cooling and climate control of the four chambers were maintained using a water chiller system (GFA Water Chiller; Industrial Frigo S.r.l., Italy). Temperature and relative humidity setpoints were maintained at 22 °C and 65%, respectively. Airflow, regulated by a mass flow controller (FlowKit FK-2000, Sable Systems International, Las Vegas, NV), was set at 700 L/min.

Gas recovery tests were performed to verify the ability of each chamber to recover injected gases. These tests consisted of injecting known volumes and flow rates of the target gases (CO_2 and CH_4) into the chamber using a mass flow meter (MC-50SLPM-D, Alicat Scientific Inc., Tucson, AZ) connected to the gas cylinder outlet and chamber inlet. Based on the recovery results, a gas-specific correction factor was calculated for each chamber and applied to the measurements obtained from each animal. The average CH_4 recovery rate in the chambers was 95.62%.

Throughout the gas measurement period, daily calibrations were performed for the CO_2 analyzer (CA-10 Carbon Dioxide Analyzer) and the CH_4 analyzer (MA-10 Methane Analyzer), while calibrations of the O_2 analyzer (FC-10 Oxygen Analyzer) and the water vapor analyzer (RH-300 Water Vapor Analyzer) were conducted biweekly. All analyzers were manufactured by Sable Systems International. Data acquisition and analysis were carried out using ExpeData software (version 1.7.5, Sable Systems International) throughout the entire measurement period.

Between consecutive steers within the same group, chambers were shut down, dry-cleaned, and bedding materials were replaced with clean ones. After cleaning, each chamber was ventilated for approximately 15 minutes to enhance air circulation. Between experimental groups, chambers were washed, dried, and ventilated. This protocol ensured a hygienic environment and minimized contamination by gases from sources other than the steers' enteric production.

To validate individual measurements, two respiratory exchange assessments were performed for each cattle in different chambers, allowing a variation of up to 15% in natural matter intake relative to the mean intake observed during the metabolic trial period. Steers entered the chambers at 10 h, after stabilization of temperature and relative humidity, and exited at 08 h the following day, resulting in approximately 22 h of continuous measurement (Figure 4). Gas exchange data collected during this period were extrapolated to a 24 h basis. Final values for each animal were calculated as the mean of the two measurements. Dry matter intake over the period was used to calculate methane yield (g CH_4 kg^{-1} DMI), whereas average daily gain was used to estimate methane emission intensity (g CH_4 kg^{-1} ADG).

Figure 4. Measurements of methane production in the respiration chamber.

3.7. Blood Sampling and Processing

Blood samples were collected on day 87 of the experimental period at two time points: 1.5 h and 3 h after supplement administration. These sampling times were specifically established for the determination of blood methemoglobin concentrations. For hemoglobin analyses, samples from a single collection time point were used.

Samples were obtained by puncture of the coccygeal vein or artery using evacuated tubes. Tubes without anticoagulant were used for serum separation, whereas tubes containing K_3-EDTA were used for plasma separation. Immediately after collection, EDTA tubes were wrapped in aluminum foil to protect samples from light exposure and were sent for methemoglobin analysis by UV/VIS spectrophotometry at Laboratório Cavalieri (Juiz de Fora, MG, Brazil).

Hemoglobin analyses were performed using EDTA-treated blood samples to determine the concentrations. Analyses were conducted using an automated hematology analyzer (MHLab BH-5100).

3.8. Rumen Fluid Collection and Processing

Rumen fluid samples were collected on days 87 and 88 of the experimental period for the evaluation of volatile fatty acids (VFAs), specifically acetate, butyrate, and propionate. Steers were divided into two groups to reduce the time interval between samplings. Rumen content was collected using an esophageal probe connected to a vacuum system, three hours after supplement provision. An initial volume of the collected fluid was discarded to reduce contamination with saliva.

Immediately after collection, samples were filtered through a sieve lined with four layers of gauze. Rumen pH was measured using a portable digital potentiometer (MS Tecnopon®, MPA 210, Piracicaba, SP, Brazil). Subsequently, a 5 mL aliquot was transferred into a Falcon tube containing 1 mL of metaphosphoric acid (20% v/v) for VFA analysis. Volatile fatty acid concentrations were determined using a liquid chromatograph (Waters Alliance e2695 Chromatograph, Waters Technologies do Brasil LTDA, Barueri, SP, Brazil). A second 5 mL aliquot was transferred into a Falcon tube containing 2 mL of sulfuric acid (0.1 N; 50% v/v) for the determination of ruminal ammonia nitrogen (NH_3–N), following the Kjeldahl method (EMBRAPA, 2006).

3.9. Chemical Analyses

Samples of hay and supplements (offered and individual refusals) from the productive performance and metabolic trial periods were collected daily, oven-dried in a forced-air circulation oven at 55 °C, pooled, and quartered to obtain a composite sample representative of each experimental period. Likewise, total fecal samples were pre-dried immediately after collection and stored for subsequent analyses.

All samples were ground using a Wiley knife mill equipped with 1 mm and 2 mm sieves. For fecal material, a composite sample was prepared for each cattle based on the five consecutive days of total feces collection. Chemical analyses were performed at the Feed Analysis Laboratory of Embrapa Gado de Leite (Juiz de Fora, MG, Brazil). Indigestible neutral detergent fiber (iNDF) analyses were conducted at the in vitro incubation and ruminant feed evaluation laboratory (RuminaLab), Department of Animal Nutrition and Pastures, Federal Rural University of Rio de Janeiro (Seropédica, RJ, Brazil).

