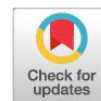


Research Article



Methane Production and Performance of Steers Supplemented with Different Levels of Calcium Nitrate

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Abstract | Methane emissions in ruminant livestock significantly contribute to greenhouse gas emissions. Various nutritional strategies have been explored to reduce CH₄ emissions, one of which involves the use of nitrates. This study aimed to evaluate the effects of calcium nitrates inclusion on enteric methane production and steer performance. Two experiments were conducted: Trial 1 (*in vitro* assay) assessed methane production and fermentation of three experimental diets containing different levels of calcium nitrate: 0%, 1%, and 2%. Trial 2 (*in vivo* assay) examined the impact of nitrate on the production performance of steers. Both experiments utilized a completely randomized design. In Trial 1, maximum gas production (Gmax) and methane production decreased by 25% and 43% with the 2% calcium nitrate supplementation respectively compared to the control ($P < 0.05$). In Trial 2, final weight and average daily gain (ADG) increased by 4.8% and 13%, respectively, with T3 compared to the control ($P < 0.05$). A similar 43% reduction in methane production was observed in steers, aligning with the *in vitro* findings. Calcium nitrate supplementation not only enhanced productive parameters but also reduced enteric methane emissions in steers. Therefore, supplementation with 2% calcium nitrate in cattle presents a viable strategy for decreasing methane emissions without negatively impacting animal productivity.

Keywords | Methane, Productive parameters, Gas production, Nitrate, Silage, Nitrogen

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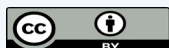
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INTRODUCTION

Drought has become into a frequent worldwide issue in the latest years. Unfortunately, developing countries

have been struggling with agriculture production and the loss of animals due to the lack of water; droughts reduced yields in most of the crops whereas others were lost (NCEI, 2023). In addition, extensive livestock farming has been

alarmingly decimated since feed and water supply is more difficult for farmers. Likewise, Mexico is facing one of the driest years in decades. According to the National Weather System, approximately 58 % of the total municipalities in the country present a certain level of drought; Durango reached the highest level of drought in some areas (NCEI, 2023; SMN, 2023). Additionally, states as Chihuahua, Zatecas and San Luis Potosí recorded a loss of 411,000 ha where crops did not germinate; this number increased over 600 % since 2022 (Agrolatam, 2023).

Otherwise, since roughage and nutrients are limited because of droughts, livestock farmers are in the need to supplement nutrients to avoid weight and animal losses. Moreover, N is the nutrient which is principally limited during extended dry seasons for grazing animals (Luo et al., 2019). Consequently, additives with N as urea are used to increase the roughage intake (Hennessy et al., 2000).

It is well known that drought is an event which occurs naturally as a part of hydrological cycles. However, this event occurs even more often and duration and intensity has increased as well. In this sense, a raise in global temperature as an effect of climate change may lead to terrifying scenarios in the future (Mukherjee et al., 2018). The accumulation of greenhouse gases in the atmosphere is a great issue to deal with; agriculture is the economic activity which contributes widely to the climate change effect (IPCC, 2019). Carbon dioxide and methane are the main responsible gases of the greenhouse effect. Additionally, different N sources, as nitrates, are used as competitors in the methane synthesis; nitrate reduction to nitrites and eventually to ammonium become into a natural sink to free H⁺ in the rumen (Olijhoek, et al., 2016). According to published research, ammonium formation through nitrates reduction yields more energy than methanogenesis; nitrate reduction pathway is more expectable than methanogenesis (Ungerfeld and Kohn, 2006; Olijhoek, et al., 2016). As a matter of fact, Klop et al. (2016) reported a methane reduction above 16% when administered 21g of nitrite per kg of DM to dairy cattle. Consequently, scientists worldwide are looking for feedstuffs or additives which may help to reduce greenhouse gases emissions, mainly methane, and to increase performance in steers going through extended droughts. However, there is some controversy in performance of steers supplemented with nitrates. Therefore, this study aimed to evaluate the level of calcium nitrate in enteric methane production and performance steers.

