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Technical note

In vitro evaluation of a protected ruminant nitrate source: effect on dry matter degradation and methane production

María Elizabeth Rendón-Correa [a]

Sandra Lucia Posada-Ochoa [a]

Jaime Ricardo Rosero-Noguera [a*]

[a] Universidad de Antioquia. Facultad de Ciencias Agrarias. Grupo de Investigación en Ciencias Agrarias – GRICA Calle 70 No. 52 – 21, Apartado aéreo 1226. Colombia.

[*]Corresponding author: jaime.rosero@udea.edu.co

Abstract:

The objective was to evaluate a method to reduce the calcium nitrate release rate in a simulated rumen fermentation environment, and to determine its effect on dry matter degradation and methane production. In the *in vitro* experiment, kikuyu grass (*Cenchrus clandestinus, Hochst ex Chiov*) (KK) was used as the base feed and the addition of protected nitrate (PN), free nitrate (FN) and urea (KU) to the fermentation environment. The amount of nitrate added corresponded to 3 % of the incubated dry matter. The data were analyzed with repeated measures over time considering treatment and time as fixed effects and the rumen inoculum donor animal as a random factor. After 24 h of incubation, FN and PN reduced dry matter degradation by 11.4 and 15 %, respectively. The addition of nitrate significantly reduced methane production. The difference in methane production rates expressed in ml/g of degraded dry matter between the FN (21.0) and PN (31.2) treatments at 48 h of incubation indicates a lower nitrate release rate as a consequence of the protection method employed. The results of this trial show that the inclusion of protected nitrates at levels corresponding to 3 % of the incubated dry matter can reduce methane production by 53 %.

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Among the greenhouse gases (GHG) caused by human activity, methane (CH_4) is the second most emitted gas, after carbon dioxide (CO_2), although CH_4 remains in the atmosphere for a shorter period of time and is emitted in smaller quantities. Its global warming potential is 25 to 34 times greater than that of CO_2[1]. CH_4 accounts for 30 % of the global enteric emissions of this gas. Because CH_4 is a short-lived climate pollutant, reducing enteric CH_4 emissions can help mitigate climate change within our current lifetime[2].

In ruminants, CH_4 production occurs during the enteric fermentation of organic matter, due to the need to remove hydrogen from the rumen in order to maintain a low redox potential at the fermentation site. Nitrate (NO^{3-}), an electron acceptor, has been studied as a potential pathway to route reduced equivalents away from methanogenesis, presenting itself as a hydrogen dissipating pathway that is useful to the animal and to the environment[3]. In the rumen, NO_3^- is reduced to nitrite (NO^{2-}) ($NO^{3-} + H_2 \rightarrow NO^{2-} + H_2O$), which in turn is reduced to the ammonium ion (NH_4^+) ($NO^{2-} + 3H_2 + 2H^+ \rightarrow NH_4^+ + 2H_2O$) —a process that captures four moles of hydrogen per mole of reduced NO^{3-}[4]. The reduction of NO^{3-} to NO^{2-} has a ΔG= -130 kJ, while that of NO^{2-} a NH_4^+ exhibits a ΔG= -371 kJ, which is energetically more favorable than the production of methane (ΔG= -67 KJ)[5]. The reduction of NO_2^- to NH_4^+ is a slow step, due to the low production of the enzyme nitrite reductase by rumen microorganisms, which can lead to an increase in nitrites at the rumen level. These nitrites cross the rumen wall and pass into the blood circulation, binding to hemoglobin and forming methemoglobin, which affects oxygen transport in the blood and may eventually lead to death by hypoxia[6]. Considering that the supply of pure NO^{3-} can present risks to animal health, several studies have been carried out with encapsulated NO^{3-} to release it slowly to ruminal microorganisms and reduce its potential toxic effect[7,8,9]. The purpose of this work was to evaluate a method for reducing the calcium nitrate release rate in a simulated rumen fermentation environment and determining its effect on dry matter degradation and methane production.

The experiment was carried out in the Nutrilab-Grica laboratory, at the University Research Headquarters (SIU) of the University of Antioquia - Colombia.

A sample of kikuyu grass (*Cenchrus clandestinus*, Hochst ex Chiov) at 45 d of regrowth was collected at the "La Montaña" farm located in the municipality of San Pedro de los Milagros (Antioquia - Colombia), at an altitude of 2,470 m asl and an average temperature of 16 ºC, corresponding to a Low Montane Humid Forest life zone.