Hay samples (offered and refusals) were analyzed for air-dried matter (ADM; 55 °C for 24 h), oven-dried matter (DM; 105 °C for 3 h), and ash content (Ash; gravimetric method; Silva and Queiroz, 2006). Crude protein (CP) was determined by the Kjeldahl method (Detmann et al., 2025), using a conversion factor of 6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined sequentially using an Ankom 220 Fiber Analyzer (Van Soest method adapted; Ankom, 2006). Neutral detergent insoluble protein (NDIP; Kjeldahl; Detmann et al., 2025), neutral detergent insoluble ash (NDIA; gravimetric method; Silva and Queiroz, 2006), acid detergent insoluble protein (ADIP; Kjeldahl; Detmann et al., 2025), ether extract (EE; Ankom, 2009) and gross energy (GE; Parr adiabatic calorimeter).

Supplement samples (offered and refusals) were analyzed using the same methodologies described above for ADM (2 h), DM, ash, CP, NDF, NDIP, NDIA, EE, GE. Fecal samples were analyzed for ADM (72 h), DM, ash, CP, NDF, ADF, EE, GE. Samples of hay and supplement offered were analyzed for indigestible neutral detergent fiber (iNDF; Detmann et al., 2021) to characterize the material.

Urine samples were thawed and analyzed to determine nitrate and nitrite concentrations using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical). The method is based on a two-step colorimetric reaction allowing the determination of total nitrate + nitrite and nitrite alone. Urinary urea excretion was quantified using a commercial Urea CE analytical kit (Labtest, REF. 35, Lagoa Santa, MG, Brazil). In both analyses, absorbance was measured using an EON Microplate Spectrophotometer (BioTek), operated with Gen5 software for sample quantification. In addition, total nitrogen concentration in urine was determined by the Kjeldahl method (EMBRAPA, 2006). These analyses were performed for all urine samples collected at each sampling time for each cattle.

For the remaining analyses, a composite urine sample was prepared from the eight collection times. Urinary creatinine and uric acid concentrations were determined using commercial colorimetric methods (Labtest, REF. 35, Lagoa Santa, MG, Brazil). Urine samples were also analyzed for gross energy using a Parr adiabatic calorimeter.

Total urinary volume (L/day) was indirectly estimated using creatinine as a marker from *spot* urine samples. Calculations were based on urinary creatinine concentration (UC; mg/dL) and the assumed daily creatinine excretion. Daily creatinine excretion was estimated using the equation proposed by Silva et al. (2012):

$$UCE = 0.0345 \times SBW^{0.9491}$$

where UCE represents urinary creatinine excretion (g/day) and SBW represents shrunk body weight (kg).

Shrunk body weight (SBW) was estimated according to the equation proposed by Valadares Filho et al. (2023) for zebu and crossbred beef cattle:

$$SBW = 0.8915 \times BW^{1.0175}$$

Total urinary volume (UV; L/day) was estimated by dividing daily urinary creatinine excretion by creatinine concentration in *spot* urine samples, as follows:

$$UV = UCE / UC \times 0.01$$

where UCE is urinary creatinine excretion (g/day), UC is urinary creatinine concentration (mg/dL), and 0.01 is the conversion factor from mg/dL to g/L.

3.10. Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS, according to a completely randomized design, with initial body weight included as a covariate. Data related to individual intake patterns were analyzed as repeated measures over time, with animal included as a random effect. Orthogonal contrasts for treatment levels were generated using the PROC IML procedure (ORPOL function), and linear and quadratic effects of calcium nitrate inclusion levels were tested. Statistical significance was declared at α=0,05.

4 RESULTS

In the present study, the effect of supplementation with increasing levels of calcium nitrate was evaluated in steers fed low-quality forage. The additive was used as a source of non-protein nitrogen in the diet, presenting an approximate concentration of 15.56% N, with a nitrate content of 75.76%. The average NO_3^- intake over the experimental period was equivalent to 0, 6.14, 11.12, and 20.96 g of $NO_3^- kg^{-1}$ of total dry matter intake, for the respective treatments. The diet was composed of a high proportion of low-quality forage, simulating typical conditions of tropical pastures during the dry season, with an average forage: concentrate ratio of approximately 75:25.

There was no treatment effect ($P > 0.05$) on initial body weight (Table 2). However, a quadratic effect was observed for final body weight ($P = 0.045$) and average daily gain ($P = 0.045$), with the highest value recorded in the INT treatment, being 54% higher compared to the CON treatment.

The inclusion of different levels of the additive did not affect total dry matter intake (DM), nor hay intake. However, calcium nitrate inclusion influenced supplement intake, showing a quadratic effect ($P = 0.011$), with the lowest intake observed at the highest level of additive inclusion. In this context, the analysis of the temporal profile of individual supplement intake (Figure 5) showed a significant interaction between treatment and time after feeding ($P < 0.001$). This indicates that, in addition to differences in total supplement intake, there were also differences in the intake dynamics during the hours following supplementation. Steers receiving the diet HIG exhibited lower immediate intake, with a greater dispersion of supplement consumption over time, maintaining refusals for more than six hours after feeding, whereas the other treatments concentrated most of the intake within the first two to three hours.

