NITRATE: ITS ROLE ON RUMINAL FERMENTATION OF BEEF CATTLE UNDER INTENSIFIED GRAZING SYSTEM

Willian Rufino Andrade[1], Flávio Perna Junior[1], Analisa Vasques Bertoloni[2], Ana Laura Januário Lelis[2], Murilo Trettel[2], Patrícia Perondi Anchão de Oliveira[3], Paulo Henrique Mazza Rodrigues[1].

[1]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (FMVZ/USP), Pirassununga – SP.
[2]Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo (FZEA/USP), Pirassununga – SP.
[3]Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA Pecuária Sudeste, São Carlos - São Paulo

ABSTRACT

Rumen fermentation it is one of the contributors to the greenhouse gases emission (GHG) that causes global warming, and due to that reason livestock production is scrutinized and questioned all around the world on how GHG emissions from this subsector can be mitigated. A range of techniques have been studied by animal scientist, at nutritional level, to mitigate methane emission. Nitrate is of them, and it has a double important role on rumen fermentation, acting as a source of non-protein nitrogen, and as an electron sink path that allows the redirection of hydrogen to nitrate reduction, instead of being used in the methanogenesis process, and thus reducing methane emission. The partial results here presented shows that nitrate does not affect forage, supplement, and total dry mater intake, and neither short chain fatty acids production, however, methane emission (g.kg.day^{-1}) and CO_2eq emissions per kg. head^{-1}d^{-1}were reduced when nitrate was added in the diet. Thus, nitrate has the ability in changing rumen fermentation, which besides acting as source of non-protein nitrogen, also mitigate anthropic GHG emissions from enteric fermentation.

1. Introduction

Enteric fermentation, as it is most cited in different reports regarding GHG emissions and global warming, is a basic physiological activity that occurs in the rumen of a ruminant animal, such as beef or dairy cattle.

Rumen has an enormous importance to ruminants as it is the main path to them acquirer energy to sustain themselves. The rumen works as fermentative chamber, which provides ideal condition to growth of anaerobic microorganisms mainly responsible to the digestion of feed's components, functioning as fermenters of fibers, starches, sugars, organic acids, and proteins to furnish useful compounds used as the main fuel to the ruminant's metabolism. As a result of fermentative process important outcome are generated such as short chain fatty acids (SCFA), hydrogen (H_2) and carbon dioxide (CO_2). Besides that, methane (CH_4), an important gas, is also generated by means the use of CO_2 as acceptor and H_2 as the electron doner.

It is important to point out that CH_4 production represents a path to the release of exceeding hydrogen ions from rumen, which it is a key process to the maintenance of ruminal pH (Nagaraja et al., 2016) and overall rumen functioning. Besides that, CH_4 production is considered an inefficiency of the process as it represents a metabolic energetic loss, responsible to about 5% of total agricultural, land-use and forestry's greenhouse gases (GHG) emissions to the atmosphere (IPCC, 2022).

Reduce beef cattle production, to mitigate the impact of it on GHG emissions, is not an option since based on data, the population growth's demand for food is going to increase 60 to 100% by 2050, and at some point, human population is going to outrun the growth of food supplies (Van der Mensbrugghe et al., 2013). Knowing that, adoption of techniques that allows increase of beef cattle productivity accompanied with mitigation of GHG emissions is needed.

Due to concerns on beef cattle productivity and the contribution to mitigate GHG emissions to the atmosphere, different approaches have been studied by the academia, such as inclusion of Ionophores, Halogen compounds, plants secondary compounds, lipids, nitrooxy compounds and nitrate as well. The last, has a very promising effect on cattle productivity and reduction on enteric CH_4 production (Hulshof et al., 2012; Lee et al., 2014; Tomkins et al., 2018 and Alemu et al., 2019).

The use of nitrate has been studied on cattle feeding but most of the data on it comes from feedlot systems which differs a lot from Brazilian beef cattle production that rely, 86% of its production, on forage-based systems (ABIEC, 2022). Moreover, considering that most of the beef cattle produced in Brazil comes from forage-based systems there is also gap to improvement in terms of feeding better forage quality, grazing management, use of supplementation, adoption of more intensified systems with use of fertilization, and increased stocking rate.

Addition of nitrate into ruminants' diet under grazing systems might be one of the paths to redirect H_2 to a more valuable substrate formation, as opposed to the CH_4 production. The incorporation of H_2 into electron sinks are nutritionally beneficial to beef cattle as it reduces digestible energy losses and also favors ruminal microbial protein synthesis (Lan & Yang, 2019). Thus, this literature review aims to bring information on use of nitrate in beef cattle under grazing method, and its effect on ruminal fermentation.

2. GHG emissions from cattle production

As previously mentioned, enteric fermentation is one of the main parameters that features the subsector of agricultural, land use and forest GHG emissions. The Intergovernmental Panel on Climate Change (IPCC) releases every 6 years the impact that each sector and subsector from anthropogenic activities directly and indirectly contributes to global warming by means releasing GHG to atmosphere. In the last report, from 2022, it was brought to the scientific community attention that anthropogenic activities were responsible to release into the atmosphere 59 gigaton of CO_2eq in the year of 2019.

As seen in the figure 1, the total anthropogenic greenhouse gases emissions in 2019, the industry sector contributed with 20.06 Gt of CO_2eq while the agriculture, the second most contributor to anthropogenic GHG emissions, released 13.57 Gt of CO_2eq to the atmosphere in 2019. However, when segmented into in each subsector we can see that Enteric fermentation contributes only with 5.0% of the total Agriculture, land-use, and forest GHG emissions, which roughly means that on total anthropogenic GHG emissions enteric fermentation releases to the atmosphere 0.678 Gt of CO_2eq, 1.15% of the total amount.

Despite of the size of Enteric Fermentation contribution to the GHG emissions to atmosphere, there is still room for reducing the impact of this subsector on GHG emission. Ranked as the second largest beef cattle herd in the world, with around 196 million cattle, Brazilian beef cattle industry occupies a prominent position (ABIEC, 2022). The late slaughter age and low intensification of forage-based systems are one of the main reasons why cattle production occupies a great slice of the total livestock GHG emissions.