The grass sample was partially dried in a forced ventilation oven at 60 ºC for 72 h, ground through a 1 mm sieve and stored for subsequent chemical analysis. Dry matter (DM), crude protein (CP), and ash concentrations were determined on the partially dried grass sample[10]. The proportions of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were

determined as described by Van Soest *et al*[10]. Table 1 describes the chemical composition of kikuyu grass.

Table 1: Chemical composition of kikuyu grass (*Cenchrus clandestinus*)

Chemical composition	Value
Dry matter (DM), %	23.5
Crude protein, % DM	20.6
Neutral detergent fiber, % DM	57.2
Acid detergent fiber, % DM	30.3
Ashes, % DM	11.5

The NO_3- source utilized in this study was calcium nitrate (CALCINIT, 15.5-0-0, YARA, Bogotá, Col.). The NO^{3-} was protected using a handmade soap produced by the saponification of commercial soybean oil with sodium hydroxide (NaOH, Merck N^o 106462)

The treatments evaluated were kikuyu grass incubated as a control treatment (KK), kikuyu grass + nitrate without protection (FN), kikuyu grass + protected nitrate (PN) and kikuyu grass + urea (KU). NO_3- treatments were adjusted to provide 3% nitrate/g DM incubated. The urea treatment was included as a control to demonstrate the effect of nitrogen addition on dry matter degradation and CH_4 production.

The nitrogen content present in the additives was determined by the Kjeldahl method[10]; for urea 48.1, unprotected nitrate 13.0, and protected nitrate 3.9.

The rumen fluid for *in vitro* incubation was obtained from three non-lactating adult Holstein cows, equipped with the one-stage ruminal cannula described by Castillo and Hernandez[11]. Donor animals were managed in a rotational grazing system with kikuyu grass, free access to fresh water, and mineral supplementation. The ruminal fluid was collected in the morning hours (0600) and transported to the laboratory in thermal containers previously heated with water at 39 °C. The ruminal fluid was gassed with CO_2 and filtered through four layers of absorbent cotton and kept in a water bath at 39 °C for the inoculation process.

One day prior to the start of the experiment, a buffer solution was prepared as described by McDougall[12]. This solution was mixed with each of the collected inocula at a 9:1 ratio (buffer: inoculum). A 0.5 g sample of kikuyu grass and the additives to be evaluated were weighed and placed in 100 ml glass bottles. Subsequently, a volume of 50 ml of the buffer-inoculum solution was added to each flask; during the process, it was continuously gassed with CO_2 to ensure anaerobic conditions and sealed with rubber stoppers. The sealed flasks were kept in a forced ventilation oven at 39 °C and removed from the incubation process at 24 and 48 h post-incubation to determine DM degradation and CH_4 production.

A total of 60 flasks were incubated: 48 flasks with substrate and inoculum (4 treatments * 3 repeats/treatment * 2 reading times * 2 repeats/schedule) and 12 flasks corresponding to the blanks (2 reading times * 3 inocula * 2 blanks per schedule). The blanks are flasks with buffer solution and inoculum without substrate or additive, whose function is to correct gas production and DM degradation generated by the inoculum.

Total gas production was measured at 24 and 48 h of incubation by measuring the pressure generated in each flask using a digital transducer (Ashcroft 2089QG- Precision Digital Test Gauges, USA) described by Posada *et al*[13]. After the measurement, a gas sample was taken to determine the concentration of CH_4 gas. A valve with three outlets was used. The first outlet was connected to a needle (0.6 mm); the second, to the pressure transducer, and the third, to a plastic syringe that was used to extract the gas sample. The needle attached to the valve was inserted through the rubber cap for pressure measurement, and, subsequently, the gases accumulated at the top of the bottle were withdrawn with the syringe to the point where the pressure recorded on the transducer reached zero. The gas collected in the syringe was stored in Clear Flex type co-extruded polyolefin bags (Baxter, USA). After finishing the sampling, CH_4 concentrations were measured by gas chromatography. A subsample of 100 μL of gas was taken from each bag with the help of a syringe to be injected into a Thermo Trace GC Ultra gas chromatograph (Thermo Scientific, USA). CH_4 production was established as the product of the total gas volume recorded over the incubation time (24 and 48 h) and the CH_4 concentration determined in the sample by gas chromatography.