Relative supplement intake, defined as the proportion of supplement consumed in relation to total dry matter intake, differed among treatments and showed a linear response to additive inclusion ($P = 0.005$). Organic matter (OM) and neutral detergent fiber (NDF) intakes did not differ among treatments. Crude protein (CP) and non-fiber carbohydrate (NFC) intakes were affected by the treatments, showing linear and quadratic reductions, respectively, at the highest level of additive inclusion ($P = 0.002$; $P = 0.003$).

No significant treatment effects ($P > 0.05$) were observed for total DM, NDF intake, or hay intake when expressed relative to body weight (BW). However, supplement intake relative to body weight decreased as calcium nitrate inclusion in the supplement increased, exhibiting a quadratic effect ($P = 0.015$).

Table 2. Intake, performance and digestibility in Nellore steers fed low quality Tifton hay and concentrate supplementation with calcium nitrate levels

Item^1	**Supplement**				**SE^2**	**P-value**	
	CON	**LOW**	**INT**	**HIG**		**Lin.**	**Quad.**
Body weight, kg							
Initial	364	366	363	358	13.8	0.691	0.848
Final	387	392	**400**	393	3.8	0.223	**0.045**
Daily gain	0.279	0.343	**0.430**	0.352	0.044	0.223	**0.045**
Intake, kg d^{-1}							
Total DM	6.52	5.99	6.60	5.88	0.262	0.182	0.594
Hay	4.77	4.30	4.74	4.56	0.224	0.823	0.691
Supplement	1.70	1.67	1.68	**1.37**	0.04	<.001	**0.011**
g g DMI^{-1}	262	278	255	**232**	9.0	**0.005**	0.182
OM	5.84	5.29	5.88	5.27	0.24	0.221	0.741
NDF	4.07	3.67	4.14	3.78	0.20	0.543	0.857
CP	0.786	0.738	0.788	**0.655**	0.0249	**0.002**	0.122
NFC	1.031	0.932	0.975	**0.797**	0.0137	<.001	**0.003**
Intake, g kg BW^{-1}							
DM	17.1	15.7	17.1	15.3	0.6595	0.127	0.658
NDF	10.66	9.64	10.72	9.83	0.489	0.438	0.925
Hay	12.6	11.4	12.7	11.8	0.6111	0.567	0.979
Supplement	4.44	4.33	4.35	3.54	0.1033	<.001	**0.015**
Digestibility coefficients, g Kg^{-1}							
DM	0.592	0.585	0.582	0.577	0.0125	0.392	0.853
OM	0.602	0.597	0.591	0.585	0.0125	0.295	0.888
NDF	0.538	0.532	0.531	0.523	0.0247	0.653	0.995
CP	0.748	0.746	0.738	**0.708**	0.0088	**0.001**	0.310
NFC	0.805	0.791	0.789	0.805	0.0293	0.253	0.113
GE	0.577	0.570	0.561	0.556	0.0139	0.235	0.763
Energy intake, Mcal d^{-1}							
GE	26.7	24.1	26.7	23.2	1.09	0.068	0.573
DE	15.1	13.7	14.8	13.5	0.73	0.210	0.932
ME	11.7	10.4	11.6	10.7	0.77	0.571	0.900

[1] DM: Dry matter; DMI: Dry matter intake; OM: Organic matter; NDF: Neutral detergent fiber; CP: Crude protein; NFC: Non-fibrous carbohydrates; BW: Body weight; GE: Gross energy; DE: Digestible energy; ME: Metabolizable energy.

[2] SE: Standard error of the mean.

Regarding nutrient digestibility, no treatment effects ($P > 0.05$) were observed for DM, OM, NDF, NFC, or GE digestibility (Table 2). In contrast, CP digestibility differed among treatments, showing a linear effect ($P = 0.001$), with an approximate 5.35% reduction in the treatment with the highest level of additive inclusion. Gross, digestible, and metabolizable energy intakes were not affected ($P > 0.05$) by the levels of calcium nitrate inclusion.