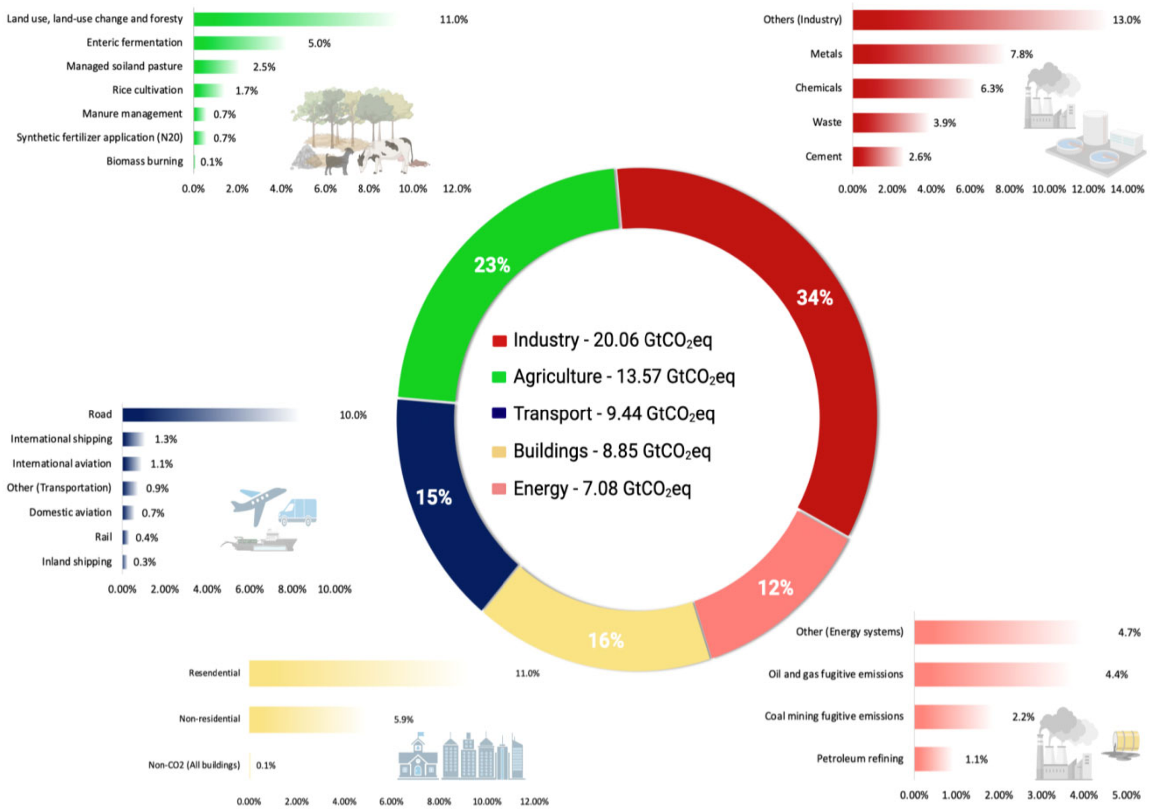

Figure 1. Total anthropogenic greenhouse gases emissions in 2019 expressed as $GtCO_2eq$. Data retrieved from the sixth IPCC report of 2022. Biorender was used to add some feature to the figure.

In 2016 the Ministry of Science, Technology, and Innovations (MCTI) release a report of all Brazilian's GHG emissions (Gt CO_2eq) to the atmosphere. According to MCTI (2016) Brazil contributed with 1,581,448 $GtCO_2eq$ emissions, being 35.8% of that from the Agricultural sector (576,146 of $GtCO_2eq$). On that scenario, according to the report, enteric fermentation was responsible for 329,228 Gt of CO_2eq, which was equivalent to 58.04 of the total agriculture and 20.81% of the total Brazilian CO_2eq emissions for 2016.

There was also a release of an estimative for 2019 in which the Brazilian GHG emission was accounting from 2.9% of World GHG emissions, and the agriculture and Enteric fermentation contributed with 0.92 and 0.51%, respectively. It is important to note that for this estimative the MCTI did not detailed the subsector information, and knowing that, the Enteric fermentation on this review was calculated using the metric from 2016 of 19.2%

It is noteworthy mention that enteric methane production, mainly released through eructation which accounts for 90% of the total methane released (Ribeiro et al., 2020), represents a loss of gross energy intake that can goes up to 12% on forage-based systems (Ungerfeld, 2020). Therefore, despite of the non-economical appeal of techniques to reduce the generation of methane, it is of interests of beef cattle producers to reduce enteric methane production as it means lower economic loss in the feeding system.

In Brazil, policies have been proposed to incentive a reduction of GHG emissions., The government based on National Policies of Climate Change (PNMC) proposed the ABC (Agriculture of Low Carbon) plan which has as main goal the reduction of GHG emissions. One of the ways that ABC's plan aims to accomplish settled goals are by means of improving the use efficiency of natural resources; promoting the increase of CO_2 fixation in the plant-soil system and incentivising the adoption of a more sustainable production systems that simultaneously to the reduction of GHG emissions allow producers to witness profit increase. Certainly, to achieve better results by means meeting sustainable and economic aspects in the system is not easy; however, as mentioned, there is a range of tangible tools that can be used to get there.

3. Grazing and supplementation in beef cattle production

The Brazilian beef cattle herd have accounts 196 million animals; it is a big herd which has annually an average of 39 million animals slaughtered that comes mostly from forage-based systems (82.81%) as compared to 18.19% from feedlot systems (ABIEC, 2022). Considering annual seasonality observed in the Tropics, especially in regions that beef cattle farms are located, finishing animals with ideal body weight signed to reduction of enteric methane production may be considered tough targets to achieve.

Forage quantity and quality tend to change through the year as observed by Lelis (2021). The author noticed that higher mass forage is produced in rainy season on deferred and rotated grazing methods (70.45 and 81.74%) as compared to dry season (29.55 and 18.26%) respectively. Knowing that fact, it becomes difficult to ensure that pastures by itself will provide to the herd the full nutritional needs, especially during dry season when forage decrease productivity and nutritional quality as well. Minerals, proteins and energy for instance might be a limiting factor to ideal ruminal fermentation and therefore overall animal performance under grazing systems (McAllister et al., 2019).

Souza et al. (2011) demonstrated that during dry season rumen kinetics parameters tend to be lower compared to the rainy season. According to the authors, the effective degradability of the dry matter (DM) of *Brachiaria brizantha* cv. Marandu from a monoculture was 21.41% lower (50.71%) than that observed on the rainy season (64.53%), and that the ruminal degradability rate followed the same trend being higher during rainy season, 4.1 $\%.h^{-1}$ as opposed to $3.1\%.h^{-1}$ in dry season. One of the reasons for that is the colonization time on forages, in that case during rainy season is 1.66 times faster than the colonization time during dry season. This trend is due to higher availability of soluble carbohydrates on forages during rainy season, while during the dry period all the soluble carbohydrates from leaf migrates to the bottom of stems.

As seasons go by and forage achieve the mature stage its nutritional values tend to pike and then decrease because of increase in undigestible components, which may be a limiting factor in terms of animal performance, culminating in loss of weight gain and decrease in the overall ruminal energetic performance (Capstaff & Miller, 2018). As observed by Lelis (2021), grazing simulation on *Brachiaria brizantha* cv Marandu pastures had lower CP (-8.45%) and higher lignin (+80.9%) concentrations on dry season as compared to the rainy. The author also noticed that the *in vitro* digestibility of the dry matter (DM) of pastures during winter were 13.3% lower than that observed on summer. That information contributes to understand how rumen environment and parameters such as rumen digestibility, degradability and consequently rumen fermentation products can be affected by forage quality.