After gas sampling at each measurement time, the flasks were opened to measure the degraded dry matter (DDM), determined by the difference in weight between the incubated DM (IDM) and the residue after incubation. In order to determine the DDM by gravimetry, the contents of each vial were filtered through glass crucibles (porosity 1, 100 - 160 μm) using a vacuum pump. The crucible-residue set was dried in a forced ventilation oven at 60 ºC for 48 h and subsequently weighed. After deducting the weight of the crucible, the value of the degraded DM was obtained as the difference between the DM of the residue and the DM of the blank, divided by the value of the initially incubated DM[14]. The liquid fraction of each incubation bottle was preserved by adding sulfuric acid (98 % v/v) drop by drop until an average pH of 2 was achieved; each sample was centrifuged at 4,000 rpm for 10 min and, finally, a subsample of 1.5 ml of supernatant was collected for the measurement of volatile (acetic, propionic, and butyric) fatty acids (VFA) by gas chromatography. The remaining liquid fraction was used to determine the ammoniacal nitrogen ($N\text{-}NH_3$) concentrations with the Kjeldahl method[10].

The effect of treatments on gas production, CH_4 and DM degradation were analyzed with a repeated measures model over time, using the PROC MIXED procedure of SAS[15] where the fixed effects corresponded to treatment and time (schedules), and the random effect corresponded to the source of rumen inoculum (animal). Comparison of means was

performed with the Tukey - Kramer test ($P<0.05$). Differences between treatments with respect to VFA and N-NH_3 production at 24 h were measured with a completely randomized model, using the GLM procedure of SAS[15]. Differences between means were determined with Duncan's multiple comparisons test ($P<0.05$).

The effect of NO_3- on DM degradation, gas production and CH_4 production *in vitro* at the 0 to 24 h and 0 to 48 h measurement intervals are presented in Table 2. NO_3- treatments showed a reduction in DM degradation at 24 h of incubation. The PN caused a 24 % reduction of DDM, while the reduction caused by the FN was 18 % compared to the control treatment (KK). At 48 h of incubation, there were no differences ($P>0.05$) between treatments. When the DDM was expressed in percentage terms, clearly the treatments that included nitrates exhibited lower degradations at 24 and 48 h of incubation ($P<0.01$) than the KK treatment ($P<0.01$). The comparison between the KU and KK treatments shows that the addition of urea to the fermentation environment had no effect on DDM or CH_4 production, indicating that the nitrogen supply in the control treatment (KK) was sufficient to maintain microbial activity during the incubation process.

Table 2: Effect of nitrate with and without protection on total gas production, methane production and degraded dry matter (DDM) in two *in vitro* fermentation schedules

Variable	Schedule	Treatments				Effects		
		KK	FN	PN	KU	T	Ti	Tx Ti
DDM, g	0 a 24 h	0.266^{a}	0.218^{bc}	0.202^{c}	0.248^{ab}	0.01	0.01	0.03
	0 a 48 h	0.271	0.27	0.267	0.30			
DDM, %	0 a 24 h	55.5^{ab}	49.2^{bc}	47.0^{c}	56.1^{a}	0.01	0.01	0.33
	0 a 48 h	64.6^{ab}	58.5^{b}	60.5^{b}	67.9^{a}			
Gas production, ml	0 a 24 h	46.9^{a}	35.2^{ab}	23.4^{b}	45.1^{a}	0.01	0.01	0.01
	0 a 48 h	84.2^{a}	57.1^{b}	60.1^{b}	84.4^{a}			
Gas production, ml/g DDM	0 a 24 h	177.8^{a}	161.0^{ab}	115.3^{b}	181.5^{a}	0.01	0.01	0.01
	0 a 48 h	313.8^{a}	211.2^{c}	225.6^{b}	279.7^{ab}			
Mehtane, ml	0 a 24 h	8.2^{a}	2.6^{b}	1.6^{b}	7.8^{a}	0.01	0.01	0.01
	0 a 48 h	17.7^{a}	5.7^{b}	8.3^{b}	16.35^{a}			
Methane, ml/100 ml gas	0 a 24 h	17.5^{a}	7.4^{b}	6.8^{b}	17.2^{a}	0.01	0.01	0.05
	0 a 48 h	21.0^{a}	9.7^{b}	13.8^{b}	19.4^{a}			
Methane, ml/g DDM	0 a 24 h	30.9^{a}	12.0^{b}	7.8^{b}	31.2^{a}	0.01	0.01	0.01
	0 a 48 h	66.0^{a}	21.0^{b}	31.2^{b}	54.2^{a}			

KK= kikuyu grass (*Cenchrus clandestinus*); FN= kikuyu grass + free nitrate; PN= kikuyu grass + protected nitrate; KU= kikuyu grass + urea; T= effect of the treatment; Ti= effect of the incubation; TxTi= effect of the interaction between the treatment and the incubation schedule.