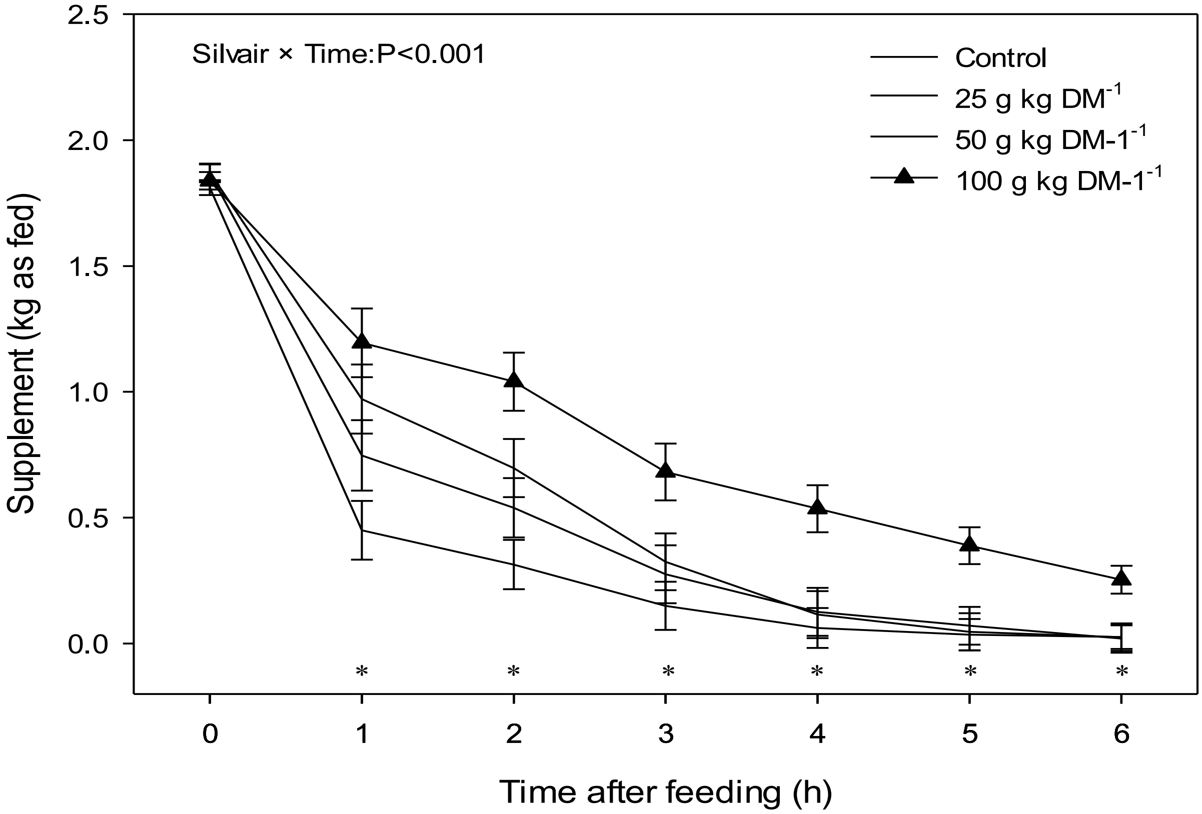

Figure 5. Supplement content on feed bunk as a function of time after feeding of Nellore steers fed low-quality Tifton hay and concentrate supplementation with calcium nitrate levels.

Based on ruminal fluid samples, no differences were observed ($P > 0.05$) among treatments in pH or in the concentrations of acetate, propionate, butyrate, and ammonia nitrogen (Table 3). However, a linear effect was observed for the acetate: propionate ratio ($P = 0.008$), and a quadratic effect was detected for the acetate: (propionate + butyrate) ratio ($P = 0.033$), indicating alterations in the volatile fatty acid (VFA) profile.

Table 3. Rumen parameters in Nellore steers fed low quality Tifton hay and concentrate supplementation with calcium nitrate levels

Item[1]	**Supplement**				**SE[2]**	**P-value**	
	CON	**LOW**	**INT**	**HIG**		**Lin.**	**Quad.**
pH	7.20	7.22	7.41	7.28	0.100	0.468	0.255
Acetate (mg mL^{-1})	29.5	29.2	28.5	34.4	2.32	0.108	0.242
Propionate (mg mL^{-1})	6.26	6.38	5.45	6.90	0.529	0.447	0.164
Butyrate (mg mL^{-1})	3.17	2.97	2.44	3.46	0.369	0.560	0.085
A:P	4.69	4.61	5.23	**5.02**	0.114	**0.008**	0.107
A:(P+B)	3.12	3.17	3.64	3.36	0.114	0.068	0.033
N-NH_3 (%)	11.86	11.34	10.30	8.86	1.493	0.124	0.978

[1] A: Acetate; P: Propionate; B: Butyrate; N-NH_3: ammoniacal nitrogen.

[2] SE: Standard error of the mean.

The inclusion of different levels of the additive did not affect ($P > 0.05$) total DM intake, hay intake, or GE, DE or ME intake (Table 4). Likewise, dietary metabolizability (q) was not influenced by the levels of additive inclusion. Supplement intake decreased linearly ($P = 0.008$) with increasing levels, with the lowest intake observed in the HIG treatment.

The actual CH_4 emissions were corrected based on the recovery rate of the chamber corresponding to each passage. Daily methane production (CH_4, L d^{-1}) decreased linearly ($P = 0.047$) as calcium nitrate levels increased in the diet. Consequently, linear effects were also observed for the CH_4:CO_2 ratio ($P = 0.002$), methane yield per unit of dry matter intake (Yield; $P = 0.007$), and methane emission intensity per unit of body weight gain (Intensity; $P = 0.017$). Methane emission intensity was reduced by approximately 47% between CON and HIG. Carbon dioxide production was not affected by treatments ($P > 0.05$). The methane conversion factor (Ym) did not differ among additive inclusion levels, although a tendency for a linear reduction was observed ($P = 0.071$).

Table 4. Energetic metabolism in Nellore steers fed low quality Tifton hay and concentrate supplementation with calcium nitrate levels

Item[1]	**Supplement**				**SE[2]**	**P-value**	
	CON	**LOW**	**INT**	**HIG**		**Lin.**	**Quad.**
DMI, kg d^{-1}	6.21	5.88	6.39	6.06	0.280	0.952	0.789
Hay	4.50	4.17	4.72	4.52	0.264	0.657	0.904
Supplement	1.71	1.71	1.67	1.54	0.046	**0.008**	0.343
GEI, Mcal d^{-1}	24.5	22.9	25.0	23.0	1.20	0.551	0.737
DEI, Mcal d^{-1}	13.8	12.7	13.7	12.4	0.99	0.372	0.886
MEI, Mcal d^{-1}	10.5	9.8	10.6	10.6	0.95	0.785	0.783
q	0.774	0.759	0.774	0.792	0.0200	0.364	0.515
CH_4, L d^{-1}	203	**194**	**198**	**183**	6.8	**0.047**	0.765
CO_2, L d^{-1}	2647	2660	2800	2668	59.7	0.659	0.121
CH_4:CO_2	7.70	7.30	7.07	6.84	0.184	**0.002**	0.278
Yield, L kg DMI^{-1}	32.9	33.3	31.1	28.6	1.27	**0.007**	0.551
Intensity, L kg BWG^{-1}	838	585	486	443	111.1	**0.017**	0.140
Y_m	7.88	8.12	7.51	7.08	0.399	**0.071**	0.663