As observed by Maciel (2016), the *in vitro* digestibility of the DM of *Brachiaria brizantha* cv Marandu reduces 7% from transition season (rainy to dry season) to dry season. Same trend was noticed for the effective degradability of the DM, at rate passage of 2 and 5% h^{-1}, with reduction of 9 and 9.69%, respectively. The consequences of rumen fermentation dynamics are the changes in the profile of fermented products. Canesin (2009) showed that acetic, propionic, butyric, and total short chain fatty acids concentration were 14.03, 27.17, 24.2 and 23.5% higher in the very early begging of rainy season compared to the dry season, respectively.

Dry season usually leads to lower crude protein levels in the forage, especially when fertilization is not a common practice, and lower availability of soluble carbohydrates, which can directly affect the rumen fermentation as there is an inadequate apport of nitrogen ammonia, causing instability on rumen microbial population and can lead to reduction of digestibility, degradability, and feed intake. Coupled with nutritional aspect, there are also some other variables that might influence beef cattle productivity in the Tropics under grazing systems such as soil amendment and fertility, forage specie, quality, and its availability (Delevatti et al., 2019). Understanding that, becomes way clear that beef cattle production under grazing systems is not as easy as it seems, especially for those that runs beef cattle systems in Tropical regions.

With that under perspective, supplementation of animals under grazing systems is an alternative to improve deficiencies that grazing systems may result. The main point of adopting supplementation is to have a better utilization of the nutrients by the ruminal microorganism’s synchronization in terms of protein and energy intake which directly affect the overall performance. Asizua et al. (2018) showed that the use of supplementation for grazing beef cattle improved in 29.6% the degradability rate from 2.7 to 3.5 $\%.h^{-1}$, while Dorea (2010) demonstrated that the effective degradability of the dry matter increases from 75.7 to 80.4% when grazing beef cattle were supplemented.

Adoption of supplementation is an alternative to improve feed efficiency in grazing-based beef cattle systems. It is a strategic tool to increase feed intake, improves cellular wall digestibility, and increase the passage rate in the rumen; thus, not only improvements in terms of performance might be achieved but also potential reduction of methane emission as the ingested feed becomes more digestible.

3.1.Urea and nitrates on beef cattle's diet

Non-protein nitrogen (NPN) supplementation in beef cattle production is a strategy taken to increase the apport of protein into the diet and meet the requirement of ammonia for microbial protein synthesis in the rumen. Notably, urea is one of the most well-known NPN sources that can be efficiently used in beef cattle diets. However, the use of alternative sources such as nitrate it has also been done.

Urea and nitrate, despite of being very important when added into beef cattle's diet since it is a NPN source, it can limit the feed intake as they are not palatable. For urea intake of 2% in DM basis of diet is a recommended limit edged; however, great results are found when added 1.34 to 2.26% on total diet DM (Paixão et al., 2006). When it comes to nitrate, as found by Cassiano (2017), inclusions of calcium nitrate on Nellore beef cattle diet at 1.0 or 2.0% on DM did not cause sides effect on limiting feed intake. However, 3.0% on DM can cause slight DM intake. Nitrate taste biter and it is known to reduce palatability (Yang et al., 2016), and that might be the causes of reduction on DMI found by Cassiano (2017).

On the same work Cassiano (2017) also showed that, despite of reduction of DMI at 3% nitrate inclusion, serum biochemistry parameters (urea, creatinine, albumin, gamma-glutamyl transferase enzymatic activity, aspartate-aminotransferase enzymatic activity, lactate concentration, calcium, total protein concentration and phosphorus concentration) did not display statistical effect for any of the nitrate inclusion.

As already mentioned, they are efficiently used as NPN sources to replace expensive plant protein feed and great results are found on literature when it comes to dry mater intake, as seen on table 2. Urea might appear to be the best economic choice in among both since it is the cheapest and in just 1 kg of it there is a total of 0.49 kg of nitrogen, which directly represents 2.875 kg of crude protein. For ammonium nitrogen, for instance, 1 kg of it has a total of 0.35 kg of nitrogen, which represents on total 2.187 kg of CP. Both sources are known for being biter and one of the main reasons for its use in beef cattle diet is the outcome in the rumen, the microbial protein synthesis. However, nowadays nitrates stand out as an important tool that might be used by nutritionist to attain not only great animal performance but also mitigation of GHG emissions to the atmosphere.

Specifically on nitrate, despite of its bitter taste (Yang et al., 2016), as observed on Table 2, data on literature shows no statistical effect on DMI when nitrate is added into beef cattle's diet, even with slight numerical difference, as shown on the following table.

Table 1. Nitrates into beef cattle's diet have no major effect on total dry mater intake

Category	**Diet**	**Nitrate**[**]	**DMI (kg.day^{-1})**		**Diff. (%)**	**Reference**
			Urea[***]	**Nitrate**[***]		
Bos indicus	80:20[*]	2.5%	8.93	8.47	- 5.42	Alemu et al. (2019)
Bos indicus	100:0[a]	1.5%	13.51	13.19	- 2.40	Salcedo et al. (2018)
Bos indicus	60:10[*]	2.2%	7.1	6.6	- 7.57	Hulshof et al. (2012)
Bos indicus	50:50[*]	4.5%	12.10	11.12	- 8.81	Borges (2018)
Bos Taurus	30:70[*]	2.0%	11.27	11.07	- 1.80	Hegarty et al. (2012)
Bos Taurus	20:80[*]	2.5%	7.8	7.2	- 8.33	Lee et al. (2017)
Bos Taurus	55:45[*]	2.15%	10.3	9.8	- 5.10	Duthie et al. (2018)
Bos Taurus	15:85[*]	2.15%	10.3	9.5	- 8.42	Troy et al. (2015)

[*] Forage: concentrate ratio; [**]nitrate inclusion is based on % of the DM; [***] Urea and Nitrate refers to the treatment that animals were assigned to; a: grazing at *Panicum maximum* during dry season and at *Brachiaria brizantha* cv Marandu during rainy season.

In spite of no major effect on feed intake, nitrate supplementation has been considered thermodynamically favourable since it is linked with ATP synthesis in some microbial species, which could increase nitrate reducing bacteria and overall flow of microbial protein in the rumen (Guo et al., 2009; Yang et al., 2016). Nitrate is ultimately converted into ammonia and thus ruminal microbial protein synthesis can be favored.

4. Effect of nitrate on ruminal metabolism

Nitrate is known as an inorganic anion with high redox potential that has negative charge and higher number of electrons (Wang et al., 2018). Due to that, it has been under investigation over the years nitrate utilization as a hydrogen sink, as a main electron-consumer competitor of the methanogenesis.