MATERIALS AND METHODS

All procedures involving animals were approved by the Livestock Protection and Promotion of the State of Durango (OF-2019-011-35). The experiment was carried out in the summer of 2023.

ANIMALS AND FEEDING

Two experimental trials were carried out at the Faculty of Veterinary Medicine and Husbandry of the Durango State Juarez University located in Durango, Dgo., Mexico. A growing steers diet was designed and three levels of calcium nitrate were added for each experimental treatment: 0, 1 and 2% (Table 1). The corn silage was obtained from barns whereas oat hay was obtained from crops; both nearby the faculty and according to drought conditions which reigned in the area.

Table 1: Ingredients and chemical composition of experimental diets.

Ingredients	T1	T2	T3
Oat hay	28%	28%	28%
Corn silage	36%	36%	36%
Ground corn	17%	17%	17%
Dry distillers grain	18%	18%	18%
Minerals mix	1%	1%	1%
Calcium Nitrate	0%	1%	2%
Nutrients	Chemical composition (%)		
Dry matter	93.87		
Crude protein	13.04		
Ether extract	2.25		
Neutral detergent fiber	56.82		
Acid detergent fiber	28.82		
Ash	6.97		
Dry matter digestibility	60.23		

TRIAL 1 IN VITRO ASSAY

Ground samples of each experimental treatment were dried in a forced-air oven at 55°C for 48 h and ground to 1 mm particles in a Wiley mill (Arthur H Thomas, Philadelphia, PA, USA) and subjected to further in vitro analyses.

CHEMICAL COMPOSITION

The determination of matter digestibility (DM), ether extract and crude protein (CP) content were calculated according to standard procedures (AOAC, 2016). The NDF and ADF concentrations were determined following recommendation of the manufacturer (ANKOM, 2018). Dry matter digestibility (DMD) was determined using a DaisyII incubator (ANKOM, Macedon, NY, USA) based on dry matter disappearance after 48 h and according to the manufacturer's procedures (ANKOM, 2018).

IN VITRO GAS AND METHANE PRODUCTION

The in vitro incubations were carried out using the ANKOM RF Gas Production System (ANKOM Technology, Macedon, NY, USA). Thus, samples of 1 g DM from experimental treatments (T1, T2 and T3) were incubated in triplicates in ANKOM glass modules equipped

with a pressure transducer. Fermentations were carried out according to procedures proposed by ANKOM Technology. The samples were incubated into a 2:1 mixture of buffer and ruminal inoculum solution. For ruminal inoculum, two Angus steers fitted with a permanent rumen cannula (10 cm i.d., Bar Diamond Inc., Parma, ID, USA) were adapted to the growing steers proposed diet for 15 days (70:30 forage:concentrate ratio), and rumen liquor was withdrawn from these steers before the morning feed (0800 h). The ruminal inoculum was collected as a pooled sample and immediately transported in thermos bottles to the laboratory, where it was filtered through four layers of cheesecloth, placed into glass modules with the samples and flushed with CO₂ (Musco *et al.*, 2015). The pressure was measured every hour during the 96 h incubation period. The kinetics of *in vitro* gas production were determined by fitting data to the Gompertz function using the following equation:

$$GP = Gmax \times \exp [-A \times \exp (-k \times t)]$$

where GP = gas production at time t (mL); Gmax = maximum gas production (mL/g DM); k = constant gas production rate (h⁻¹); and A = latency time before gas production begins (h). To measure the proportions of CH₄ and CO₂, the pressure relief valve of the modules was opened for 2 s. The expelled gas was sent through a tube to a portable gas analyzer (GEMTM5000, LANDTEC, USA) as previously described by González-Arreola *et al.* (2019). In addition, metabolizable energy (ME) of experimental treatments was estimated according to equation proposed by Menke and Steingass (1988):

$$ME = 2.20 + 0.136 (GP_{24}) + 0.057 (CP) + 0.0029 (EE^2)$$

where:

GP₂₄ = gas produced after 24 h of fermentation time (ml/g DM).