[1] DMI: Dry matter intake; GEI: Gross energy intake; DEI: digestible energy intake; MEI: Metabolizable energy intake; q: metabolizability of the diet; BWG: Body weight gain; CH_4: Methane; CO_2: carbon dioxide; CH_4:CO_2: relationship between CH_4 and CO_2; Ym: Methane conversion factor.
[2] SE: Standard error of the mean.

The inclusion of the additive did not affect ($P > 0.05$) the urinary concentration of total nitrogen (Table 5) or urea (mg/dL) among treatments (Table 5). However, a significant effect of sampling time was observed ($P < 0.001$) for both variables, reflecting the metabolic dynamics of nitrogen excretion throughout the day. No significant interaction between treatment and time was observed for these variables ($P > 0.05$). In contrast, urinary nitrate and nitrite concentrations showed a significant treatment x time interaction ($P < 0.001$).

Table 5. Urinary concentration and balance of nitrogen compounds in Nellore steers fed low quality Tifton hay and concentrate supplementation with calcium nitrate levels

Item	Supplement				SE[1]	Time (h)								SE[1]	S*T	T	P-value	
	CON	LOW	INT	HIG		2	6	10	12	14	16	18	22				Lin.	Quad.
Concentration																		
Nitrogen, %	0.542	0.694	0.459	0.614	0.1059	0.587	0.511	0.311	0.587	0.637	0.646	0.679	0.659	0.0799	0.159	<0.001	0.913	0.726
Urea, mg/dL	1378	1580	1263	1495	155.7	1490	1358	1156	1514	1356	1529	1416	1613	103.9	0.712	<0.001	0.852	0.712
Nitrate, mg/dL	0.021	0.122	0.170	0.492	0.033	0.276	0.202	0.094	0.178	0.183	0.200	0.223	0.255	0.0289	<0.001	<0.001	<0.001	0.067
Nitrite, µg/dL	0.409	0.418	0.454	0.787	0.052	0.471	0.426	0.506	0.777	0.685	0.424	0.444	0.402	0.057	<0.001	<0.001	<0.001	0.024
Uric Acid, mg/dL	17.14	15.86	9.79	14.50	2.688	-	-	-	-	-	-	-	-	-	-	-	0.405	0.160
Nitrogen balance																		
Intake, g d^{-1}	119.0	117.4	122.9	110.0	4.41	-	-	-	-	-	-	-	-	-	-	-	0.185	0.235
Fecal, g d^{-1}	30.1	30.1	32.3	31.8	1.58	-	-	-	-	-	-	-	-	-	-	-	0.348	0.657
Urinary, g d^{-1}	57.7	57.8	62.3	58.5	4.74	-	-	-	-	-	-	-	-	-	-	-	0.836	0.596
Urea, g d^{-1}	54.4	48.9	56.3	50.1	4.24	-	-	-	-	-	-	-	-	-	-	-	0.689	0.779
g gN^{-1}	945	877	941	871	92.8	-	-	-	-	-	-	-	-	-	-	-	0.680	0.958
Nitrate, mg d^{-1}	2.6	9.9	25.6	49.4	3.70	-	-	-	-	-	-	-	-	-	-	-	<0.001	0.672
mg gN^{-1}	0.009	0.039	0.098	0.190	0.0128	-	-	-	-	-	-	-	-	-	-	-	<0.001	0.680
Nitrite, µg d^{-1}	44.2	34.0	72.0	85.6	11.60	-	-	-	-	-	-	-	-	-	-	-	0.006	0.866
µg gN^{-1}	0.233	0.179	0.402	0.433	0.0713	-	-	-	-	-	-	-	-	-	-	-	0.022	0.880
Balance, g d^{-1}	31.2	29.6	28.3	19.7	5.26	-	-	-	-	-	-	-	-	-	-	-	0.119	0.688

[1] SE: Standard error of the mean.

For nitrate, a distinct temporal pattern was observed among supplementation levels (Figure 6), with values close to zero in the CON treatment, intermediate concentrations in the LOW and INT treatments and higher concentrations in the HIG treatment, especially from 12 h onward or two hours after supplement provision. Regarding nitrite, only the HIG treatment exhibited excretion peaks, with higher values between 12 h and 14 h and a smaller peak at 18 h, corresponding to two, four and eight hours post-supplementation. The remaining treatments maintained relatively stable concentrations throughout the sampling period. Urinary uric acid concentration did not differ among treatments ($P > 0.05$).

Regarding nitrogen balance, no treatment effects were observed on nitrogen intake, fecal nitrogen excretion, total urinary nitrogen excretion or urinary urea excretion, either in absolute terms (g/day) or relative to nitrogen intake (g gN^{-1}) (Table 5).

In contrast, urinary nitrate excretion increased linearly ($P < 0.001$), both in absolute terms (mg/day) and relative to nitrogen intake (mg gN^{-1}), with higher values as calcium nitrate inclusion levels increased in the diet (Table 5). A similar pattern was observed for urinary nitrite excretion, which also increased linearly in absolute terms ($P = 0.006$) and relative to nitrogen intake ($P = 0.022$).