In terms of methanogenesis' inhibition, when nitrate reaches ruminal environment it is rapidly reduced to NO_2^- and that significantly contributes to the reduction of enteric methane since by this pathway there is a consume of hydrogen generating ammonium nitrogen (NH_4^+), disposing hydrogen by a path that is considered to be thermodynamically more favourable as compared to CO_2 reduction to methane (Yang et al., 2016). As seen on Equation 1 described by Olijhoek et al. (2016), when nitrate reaches ruminal environment it is first reduced to nitrite consuming already 2 electrons (e-) as showed in the following equation:

$$NO_3^{-} + [H_2] \rightarrow NO_2^{-} + H_2O \rightarrow (\Delta G = -130\ kJ)\ Eq.1$$
$$NO_3^{-} + 2H + \mathbf{2e^{-}} \rightarrow NO_2^{-} + H_2O \rightarrow (2e^{-}\ electrons\ not\ available\ to\ methanogenesis)\ Eq.2$$

After that, nitrite is further reduced to ammonium and in this second step about 6e- electrons are used, as seen on equation 3:

$$NO_2^{-} + [3H_2] + 2H^{+} \rightarrow NH_4^{+} + 2H_2O \rightarrow (\Delta G = -371\ kJ)\ Eq.3$$
$$NO_2^{-} + 8H^{+} + \mathbf{6e^{-}} \rightarrow NH_4^{+} + 2H_2O \rightarrow (6e^{-}\ electrons\ not\ available\ to\ methanogenesis)\ Eq.4$$

According to the same authors, following the Gibbs free energy (ΔG), all this process which involves both reductions path yields way more energy as opposed to methanogenesis (Equation 5), which makes nitrate reduction a competitive H_2 sinker.

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \rightarrow (\Delta G = -131\ kJ/mol)\ Eq.5$$

Van Zijderveldet et al. (2010) explain that through this path there is an efficient sink for hydrogen since for each mol of nitrate reduced 1 mol of CH_4 is not generated, which means that nitrate preferentially directs hydrogen away from methanogenesis. Considering NO_3 reduction pathways up to NH_4 generation, four moles of hydrogen are used to generate a molecule of ammonia nitrogen (Yang et al., 2016).

It is known that quite high concentrations of NO_3^{-} in ruminants' diet can lead to nitrite accumulation; however, that can only happen when kinetics of NO_2^{-} removal is running lower than that of NO_3^{-} first step reduction, as seen on equations 2 and 4. Intense accumulation of nitrate/nitrite in the ruminal environment may alter microbe's composition, especially methanogens, which is known to be sensitive to nitrite (Iwamoto et al., 2002). Cellulolytic bacteria can also be affected by nitrate concentration in the rumen (Latham et al., 2016). There are two main paths in which nitrate undergoes, dissimilatory and assimilatory reduction, and the fate of each one regards to the way of NO_3^{-} use (Sparacino-Watkins et al., 2014).

Nitrate metabolism in a cell of a ruminant bacteria is not well described yet; most of the studies and effort regarding nitrate metabolism come from a range of bacteria that does not inhabits the rumen. But despite of that, some representation of the metabolisms is proposed on the literature, as seen in the figure 2. Since inside of membrane has a negative potential, nitrate uptake by ruminal bacteria is done by means of active transport mechanisms to allows nitrate goes through the cytoplasmatic membrane with no harm (Andrade & Einsle, 2013), as seen on figure 3.

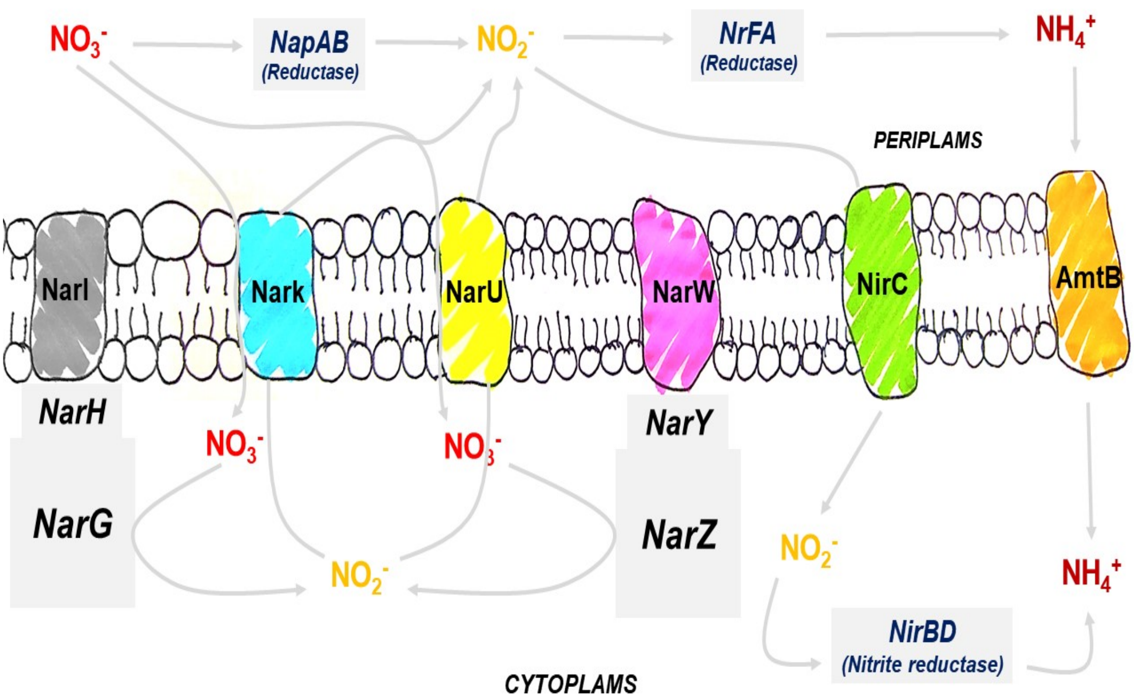

Figure 2. Schematic representation of Nitrate uptake and metabolization of it by bacterial cell. Adapted from Andrade & Einsle (2013).

As seen on the schematic figure 2, through a simplified view, nitrate reduction is catalysed by three different nitrate reductases which are assimilatory nitrate reductase (NASs), periplasmic nitrate reductase (NAPs), and membrane-bound respiratory reductase (NARs) (Andrade & Einsle, 2013; Moir & Wood, 2001). Evidently that these enzymes are distinguished themselves by some traits such as location, function, composition, and identity of their redux centre.

The NAP is a complex subunit present in the periplasm, and it is usually involved on energy dissipation or nitrate respiration. The NAR has its subunits located in the membrane and they participate in the anaerobic nitrate respiration. Nark and NarU are member of nitrate or nitrite porter, respectively, playing an important role in the facilitation of transportation, while NirC channel allows transportation of NO_2^-, and Amt/Rh family being the main transporter of NH_4^+ from out of the periplasm (Andrade & Einsle, 2013; Nolan et al., 2016).

When cattle are fed diet containing nitrate on it, and it gets in the ruminal environment, nitrate is conducted into the cytoplasm of the bacteria cell to be reduced to nitrite. This first process of nitrate reduction to nitrite occurs by means action of the subunit enzyme of the NarG complex which is attached to Narl in the inner surface of the cytoplasmatic membrane. On this complex there is biding site for oxidation of the electron donor. There, one of the NarG subunit catalyses electron transfer by means the redox cofactors embedded in the enzyme to the the molybdobis (molybdopterin) guanine dinucleotide (Mo-bisMGD), a cofactor located in the cytoplasmic NarG, site where nitrate is reduced to nitrite (Nolan et al., 2016).