^{abc} Means of treatments with different letters in the same row show differences ($P<0.05$).

Total gas production was significantly reduced (P<0.001) with the PN treatment at 24 h of incubation, compared to the KK and KU treatments. After 48 h of incubation *in vitro*, PN and FN treatments decreased total gas production by an average of 30 % compared to KK and KU treatments (P<0.05). When gas volume was expressed in ml/g DDM, the PN treatment produced 35 % and 28 % less gas than KK during 24 and 48 h *in vitro*.

The FN and PN treatments reduced total CH_4 production by 68 and 80 % with respect to KK (P<0.05) at 24 h (P<0.05). At the end of 48 h, the FN treatment maintains a 68 % reduction in CH_4 volume, and PN achieves a 53 % reduction compared to the control.

Table 3 shows the effect of the addition of protected and unprotected NO_3- on the production of VFA and ammonia nitrogen (N-NH_3) in an *in vitro* fermentation system. The production of VFA and N-NH_3 was not affected by the addition of NO_3- or urea to the fermentation environment (P>0.05).

Table 3: Effect of nitrate on the production of volatile fatty acids, and ammoniacal nitrogen (N-NH3) with and without protection at 24 hours of *in vitro* fermentation

Variables	**Treatments**				
	KK	**FN**	**PN**	**KU**	***P* value**
Acetic, mmol/L	62.9	58.9	76.7	98.2	0.29
Propionic, mmol/L	16.9	14.5	12.3	20.3	0.44
Butyric, mmol/L	8.5	7.7	7.1	8.5	0.20
N-NH_3, mg/L	14.0	10.5	10.5	11.7	0.69

KK= kikuyu grass (*Cenchrus clandestinus*); FN= kikuyu grass + free nitrate; PN= kikuyu grass + protected nitrate; KU= kikuyu grass + urea.

The decrease in DDM occurring with the PN treatment may be due to the soap used for protection. There is evidence that soy soap has a high dissociation in mediums with a pH of approximately 6.5[16]. This characteristic of soybean soap may lead to an increase in the polyunsaturated fatty acid content, which significantly depresses cell wall digestibility[17]. When a high dissociation of the soap has occurred, it enhances the release rate of NO_3-, potentially reducing the DDM[17]. Therefore, the use of PN may have prompted an additive effect of the unsaturated fatty acids and NO_2- on the reduction of DDM. On the other hand, the decrease in DDM with the FN and PN treatments may have been caused by the toxic effect of nitrites (NO_2-), which inhibit growth and promote the abundance of methanogens and other bacteria, such as *F. succinogenes* and *R. flavefaciens*, that play an important role in the degradation of dry matter in the rumen[18,19].

Mitigation of the CH_4 production through the inclusion of NO_3- *in vitro* has been reported by in previous research[8,20,21]. In the present study, the use of a 3 % dose of NO_3- with the FN treatment reduced the production of CH_4 /g DDM by 68 % during the 48 h of incubation. The

reduction in the CH_4 production observed with FN may be a consequence of the high reducing capacity of NO_3- in anaerobic media[22,23]. NO_3- behaves as an alternative hydrogen sink in the rumen, through its reduction to NH_4+ —an energetically more favorable process (ΔG =-501 kJ) than the reduction of CO_2 to CH_4 (ΔG= -67 KJ)[5]. Furthermore, the NO_2- resulting from the reduction of NO_3- may have exerted a toxic effect on the population of methanogens and certain cellulolytic bacteria[19,23], as mentioned above, which may have favored the trend in the reduction of the DDM.

The PN treatment brought about a 74 % reduction in CH_4 production in ml/g of DM, affecting DM degradation by 21 % at 24 h, compared to the control treatment (KK). Natael *et al*[18], assessed a similar dose of PN (3 % of the incubated DM), in an 80:20 (forage/concentrate) diet and found a 10 % reduction in CH_4 production during the same incubation time that did not affect the degradation of the incubated organic matter. With a 15 % inclusion of PN in 24 h *in vitro*, Lee *et al*[8] obtained a 45 % reduction in the produced CH_4 volume with respect to the control.