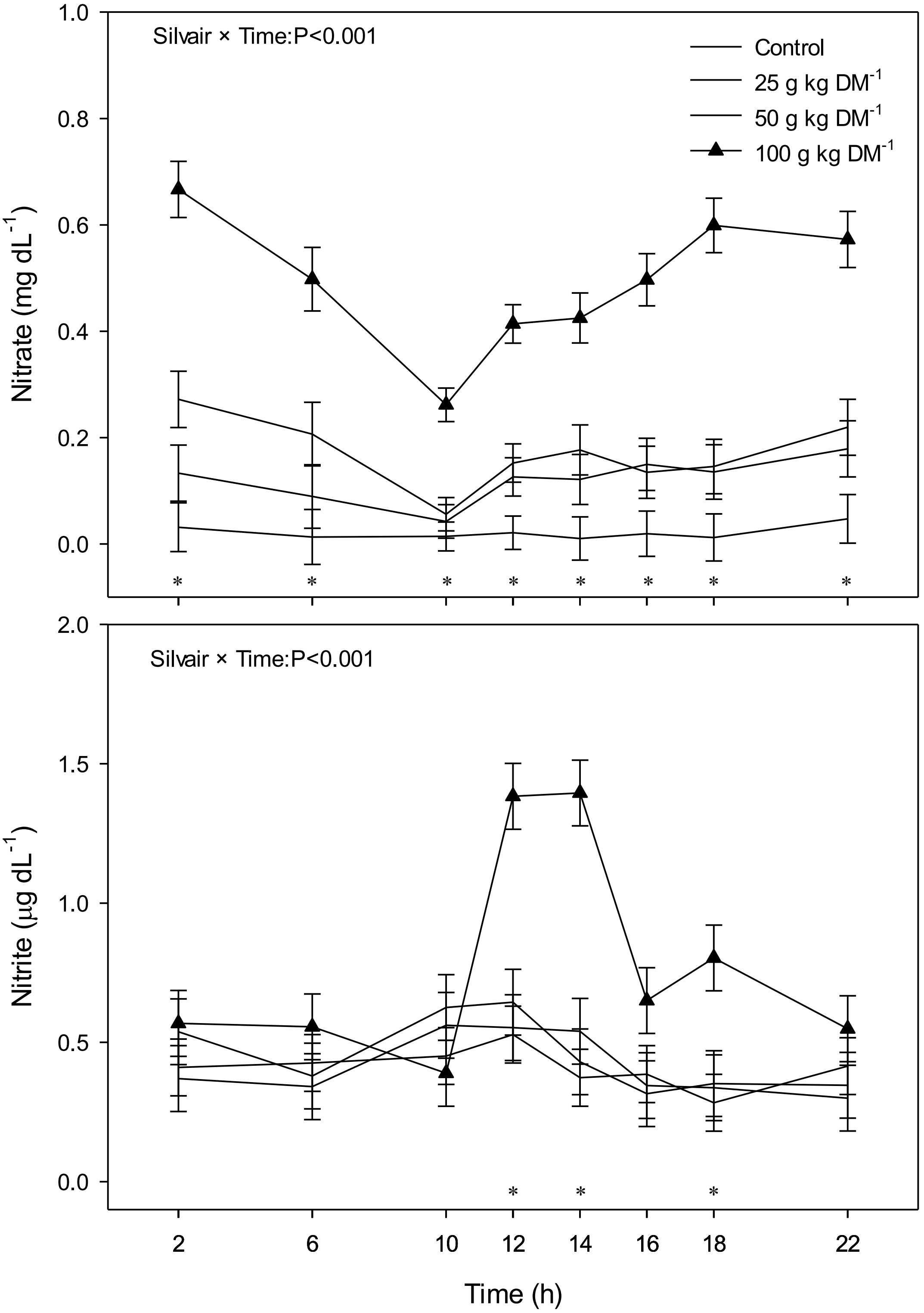

Figure 6. Urinary concentration of nitrate and nitrite in Nellore steers fed low-quality Tifton hay and concentrate supplementation with calcium nitrate levels

Additionally, the different levels of calcium nitrate inclusion in the supplement did not affect blood concentrations of methemoglobin and hemoglobin (Table 6) ($P > 0.05$).

Table 6. Blood parameters in Nellore steers fed low-quality Tifton hay and concentrate supplementation with calcium nitrate levels

Item[1]	**Supplement**				**SE**[2]	**P-value**	
	CON	**LOW**	**INT**	**HIG**		**Lin.**	**Quad.**
MetaHH (%)	0.54	0.58	0.63	0.54	0.105	0.985	0.497
HGB (g/dL)	11.0	10.9	11.2	11.6	0.498	0.265	0.735

[1] MetaHH: Methemoglobin; HGB: Hemoglobin.
[2] SE: Standard error of the mean.