After that, nitrite is then shipped into the periplasm by means antiporters Nark and NarU, preventing cytotoxicity. Nark and NarU are member of nitrate or nitrite porter, respectively, and they play an important role in the facilitation of transportation. Periplasmatic dissimilatory nitrate and nitrite reductase known as NapAB and NrfA are responsible to metabolise the excess of NO_3^- and NO_2 pumped from the cytoplasm. The ammonia generated by the metabolization of the nitrate compounds can be assimilated for bacterial polymer synthesis by means the junction action of cytoplasmatic nitrite reductase (NirND) with association to NirC, which transports NO_2 to the cytoplasm, and the AmtB, an ammonium transporter, respectively. All components that bacterial cell does not use are moved out of it (Nolan et al., 2016).

The ruminal environmental pH plays a role in nitrate reactions by bacteria. Nitrate-reducing bacteria, for instance, display a lower growth when ruminal pH is low. According to Iwamoto et al. (2002), it has to do with the fact that it happens due to limited supply of the environment by electrons as fermentation can be suppressed at low pH. Because electrons used to nitrite reduction activity is three times higher as compared to nitrate, an ideal pH coupled with steady fermentation activity is indeed needed to avoid suppression of the reducing activity and accumulation of intermediate toxic compounds in the rumen environment.

Despite of the shift caused in the hydrogen path utilization, nitrate can have direct toxic effect over the rumen suppressing the growth and activity of methanogens, decreasing then the rate of hydrogen utilization. It is consistently found on the literature reduction around 15 to 25% of CH_4 per g.kg.DMI. Wang et al. (2018) showed a reduction of 22% of methane (g.kg.DMI), which corresponded to 9.8% reduction per percentage unit of nitrate added into the diet.

4.1.Effect of nitrate on Short Chain Fatty Acids

Under normal circumstances in which cattle are under grazing having no additional supplementation to modulate the metabolic fermentation paths there will be a high amount of hydrogen not only for acetate production, which is a H_2 sinker, but also the available hydrogen from re-oxidation of reduced cofactors (NADH, NADPH and FADH) and the reduction of pyruvate to acetyl-CoA will be used to fomentation of methane synthesis. Nonetheless, with inclusion of nitrate in diet, this perspective switch as nitrate eventually sinks the energy that could be provided to the methanogen.

In fact, the incorporation of hydrogen to more valuable fermentative products are nutritionally advantageous, meaning reduced digestible energy losses from gas production (Lan & Yang, 2019). There is a consistency on literature in the regard of methane reduction when nitrate is added into diet as seen on the previous topic; however, fermentative end products such as acetate, propionate and butyrate yet seem not to be very well elucidated.

Some papers on literature associate nitrate inclusion on ruminants' diet with increased propionate production as some H_2 could be uptake into propiogenesis pathway competing H_2 with NO_3^- (Ungerfeld, 2020). In fact, that can be corroborated by some work on literature; however, reduction on propionate production have been described on literature. Despite of that, if no major change on short chain fatty acids production is detected, coupled with decreased methane production it means that nitrate brings positive results regarding its main purpose as seen on Figure 3, that draws a possible metabolic path that involves the methane reduction and a redirection of hydrogen for NO_3^- reduction to NH_3.

This schematic representation of metabolic path would fit well the occurrence of acetate and methane production that occurs on beef cattle fed high forage diet. It is known that when animals are under grazing higher acetate productions is detected and thus addition of nitrate may have a direct effect on mitigation of methane production via this metabolic path.

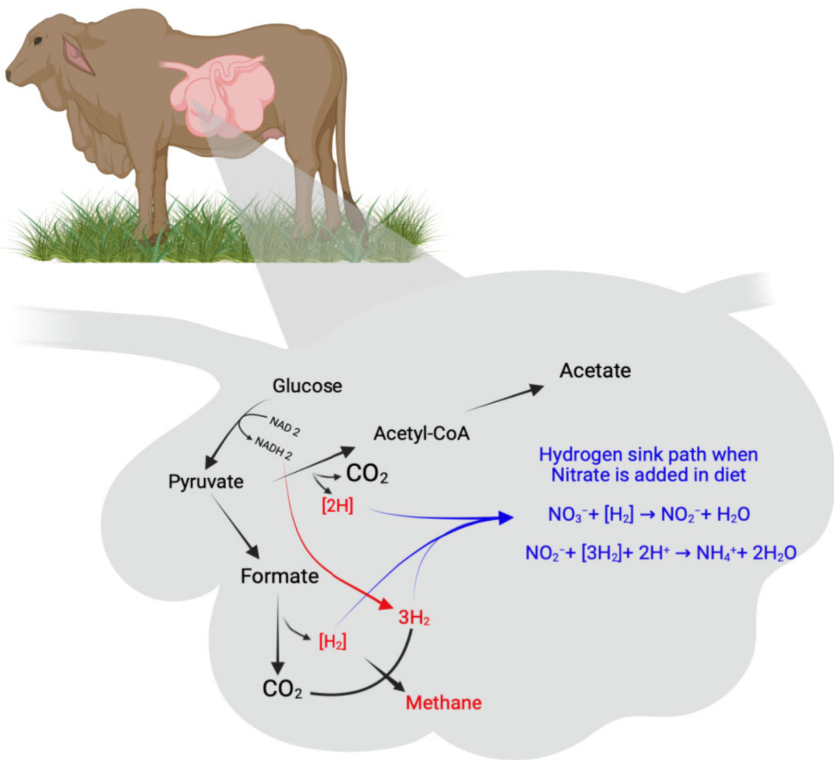

Figure 3. Simplified metabolic path for acetate production from pyruvate molecule yielding Acetyl-CoA and Formate. The excess of hydrogen generated by this path is eventually used in nitrate reduction up to ammonia highlighted, in blue, instead of being used in the synthesis of methane by means formate path highlighted in red. Schematic design created with Biorender. (Own authorship)

However, this simplified metabolic path is just a suggestion of what may occurs under the mentioned circumstances. The most recent works on literature brings divergences regarding fermented end products when nitrate is used.

Capelari (2018), working with beef cattle with average body weight of 335kg fed mixed ratio with inclusion of 15g of nitrate per kg of DM, found increased SCFA production by 6% and reduction in propionate production by 8% as compared to control treatment. In consonance with that, Duthie et al. (2018) obtained 12% reduction in propionate concentrations with beef cattle fed diet 55:45 (F:C) with inclusion of 21.5g of nitrate/kg of DM. As mentioned before, propionate molar concentration increase can be justified as hydrogen from phosphorylation process can eventually be uptake to the propionate pathway since its production is lower than its uptake and the H_2 also serves as an important source to the generation of propionate, which can directly compete with NO_3^- reduction to NH_3.

Differently from the previous authors, Henry (2017) working with beef cattle under grazing did no find effect on propionate production; however, achieved 11.9% reduction on butyrate as compared to control diet group. Tomkins et al. (2018), working with *Bos indicus* steers fed high forage diet with inclusion of nitrate (4.6 and 7.9 g/kg of DM), also did not find any significative effect regarding propionate production but encountered a reduction in the acetate of 6.4% and butyrate of 13.2%. According to Natel et al. (2019), butyrate synthesis will outcompete electrons with NO_3^- as it has high affinity to sink the available H_2, which can eventually reduce butyrate production. On the regard of reduction on acetate and no effect on propionate production is simply explained by the fact that nitrate is efficiently acting as H_2 sinker, redirecting them to NO_3 reduction and subsiding NH_3 production. In that case, nitrate use has a positive effect on the generation of valuable fermented products.