CP= crude protein (% DM).

EE= ether extract (% DM).

IN VIVO ASSAY

Thirty-six cross steers (304±10 kg) were randomly allocated among three dietary treatment groups according to each experimental treatment (Table 1). Three level of calcium nitrate were added to the experimental diet (0, 1 y 2%). Treatments were prepared weekly and supplemented with a mineral mix (12% phosphorus, 1 g/steer/day, Microfos12 MNA, Mexico). Steers were gradually adapted to nitrate diets from Day 1 to 14; the doses of dietary nitrate were increased every 2 days. Feed intake was restricted to 2.5% of live weight throughout the experiment. The daily ration was offered twice a day: at 0800 and 1500 h. Feed refusals were collected daily before the morning feeding, stored in a bag and weighed once a week. Dry matter intake was

measured on a daily basis by weighing refusals. Average daily gain (ADG) was measured by weighing each animal weekly. The trial lasted 90 days. Prior to the experiment, animals were vaccinated (Bacterina Triple Bovina, Bayer, Berlin, Germany) and treated for parasites (Ivermectin + ADE, NOVA, Mexico).

IN VIVO DETERMINATION OF CH₄ AND CO₂ PRODUCTION

The ruminal production of CH₄ and CO₂ was estimated based on DMI of each steer and the *in vitro* production of methane and carbon dioxide of experimental diet as proposed previous research (Herrera-Torres *et al.*, 2022; Table 1).

STATISTICAL ANALYSIS

The obtained data for the *in vitro* trial of gas production kinetics, methane and CO₂ production, were analyzed using a completely randomized design. The obtained data from animal performance were analyzed using a completely randomized design with PROC GLM of SAS. Each animal was treated as an experimental unit in the *in vivo* experiment, considering the treatments as fixed effects and random errors associated with each observation. Initial weight was introduced as a covariate using the procedures of SAS (SAS Software ver. 9.4, SAS Institute; Cary, NC, USA). Means of treatments were compared using the Tukey test for both trials (P < 0.05).

RESULTS AND DISCUSSION

Maximum gas production (Gmax) and constant rate of gas production decreased 25% and 75%, respectively, by supplementing 2% of calcium nitrate when compared with T1 (control) (P<0.05). However, no changes were observed when supplementing 1% in T2 when compared to T1 (P>0.05) in the same variable (k). These results suggest that microorganisms may take more time in degrading the nutrients when calcium nitrate is present. Zhou *et al.* (2012) affirmed that two of the major cultured cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) decreased their relative abundance when added sodium nitrate to the diet. These changes may affect the degrading rate since less bacteria are working in degradation of feedstuffs. However, the same authors reported no changes in abundance of total bacteria with the addition of sodium nitrate. This suggest that other bacteria may be increasing their relative abundance. As a matter of fact, Zhao *et al.* (2015) observed an increase in nitrate-reducing bacteria, which can later produce ammonia in rumen. Regarding to the latter, Olijhoek *et al.* (2016) observed an increase in ammonia when supplementing nitrates through the nitrate reduction pathway. In addition, Zhao *et al.* (2015) reported an increase in activity of *Selenomonas ruminantium*, *Veillonella parvula*, *Wolinella succinogenes*, *Campylobacter fetus*,

and *Mannheimia succiniciproducens* when nitrates were added into the diet. However, no changes were observed in lag phase and gas production after 24 h (GP24) (Table 2, $P>0.05$).

Table 2: Gas production parameters and methane *in vitro* of experimental diets.