5 DISCUSSION

Dry matter intake is an important indicator of possible effects associated with nitrate inclusion in the diet (Bruning-Fann and Kaneene, 1993). Reductions in intake have been related to increases in blood methemoglobin concentration when levels exceed 20% (Cockrum et al., 2010). However, studies conducted with total mixed rations (TMR) or partial mixed rations indicate that moderate reductions in intake may occur even in the absence of toxicity signs. Bharanidharan et al. (2025), when evaluating the inclusion of 1.578% NO_3^- in the dry matter of steer diets, observed a 4.5% reduction in dry matter intake. This response was mainly attributed to the organoleptic properties of the nitrate containing concentrate, since methemoglobin levels remained below 2.5%. The authors highlight that NO_3^- intake under these conditions may have been insufficient, especially during the first six hours after feeding, which is the period when CH_4 production is higher and mitigation is more desirable. Similarly, in the present study, it was possible to verify that higher levels of calcium nitrate inclusion also compromised supplement intake, without affecting hay intake or total dry matter intake. When comparing the CON treatment with the HIG, a reduction of approximately 19% and 10% in supplement intake was observed during the productive performance evaluation and metabolic trial periods, respectively.

Complementarily, the analysis of the supplement intake profile (Figure 5) indicated that the HIG altered intake dynamics over time, contributing to the reduction in total daily intake observed. Similar results were reported by Lee et al. (2017b), who observed changes in the feeding behavior of steers fed TMR and supplemented with encapsulated nitrate (2.5% DM) and non-encapsulated nitrate (2.3% DM), causing an increase in forage intake due to concentrate selection, with an increase in NO_3^- in the refusals. This change in ingestive behavior causing reduction in supplement intake and had direct effects on nutrient intake in the present study causing reduction in CP and NFC intake, whereas nutrient digestibility was not affected by treatments, except for CP ($P = 0.001$), which decreased in the same treatment. In contrast, Lee et al. (2015) did not observe differences among treatments in nutrient intake, and dry matter digestibility increased linearly ($P = 0.03$) and OM digestibility tended to increase ($P = 0.06$) with the supply of encapsulated nitrate.

Alemu et al. (2019), when supplying 1.785% NO_3^- in dietary DM to crossbred beef steers, reported no effect of encapsulated nitrate on dry matter intake, average daily gain, or feed efficiency. In agreement, no effect on total dry matter intake was reported in intensive growing systems, with supplementation of 22.5 g NO_3^- Kg^{-1} of DM, without interference in animal productive performance (Lee et al., 2017a). According to Lee et al. (2015), when evaluating beef cattle fed diets with a 55% forage and 45% concentrate proportion, supplemented with increasing doses of the additive, up to 2.48% encapsulated NO_3^- in dietary DM, there was a tendency of reduction ($P = 0.06$; linear) in dry matter intake, with no effect on performance. In the present study, the quadratic effect observed for ADG, with the highest value at the INT, suggests that in diets with a high forage proportion, moderate additive levels, up to 11.12 g NO_3^- kg^{-1} DM, may improve energy utilization associated with methane mitigation, whereas higher inclusions (20.96 g NO_3^- kg^{-1} DMI) limited productive performance, possibly due to changes in intake and supplement intake dynamics. Similarly, Araujo et al. (2021) reported a reduction in dry matter intake when Nellore steers were supplemented with increasing doses up to 19.5 g NO_3^- Kg^{-1} DM in a diet with a high concentrate proportion.

In the present study, calcium nitrate inclusion did not alter ruminal pH or individual concentrations of acetate, propionate, butyrate, and NH_3–N, indicating that ruminal fermentation was maintained within a stable range among treatments. However, significant effects were observed on VFA ratios, with a linear increase in the acetate: propionate ratio and a quadratic effect on the acetate:(propionate + butyrate) ratio, evidencing subtle changes in the fermentative profile. Similar results were reported by Troy et al. (2015), in which higher molar proportions of acetate and lower proportions of propionate were observed in steers supplemented with nitrate, resulting in a significant increase in the acetate: propionate ratio.

Likewise, Bharanidharan et al. (2025) reported increases in acetate concentration and in the acetate: propionate ratio, with no effect on ruminal pH, total VFA concentration, or NH_3–N.

The change in the VFA profile observed across studies is consistent with the mechanism proposed for nitrate in the rumen. The reduction of NO_3^- to NO_2^- and subsequently to NH_3 acts as an alternative hydrogen sink, directly competing with methanogenesis. However, this redirection of H_2 does not affect only the reduction of CO_2 to CH_4, but also other hydrogen dependent fermentative pathways, such as propionate and butyrate formation (Ungerfeld and Kohn, 2006). In this context, it has been reported that NO_3^- inclusion shifts fermentation toward greater acetate production (Farra and Satter, 1971). An increase in the population of *Ruminococcus sp.*, which are cellulolytic bacteria related to acetate production, was observed when nitrate was included in cattle diets (Bharanidharan et al., 2025). In this context, the increase in the acetate: propionate and acetate: (propionate + butyrate) ratios observed in the present study is consistent with a redirection of ruminal fermentation toward a more acetogenic profile, without causing interference in the stability of the ruminal environment.

Although nitrate is reduced to NH_3 in the rumen, no differences were observed in ruminal N-NH_3 concentration among treatments. A possible explanation is that nitrate reduction may have occurred gradually. This result is in agreement with Bharanidharan et al. (2025), who also did not observe an increase in N-NH_3 with NO_3^- supplementation. In contrast, Lee et al. (2015) reported a dose-dependent reduction in ruminal N-NH_3 concentration with encapsulated nitrate inclusion, even in isonitrogenous diets, attributing this effect to the use of a slow-release nitrate source. In addition, Leng (2008) highlighted that nitrate reduction in the rumen may not be associated with immediate NH_3 release into the medium, since this process may occur intracellularly, resulting in slower NH_3 release to the ruminal environment. Another hypothesis proposed by Wang et al. (2018) is that NH_3 derived from nitrate is rapidly incorporated into microbial biomass, which may limit significant differences in N-NH_3 concentration among treatments.