Troy et al. (2015) had beef cattle fed 50:50 mixed ration with inclusion of 21.5g of nitrate/kg of DM and detected a decrease in propionate production as compared to control treatment of 19.2%, while butyrate production was 12.1% higher. Higher butyrate reduction of 23.5% was also detected by Villar et al. (2020) with beef cattle steers fed 3.4g of nitrate/kg of DM. They also detected reduction for propionate while acetate was higher than that of control group by 7.5%. Eventually, higher butyrate production has to do with the fact that butyrate producing bacteria are capable to metabolize several carbohydrate sources as the sole source of energy, converting them (for instance: polymers start; xylan; glucose, arabinose, xylose and cellobiose) into butyric acid (Miguel et al., 2019).

4.2.Effect of nitrate on methane production

Despite of many *in vivo* and *in vitro* reports on literature certifying the effectiveness of nitrate as H_2 sinker, a few papers do not corroborate with that. The extent of decrease in methane production will depend on the level of nitrate added in the diet and how it is administered in the diet. However, is a consensus that when nitrate is added into the diet a systemically inhibition of methanogens microorganism activity coupled with the pick-up of H_2 will happen and thus methane production will decrease. According to stoichiometric calculus of Van Zijderveld et al. (2010), 100 g of dietary nitrate reduced to ammonia in the rumen should lower CH_4 emissions by 25.8 g.

On table 1 it is possible to visualize the positive effect of nitrate over the decrease of enteric methane emission. It is also important to point out that all references used to build up that compiled regard from different Laboratory research groups which does not use the same technique to evaluate enteric methane emissions. In fact, the compiled information on table 1 has as its main purpose make visual the nitrate effect on CH_4 reduction.

Hulshof et al. (2012), evaluating the effect of 22 g of nitrate/kg of DM in diet of Nellore x Guzera (*Bos indicus*) beef cattle fed freshly chopped sugarcane and concentrate (60:40 on DM basis) as a mixed ration on animals for 46 days, achieved 20% of methane reduction, which was detected by means the sulfur-hexafluoride technique (Table 3).

Table 3. Description of experiments conduction in which nitrate encapsulated or not was used in the supplementation as a H_2 sinker. In each case inclusion of nitrate, days or experimental run, methane reduction and sources are described.

Nitrate (g.kg.DMI)[**]	Days	CH_4 red.[*] (%)	Method	Reference
22	46	27	SF_6[1]	(Hulshof et al., 2012)
6 to 30	111	12 to 29	SF_6	(Newbold et al., 2014)
10 to 30	112	4.2 to 18	R.Chamber[2]	(Lee et al., 2014)
21	91	8	R.Chamber	(Duthie et al., 2018)
4.6 to 7.9	112	15	R.Chamber	(Tomkins et al., 2018)
21.5	84	22.62	R.Chamber	(Troy et al., 2015)
15	84	8.6	R.Chamber	(Capelari, 2018)
25	112	17	R.Chamber	(Alemu et al., 2019)
Average data			-	-
12.5	**94**	**16.1**	-	-

[1]SF_6: Sulphur-hexafluoride; [2] - R. Chamber: Respiratory chamber. Days- represents the amount of day animals were submitted to the treatment; [*] methane reduction; [**]grams per kg of dry matter intake

Lee et al. (2014), assessing encapsulated nitrate on ruminal-cannulated beef heifers (451 kg BW), obtained maximum CH_4 reduction of 18%, and a linear effect as in function of nitrate inclusions (1, 2 and 3% DM basis) into the diet. With inclusion of 21.5g of nitrate/kg of DM in basal diet with 550 forage (grass and whole crop barley silages) 450 concentrate to cross-bred steers for 84 days, Duthie et al. (2018) found methane reduction of 8% as opposed to control treatment. Tomkins et al. (2018) encountered mitigation on methane release by means the respiratory chamber method of about 15% when canulated *Bos indicus* steers under grazing were supplemented with 4.6 or 7.9 g of NO_3/kg DM.

The same was observed by Duthie et al. (2018) but using a different technique to assess methane emission. Troy et al. (2015) also worked with nitrate inclusion of 21.5g of nitrate/kg of DM in the diet of cross-bred steers (*Bos taurus*) fed forage: concentrate (50:50) and they achieved a much higher methane emission reduction, of 22.6% as compared to control group.

Capelari (2018), working with angus crossed steers with average body weight of 335kg fed mixed ratio (50% high moisture corn; 30% of silage; 15% of corn dry distiller’s grains) with inclusion of 15g of nitrate/kg of DM for 64 days, achieved reduction in methane of 8.6%. Alemu et al. (2019) encountered 17% of methane reduction when cross-bred steer fed high forage diet had inclusion of 25 g of nitrate/kg of DM.

As seen on table 3, methane generation from beef cattle fed nitrate diet have positive effect on methane emission reduction. Certainly, the extent of reduction results from the NO_3 depends on dosage, fed, and animal intrinsic factors as already said. however, the extent of methane reduction can go up to 29% on beef cattle fed nitrate in the diet. Despite of differences in the methods to attain the data all of them have precise techniques.

Some findings on literature point out that the replacement of urea with nitrate has not benefit in terms of productivity of cattle (Troy et al. 2015). According to Olijhoek et al. (2016), the increment of ammonia generated using nitrate reduction in the ruminal environment may not be necessarily beneficial (in terms of performance) Nevertheless, when assessed data from methane production, Wang et al. (2018) showed that a linear decrease of methane (from 6 to 23%) is detected when nitrate is increasingly added into the diet from the level of 5.3 to 21.%.

5. Results and discussion

The partial results here presented are from ongoing research, which are part of the FAPESP Thematic Project (2017/20084-5), "Strategic practices for mitigating greenhouse gas emissions in grassland systems of the Brazilian Southeast", having as partners the Faculty of Veterinary Medicine and Animal Science (FMVZ/USP – Pirassununga/SP), the Institute of Animal Science (IZ - Nova Odessa/SP) and the Embrapa Pecuária Sudeste (São Carlos/SP).

Effect of intensified grazing systems using urea and nitrate as supplementation on feed intake of Nellore beef cattle

Forage dry matter intake (DMI_F) had a clear interaction effect of grazing and season. As seen on Figure 4, higher dry matter intake was observed in summer within grazing methods while the lowest mean values were detected during winter.

When contrasting grazing methods within season it is possible to notice that during spring animals under deferred grazing method had higher DMI_F ($kg.day^{-1}$) as compared to animals under rotated method. The trend on DMI_F ($kg.day^{-1}$), when analyzing data within grazing method, was already expected as forage availability and digestibility is higher during rainy season as opposed to other seasons.

Table 4. Average dry matter intake of Nellore cattle submitted to grazing methods and nitrogen sources during different seasons by two years.