	T1	T2	T3	SEM
Gmax (mL)	101.1±0.80 ^a	94.9±2.36 ^{ab}	76.6±9.80 ^b	11.92
A (%)	3.28±0.16 ^a	3.42±0.14 ^a	4.61±0.06 ^a	0.114
k (mLh ⁻¹)	0.09±0.002 ^a	0.09±0.001 ^a	0.020±0.005 ^b	0.0001
GP ₂₄ (mL)	78.4±3.68 ^a	65.3±0.58 ^a	75.2±5.40 ^a	3.09
Methane(m-Lg ⁻¹ DM)	15.4±1.58 ^a	13.8±1.40 ^a	8.9±1.63 ^b	0.270
ME (Mcalkg ⁻¹ DM)	3.0±0.93 ^a	2.8±0.69 ^a	2.9±0.52 ^a	0.09

^{abc}Means with letter different in row are not equals ($P<0.05$). Gmax= maximum gas production, k is constant rate of gas production, A represent lag phase before, GP₂₄ is volume of gas produced at 24 h, ME is metabolizable energy, SEM: standard error between the means.

Presumably, effects of calcium nitrate in gas production may be observed after 24 h of fermentation time, in fact, Zhou *et al.* (2012) observed a reduction of biogas after 48h in fermentation of diets with addition of sodium nitrate.

Regarding to methane production, supplementation of calcium nitrate decreased methane production by 43% with T3 when compared to T1 ($P<0.05$). The above can be attributed to the fact that ammonia captured hydrogen and this inhibited the methanogenesis pathway from the capture of hydrogen by CO₂ instead (Feng *et al.*, 2020). Additionally, nitrates affect the presence of archaea which make methanogenesis possible. Previous research observed a decrease in archaea:bacteria ratio which indicates that methane production is reduced by a collaborative action in reducing methanogens and promoting a different pathway for hydrogens caption (Zhou *et al.*, 2012). Moreover, Sar *et al.* (2005) showed a decrease in ruminal protozoa when supplementing nitrate so a reduction in hydrogen production may be expected as well. Otherwise, no changes were observed in ME with supplementation ($P>0.05$; Table 3).

Performance of growing steers supplemented with calcium nitrate is given in Table 3. As can be observed, final weight changed with supplementation ($P<0.05$); 2% of calcium nitrate increased final weight and ADG by 4.8% and 10%, respectively, when compared to T1. Otherwise, supplementation of calcium nitrate did not affect DMI when compared to T1 ($P>0.05$). Since DMI was not increased, it is possible that calcium nitrate improved nutrients utilization by increasing ADG. As a matter of fact, an

improved utilization of nutrients may be justified by an increase in the IVDMD; supplementation of 2% of calcium nitrate increased IVDMD 4.7 and 9.8% when compared T3 to T1 and T2, respectively. Previous research observed a decrease in FW and ADG when supplementing nitrates (Pesta *et al.*, 2016). Several studies have shown a decrease in DMI, ADG and final weight when supplementing nitrates (Duthie *et al.*, 2017; Duthie *et al.*, 2016; Hegarty *et al.*, 2016); however, these reports have added nitrates as a part of the diet in a TMR. Contrarily, in this case, nitrates were added as supplement into a diet. Almeida *et al.* (2022) found that DMI decreased when adding nitrates to a diet and attributed these changes to the palatability. However, this effect is not observed in the present study. Consequently, an enhancement in nutrients utilization may explain rises in FW, ADG and IVDMD without changes in DMI and FC. On the contrary, various studies have reported a decrease in ADG and DMI when supplementing calcium nitrate to growing steers. However, most of them are fed with high-forage diets (Hegarty *et al.*, 2016; Olijhoek *et al.*, 2016). The diets used in the present study are offering about 50% of forage in a TMR. On the other hand, Bharanidharan *et al.* (2024) reported non toxic effects in a long-term use of calcium nitrate after a 180 d trial with Hanwoo steers.

Table 3: Effect of supplementation with calcium nitrate on performance parameters and methane emissions of growing steers.