Although the method of respirometric chambers is widely recognized as the gold standard, some disadvantages associated with the characteristic confinement of animals in respiration chambers may occur (Storm et al., 2012). In some studies, the reduction in methane production has been mainly related to decreases in dry matter intake, as Bharanidharan et al. (2025), for example, observed a reduction in daily CH_4 production of up to 43% after 21 days of supplementation; however, there was also a 17.4% decrease in intake when cattle receiving nitrate diets were confined in chambers, without a consistent effect on methane yield. In the present study, when intake interference due to the chamber environment was observed, the data were not considered and the procedure was repeated. However, low occurrence was observed due to the prior adaptation performed. Daily methane production showed a linear reduction with increasing levels of calcium nitrate inclusion in the diet, with an approximately 10% decrease between the control treatment and the treatment with the highest additive level. The results obtained are in agreement with Feng et al. (2020), who, based on a meta-analysis, reported that dietary NO_3^- supplementation promoted average reductions of 20.4% and 10.1% in CH_4 production in dairy and beef cattle, respectively. According to the authors, the magnitude of the additive response may vary according to animal category. This variation in results has been attributed to the nitrate dose offered and its form of delivery in the diet, with beef cattle generally requiring higher inclusion levels to achieve mitigations similar to those observed in dairy cattle (Lee and Beauchemin, 2014).

Lee et al. (2017a), when supplying encapsulated and non-encapsulated nitrate to cattle in an intensive rearing system, observed a tendency toward reduced CH_4 production, however, neither treatment affected CH_4 yield. The authors also reported greater forage intake as a result of selection against the nitrate-containing concentrate, evidenced by higher nitrogen and lower NDF concentrations in the refusals. In the present study, however, in addition to the reduction in absolute production, reductions were observed in the CH_4:CO_2 ratio, in yield by 13%, and mainly in methane emission intensity, by approximately 47%, indicating that the effect of nitrate was not limited to behavioral changes in intake. Similar results were reported by Troy et al. (2015), who observed reductions in both methane production and yield (by 17%) when beef cattle were supplemented with nitrate, without impact on dry matter intake. Complementarily, Lee et al. (2015) observed that absolute methane production and methane yield decreased with increasing nitrate intake, showing a dose–response relationship. The same authors reported that removal of nitrate from the diet resulted in rapid recovery of CH_4 production, with heifers receiving nitrate reaching values similar to the control on the first day after withdrawal of the additive. These results reinforce that the mitigating effect of nitrate is dependent on its continuous presence in the rumen.

The reduction in methane emissions associated with nitrate supplementation is related to its role as an alternative hydrogen acceptor in the rumen, competing more favorably with the reduction of CO_2 to CH_4 and, consequently, reducing methanogenic activity (Lee and Beauchemin, 2014). This mechanism helps to explain the absence of treatment effects on CO_2 production observed in the present study, suggesting that the effect of nitrate occurred specifically on the methane formation pathway, without compromising ruminal fermentation.

Lee et al. (2015) reported that the main difference in energy losses between heifers supplemented with encapsulated nitrate and the control was attributed to enteric methane production, indicating that lower CH_4 emission resulted in reduced dietary energy loss. In the present study, although the methane energy conversion factor (Ym) did not differ statistically among treatments, a linear decreasing trend ($P = 0.071$) was observed with increasing inclusion of the additive, which is consistent with the reductions observed in methane production and emission indicators.

The increase in urinary nitrate and nitrite excretion with additive inclusion suggests that at HIG, part of the ingested nitrate was not completely reduced in the rumen and was eliminated via urine. However, this pattern was not reflected in changes in nitrogen metabolism, since no effects were observed on total urinary N excretion, urea, or nitrogen balance. In contrast, Lee et al. (2017b) observed an increase in N retention with encapsulated nitrate supplementation, suggesting greater efficiency of NO_3^- utilization compared with urea. Additionally, increases in nitrate concentration in urine and milk have been described in dairy cows fed nitrate-containing diets (Petersen et al., 2015; Olijhoek et al., 2016). The results indicate that urinary nitrate and nitrite excretion reflects a partial limitation of ruminal reduction at higher inclusion levels, but does not compromise nitrogen utilization efficiency.

Despite the reductions observed in supplement intake, methemoglobin levels in the present study did not differ, remaining very similar among treatments and below 1% of total hemoglobin. Alemu et al. (2019) reported that steers did not present clinical signs of methemoglobinemia, with a maximum methemoglobin concentration of 4.1%, which does not represent a problem for animal health and welfare. Additionally, Araújo et al. (2021) observed an increase with a linear effect ($P < 0.01$) as dietary nitrate levels increased; however, blood concentrations remained lower than 3.57% of total hemoglobin. Taken together, these results indicate that intake reductions associated with nitrate inclusion may be more related to physiological adjustments or diet palatability than to toxic effects.

6 CONCLUSION

Supplementation with calcium nitrate was effective in mitigating enteric methane emissions on steers fed low-quality tropical forage, promoting reductions in daily production, yield, and emission intensity of CH_4, despite the decrease in supplement intake at the highest level of inclusion evaluated. The results demonstrate that calcium nitrate is a viable strategy to reduce enteric methane in systems based on low-quality forages.

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