Fixed effects			Variables			
Grazing	N source[a]	Season	DMI_F ($kg.d^{-1}$)	DMI_S ($kg.d^{-1}$)	DMI_T ($kg.d^{-1}$)	NPN_{Eq} ($kg.d^{-1}$)
Deferred			5.41	0.44	5.91	0.18
Rotated			5.20	0.67	5.87	0.25
	Nitrate		5.29	0.47	6.03	0.17
	Urea		5.32	0.65	4.60	0.26
		Winter	4.08	0.52^{ba}	4.60	0.19
		Spring	4.39	0.65^{a}	5.05	0.24
		Summer	6.77	0.40^{b}	7.18	0.15
		Autumn	5.97	0.67^{a}	6.74	0.28
SEM			0.267	0.053	0.276	0.022
Statistics Probabilities						
Grazing			NS	NS	NS	NS
N source			NS	NS	NS	NS
Season			<.0001	0.013	<.0001	NS
Grazing * N source			NS	NS	NS	NS
Grazing * Season			0.020	NS	0.038	NS
N source * Season			NS	NS	NS	NS
Grazing * N Source * Season			NS	NS	NS	NS

a: nitrogen source; DMI_F: forage dry mater intake; DMI_S: supplement dry mater intake DMI_T: Total dry mater intake; NPN_{eq}: non-protein nitrogen. NS: non-significant at $P<0.05$.

Despite of that, as seen in the figure 4, DMI_F displayed a decrease of 25.76% in autumn from animals under deferred grazing method, which might be evidence of reduction of quality or availability of forage during autumn since the content of stems and dead material increases while leaf reduces. As it is a season of transition, low precipitation associated to less sunlight might lead the pasture to a lowered forage production, which can cause lowered feed intake since the herd starts to be more selective as stems and dead material are less palatable than green leaves.

The reduction (25.76%) in forage dry matter intake on autumn from deferred grazing (Figure 4) and the higher supplement dry matter intake observed on autumn, as seen on table 4, with values 40.08% higher than summer, evidence the previous statement about the reduction of forage quality.

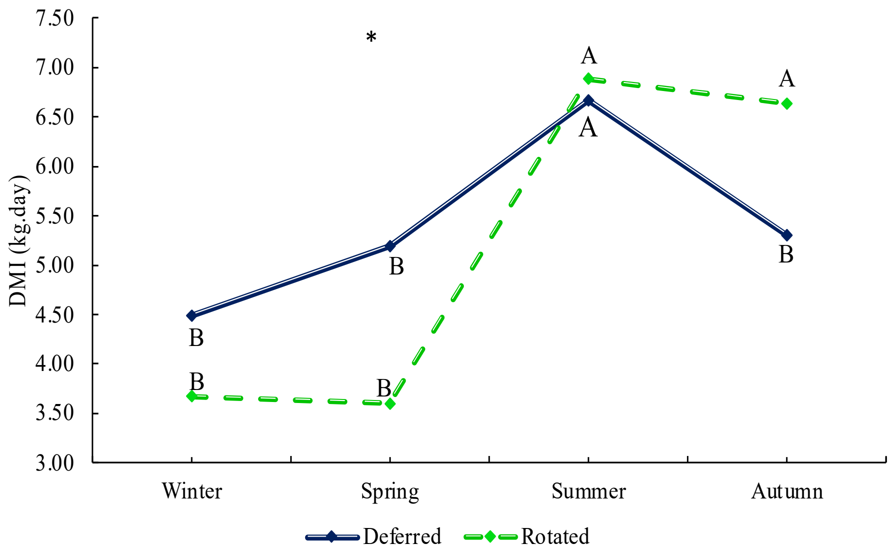

Figure 4. Interaction effect of grazing systems and season on forage dry matter intake of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. Capital letters within grazing methods differs at P<0.05; * indicates statistical difference within season at P<0.05

As seen on table 4, supplement intake had season effect with lower intake during summer as opposed to spring, for instance, season that is still transitioning from dry to rainy. It was also detected interaction effect of grazing and season for total dry matter intake ($kg.day^{-1}$) as seen on the figure 5. Within grazing rotated method animals had higher DMI_T on summer and autumn as compared to spring and winter. The higher total dry matter intake in summer and autumn reflects the higher availability and quality of forage on rainy season.

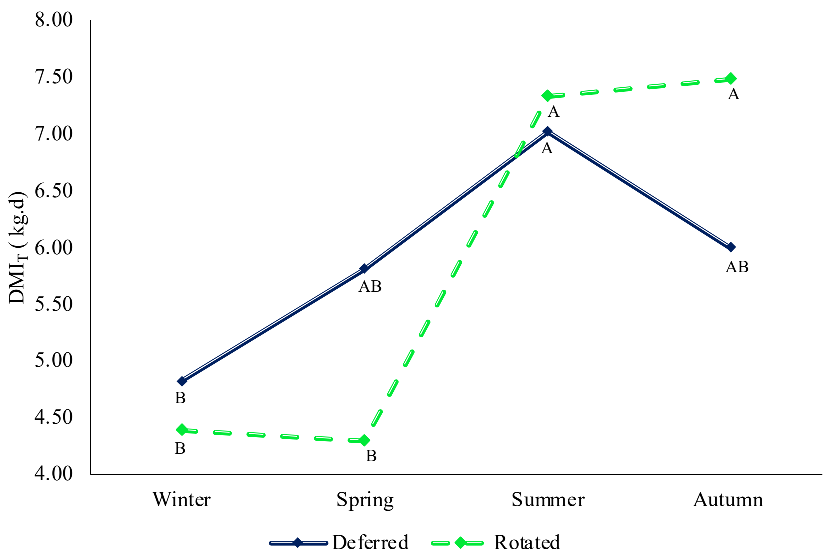

Figure 5. Interaction effect of grazing systems and season on total dry matter intake (kg.day). of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. Capital letters within grazing methods differs at P<0.05; * indicates statistical difference within season at P<0.05

Lower DMI_T intake values were detected in rotated and deferred grazing methods during winter. NPN protein equivalent intake had no significant effect.

Effect of intensified grazing systems using urea and nitrate as supplementation on fermentative parameters and GHG (CO_2eq) emissions of Nellore beef cattle

On Table 5 it is displayed the obtained data for short chain fatty acids (SCFA) production from ruminal content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

For SCFA production in g.kg.dia^{-1} it was not observed grazing or nitrogen source effect. Assumpção (2021), working with Nellore beef cattle fed concentrate:roughage (corn silage) at ratio of 60:40 with inclusion of calcium nitrate, also did not detect significant effect of inclusion of nitrate on short chain fatty acids production.

Effect of season was detected for acetic, propionic, butyric, and total acids production. For the variables propionic, butyric, and total acid production (g kg^{-1}day^{-1}), higher values were detected in summer as compared to spring and winter.

Table 5. Average short chain fatty acids production by of Nellore cattle submitted to grazing methods and nitrogen sources during different seasons by two years.