	T1	T2	T3	EEM
Initial weight, kg	300±3.5	308±4.12	310±3.5	-
Final weight, kg	461.69±0.85 ^b	462.61±1.07 ^b	483.93±1.40 ^a	0.920
DMI, kg/d	15.11±0.15 ^{ab}	15.06±0.10 ^b	15.70±0.15 ^a	0.112
ADG, kg/d	1.74±0.03 ^b	1.70±0.004 ^b	1.92±0.17 ^a	0.016
FC	8.66±0.10 ^a	8.65±0.22 ^a	8.57±0.15 ^a	0.137
IVDMD, %	73.91±0.92 ^b	70.44±0.69 ^b	77.39±0.25 ^a	0.557
Methane, kg/d	230.8±1.10 ^a	205.93±1.35 ^b	133.86±2.69 ^c	1.516

^{abc}Means with different superscript are statistically different ($P<0.05$). DMI: Dry matter intake; ADG: Average daily gain; FC: Feed conversion; kg DM/kg live weight; IVDMD: *In vitro* dry matter digestibility.

In relation to observed reductions in methane production, this study can be explained by conversion of nitrates into ammonia by captation of hydrogens as previously mentioned; other studies have found similar results by reporting a reduction of 17% (4.4 g/kg DMI) in CH₄ yield (80% of theoretical maximum) in similar conditions to those used in this study (Troy *et al.*, 2015). Likewise, the meta-analysis of Lee and Beauchemin (2014) predicted that the amount

of nitrate used would have reduced CH₄ emissions by 18%. A more recent analysis of the efficacy of nitrate, including the studies cited above (Rooke *et al.*, 2016) found that a mean inclusion of 21 g nitrate/kg DMI, reduced 21% the mean CH₄ (g/kg DMI). Nevertheless, this study observed a reduction of 42 and 35% when supplementing 2% of calcium nitrate when compared to none and 1% of supplementation, respectively. Apparently, an affectation in ruminal protozoa reduced drastically hydrogen production and neither methane or propionate were produced which may reduce gas production as observed in Table 2. If this is true, an increased reduction in methanogenesis would be expected. Duthie *et al.* (2017) showed an 11% reduction in methane yield when included nitrates as a part of the die in a TMR. Presumably, supplementation of nitrates increases antimethanogenic activity of nitrates above including nitrates as a part of a TMR. Hulshof *et al.* (2012) obtained similar results to those obtained in the present study when supplementing 2% of nitrates to beef cattle. Additionally, Andrade *et al.* (2022) reported reduction in methane yield above 30% in beef cattle supplemented with nitrates among seasons of the year. The latter may promote the worldwide commercial use of calcium nitrate to reduce ruminal enteric methane in livestock systems.

CONCLUSIONS AND RECOMMENDATIONS

Supplementation with 2% calcium nitrate in cattle increased productive parameters such as daily weight gain and decreased enteric methane emissions, which translates into a sustainable livestock practice. In addition, this study reports that *in vitro* methane production is similar to the one obtained in the *in vivo* assay, which implies that the method and equipment used are effective and precise. Finally, it is advisable to calculate the economic benefit of using this type of diet where the basis of the feed is corn silage, which is a low-cost ingredient and is produced worldwide.

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NOVELTY STATEMENT

In this study, we investigate the effect of different levels of calcium nitrate on parameters productive of cattle and methane production. Methane is a greenhouse gas, and it is important to try to reduce its concentration in the at-

mosphere. An alternative is to include nitrates in diets for ruminants, but it is important to define an adequate level that reduces methane emissions without reducing productive parameters in cattle production.

AUTHOR'S CONTRIBUTIONS

HTE conducted the experiment and wrote part of the manuscript; ARE conducted Trial 1; SFD co-conducted Trial 2; ACC co-conducted Trail 2; PCG developed statistical analysis and wrote and translate the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the published article.

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