The purpose of utilizing PN is to slow down the dissolution rate of NO^{3-} in order to favor the growth of NO^{3-} and NO^{2-} that reduce the bacteria which accelerate NH_4+ formation[8]. The increase in this type of bacteria favors the reduction rate of NO_3- to NO^{2-} as well as of NO^{2-} to NH_4+, which would imply a decrease in the risk of NO_2- toxicity for both for ruminal microorganisms and the host animal. Theoretically, the reduction of 0.015 g of NO^{3-} should result in a decrease of CH_4 by 5.32 ml[24,25]; however, with PN, a total reduction of 9.4 ml of CH_4 was obtained, which is 76 % more than expected. This behavior was possibly due to a factorial effect of polyunsaturated fatty acids resulting from the dissociation of soap and NO^{2-} from the reduction of NO^{3-}, on the decrease in DDM, which finally favored the reduction in CH_4 production *in vitro*. The dissociation of the soap made with soybean oil may have favored the reduction in CH_4 production, as was the case in another study[26] where a strong correlation was found between the high degree of soybean oil establishment and a significant reduction in the number of methanogens and density of rumen protozoa. This correlation favored the reduction in CH_4 production by 60 % with respect to the control at 36 h *in vitro*. In an analysis of eight *in vitro* and four *in vivo* experiments on the potential of medium-chain fatty acids on CH_4 production, Machmüller[27] reported a significant decrease in the number of methanogens and a reduction of up to 40 % in CH_4 release with the use of soybean oil.

The present study found no significant differences (P>0.05) in the fermentation profile due to the use of NO_3-; however, there was a numerical difference of 28 % in the production of propionic acid with FN, and 40 % with PN, compared to the treatment with urea (Table 3). The addition of NO_3- in the rumen can reduce the production of CH_4 and of propionate, as it diminishes the availability of hydrogens, since many NO^{3-} reducing bacteria can utilize them as a substrate[28]; Therefore, this may generate competition not only with methanogenesis but also with propiogenesis[29]. The inclusion of protected NO_3- at the rate of 3 % of the incubated

DM in an 80:20 (concentrate/forage) diet reportedly[18] resulted in a linear reduction in propionic acid production and an increase in acetic acid production. Contrary to this, Lund *et al*[30] report that VFA production was not statistically affected by the addition of NO_3- at any of the concentrations used (6.66, 13.3, and 20 g/kg DM).

The concentration of N-NH_3 at 24 h of incubation did not vary between treatments. Contrary to what was found in other studies[8,26], where diets containing urea showed an increase in N-NH_3 concentration compared to treatments containing FN and PN. The KU treatment did not show a significant increase in NH_3 concentration after 24 h possibly because, although urea is a highly available source of nitrogen, it is rapidly hydrolyzed to NH_3 and is utilized by ruminal microorganisms for growth and development during the first three hours of incubation[31]. This causes a reduction of NH_3 levels and, possibly, an increase in the bacterial population and fermentative activity —a behavior that coincides with the increase in DDM observed with KU.

The fact that there were no differences in N-NH_3 concentration between the FN and PN treatments with respect to the control may be due to the type of metabolism of NO^{3-}. The rumen NO^{3-} is metabolized mainly by assimilatory reduction to NH_3; however, depending on the balance of enzymatic activities, nitrous oxide (N_2O) can be formed through denitrification. Because the rumen inoculum used in the current study was obtained from animals that were not adapted to NO^{3-}, NO^{2-} may have been accumulated in the system and, instead of being reduced to NH_3, it was diverted to the denitrification pathway, converting NO^{2-} to N_2O; this is the main source of N_2O under anaerobic conditions[32]. With an inclusion of 2 and 2.5 % NO^{3-} in the total DM incubated for 24 h, Welty *et al*[33] observed that NO^{3-} had a minimal effect on NH_3 concentration, which registered a significant increase only one hour after starting the *in vitro* test, while the values decreased the rest of the time and remained low during incubation.

The results of this *in vitro* test show that the inclusion of protected nitrates at levels corresponding to 3 % of the incubated dry matter can reduce methane production by 53 % after 48 h of *in vitro* incubation. The use of soaps with soybean oil as a nitrate protection method should be considered in greater detail, as the dissociation of the soap with a pH of approximately 6.5 favors the release of unsaturated fatty acids, potentially altering thereby the dynamics of the fermentation and degradation of feed in the rumen.

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