Fixed Effects			Variables			
Grazing	N source	Season	Acetic	Propionic	Butyric	Total
			(g kg^{-1}day^{-1})			
Deferred			239.45	73.65	49.36	372.65
Rotated			230.35	78.52	44.35	326.09
	Nitrate		229.33	70.87	42.74	341.51
	Urea		240.47	81.29	50.97	357.23
		Winter	182.91^{c}	51.78^{c}	35.23^{b}	258.73^{c}
		Spring	211.96^{b}	74.08^{b}	56.78^{a}	337.86^{b}
		Summer	271.26^{a}	91.19^{a}	58.75^{a}	410.78^{a}
		Autumn	273.46^{a}	87.27^{ab}	36.65^{b}	390.11^{ab}
SEM			9.74	3.62	2.88	14.17
Statistics Probabilities						
Grazing			NS	NS	NS	NS
N source			NS	NS	NS	NS
Season			0.0006	<.0001	0.0002	<.0001
Grazing * N source			NS	NS	NS	NS
Grazing * Season			NS	NS	NS	NS
N source * Season			NS	NS	NS	NS
Grazing * N Source * Season			NS	NS	NS	NS

N source: nitrogen source; NS: non-significant at P<0.05.

The observed trend on SCFA production shows that nitrate does not influence the reduction or increase of any fermented product from Nellore beef cattle under intensified grazing system, despite of numerical reduction.

Winter was the season which based on total amount of fermentation products had the lower fermentation activity, differently from the other season where we see an increase in total SCFA production.

In terms of methane yield it was possible to identify significant effect of season, nitrogen source and interaction of grazing method and season as seen on the table 6.

Table 6. Average methane emission, relative energy loss, and CO_2eq emissions by of Nellore cattle submitted to grazing methods and nitrogen sources during different seasons by two years.

Fixed Effects			Variables			
Grazing	N source[a]	Season	CH_4 g (kg d^{-1})	Rel (%)	CO_{2eq} (kg.$head^{-1}d^{-1}$)	CO_{2eq} (kg.DMI_T.d^{-1})
Deferred			22.10	18.81	9.87	1.85
Rotated			20.80	20.28	9.32	1.87
	Nitrate		20.44	18.82	9.12	1.80
	Urea		22.46	20.27	10.07	1.92
		Winter	15.23^b	17.78	7.13^a	1.69^b
		Spring	23.12^a	19.51	10.59^b	2.55^a
		Summer	23.79^a	20.43	10.39^b	1.57^b
		Autumn	23.64^a	20.45	10.28^b	1.65^b
SEM			0.65	0.39	0.28	0.11
Statistics Probabilities						
Grazing			NS	NS	NS	NS
N source			0.0344	0.0451	0.0263	NS
Season			<.0001	0.0120	<.0001	0.0077
Grazing * N source			NS	NS	NS	NS
Grazing * Season			NS	0.0068	NS	NS
N source * Season			NS	NS	NS	NS
Grazing * N Source * Season			NS	0.0089	NS	NS

a: nitrogen source; Rel (%): relative energy loss; CO_{2eq}: carbon dioxide equivalent. NS: non-significant at P<0.05.

As seen on table 6, methane emission (g.kg.day^{-1}) was affected by the nitrogen source. Ammonium nitrate was responsible to a reduction of 9%. Similar results were found by Capelari (2018) who fed steers with 1.5% of calcium ammonium nitrate on DR basis and attained a significant reduction of 9.49% of CH_4 (g/kg DMI). As previously mentioned, the reduction of nitrate in the rumen is considered thermodynamically more favourable as compared to CO_2 reduction to methane (Yang et al., 2016), and that makes nitrate an electron-consumer competitor of the methanogenesis leading to a lower methane production.

Besides that, it was also detected significant effect for season with methane yield being 36% lower during winter as opposed to summer, when it was identified the highest methane yield. It is noteworthy to point out as well that the Relative Energy Loss (%) represents the amount of energy dispended under the fermentation process that is not converted into an energetic source (such as acetic, butyric and propionic acid) to animals' metabolism.

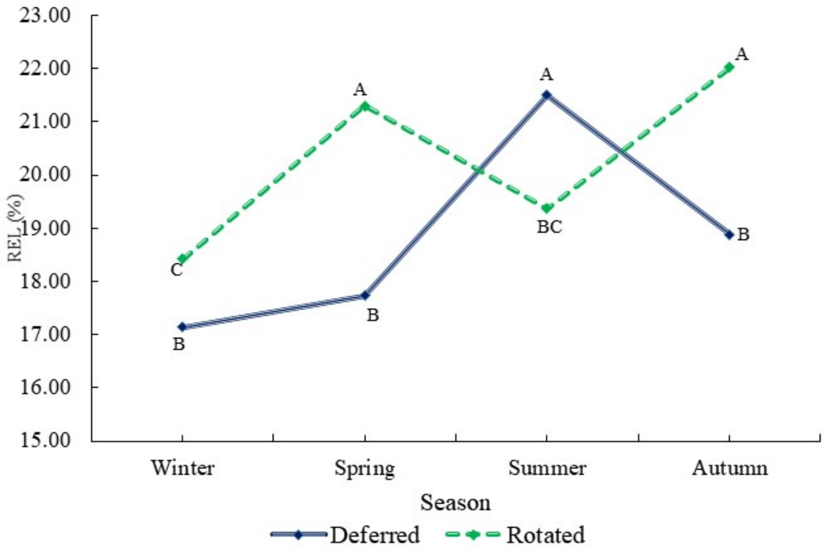

Figure 6. Interaction effect of season and grazing method on relative energy loss (REL) from ruminal content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05

The interaction for relative energy loss was decomposed (Figure 6) and it is possible to noticed that within grazing method animals under deferred grazing showed lower relative energy loss during winter, spring, and autumn, while within rotated method the lowest relative energy loss was observed from ruminal content of animal in winter and higher in spring and autumn. When assessed the differences within season comparing the grazing methods, no effect was detected.

For the metric CO_2eq emissions per kg. head^{-1}d^{-1} it was noticed effect of nitrogen source and season. The adoption of nitrate into beef cattle diets was responsible to reduce CO_2eq emissions by 10.41%, while the effect by season demonstrated that spring, summer, and autumn had on average 46.11% more CO_2eq emissions than that observed in winter, which can be simply justified by the intensity of fermentation that occurs on rumen in different seasons. No statistical effect was detected for emissions of CO_2eq (kg. DMI_T. d^{-1}) for winter, summer, and autumn. However, higher methane emission was detected on spring. Overall, ammonium nitrate was effective on the reduction of methane and CO_2eq emissions kg. head^{-1}d^{-1}.

6. Conclusion

Based on partial results, we can understand that inclusion of ammonium nitrate as a non-protein nitrogen source for Nellore beef cattle diet under intensified grazing systems did not cause major effect on forage, supplement, and total dry mater intake. Nitrate was effective on reducing enteric methane and CO_2eq emissions kg. head^{-1}day^{-1}. Ammonium nitrate can be used as a strategic tool to provide a source of non-protein nitrogen that concomitantly changes the rumen fermentation, specifically the methanogenesis, and thus mitigate anthropic GHG emissions from enteric fermentation.

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