WILLIAN RUFINO ANDRADE

Metabolism and rumen microbiome of Nellore beef cattle submitted to intensified grazing systems during different seasons

Pirassununga

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ETIC COMMIT CERTIFICATION

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

São Paulo, 12th May 2022

CERTIFIED

We certify that the Research "Metabolism and rumen microbiome of Nellore beef cattle submitted to intensified grassland systems during different seasons", protocol number CEUAx 2347040422 (ID 002144), under the responsibility Paulo Henrique Mazza Rodrigues, agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of School of Veterinary Medicine and Animal Science (University of São Paulo), and was approved in the meeting of day April 14, 2022.

Certificamos que o protocolo do Projeto de Pesquisa intitulado "Metabolismo e microbioma ruminal de bovinos de corte Nelore submetidos a sistemas de pastejo intensificados durante diferentes estações", protocolado sob o CEUAx nº 2347040422, sob a responsabilidade de Paulo Henrique Mazza Rodrigues, está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, e foi aprovado na reunião de 14 de abril de 2022.

Prof. Dr. Marcelo Bahia Labruna
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Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Committee member

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Prof. Dr. _____

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Prof. Dr. _____

Institution:_____Result:_____

DEDICATION

This work is dedicated to everyone which, genuinely, were in my side contributing with my daily routine, my well-being, my work, and my existence. I owe everyone as much appreciation as possible! When we walk with good people around us, it gets easier to go far and achieve good results!

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EPIGRAPH

“Nature is cruel, but we don’t have to be.”

Temple Grandin

RESUMO

ANDRADE, W. R. **Metabolismo e microbioma ruminal de bovinos nelore submetidos a sistema de pastejo intensivo durante diferentes estações.** 2023. 208 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2023.

Com intuito de avaliar alterativas que possibilitem a mitigação do impacto ambiental pela bovinocultura de corte, o presente trabalho propôs avaliar se a intensificação do sistema de pastejo juntamente com a utilização da suplementação com nitrato de amônio para bovinos de corte pode alterar o metabolismo ruminal e mitigar o metano gerado por animais mantidos em sistemas de pastejo. Teve-se como objetivos específicos aferir o efeito dos métodos de pastejo e suplementos quanto ao consumo de matéria seca, digestibilidade, degradabilidade, pH ruminal, concentração e produção de metabólitos da fermentação, síntese e eficiência de síntese de proteína microbiana, e o microbiota ruminal. Oito vacas Nelore canuladas foram distribuídos aleatoriamente em oito piquetes em um delineamento de blocos casualizados em arranjo fatorial formado pela fonte de nitrogênio (nitrato de amônio ou ureia), método de pastejo (rotacionado ou diferido) e estações do ano. Os tratamentos foram compostos pela combinação de dois métodos de pastejo, diferido ou rotacionado, com a suplementação de nitrato de amônio ou ureia. As variáveis de consumo de matéria seca, digestibilidade, parâmetros de degradabilidade da matéria seca, proteína e fibra em detergente neutro, esvaziamento ruminal, produção e concentrações de ácidos graxos de cadeia curta, metano, nitrogênio amoniacal, parâmetros urinários, contagem de protozoários foram avaliados usando o procedimento misto (PROC MIXED) do SAS 9.4. No entanto, para a obtenção dos parâmetros de degradabilidade foram adotado o uso do procedimento linear (PROC NLIN). Os dados referentes ao microbiota ruminal foram analisados usando o software Mothur, R e SAS 9.4. Os resultados mostraram que os animais mantidos em pastejo rotacionado apresentaram maior consumo de nitrogênio não proteico, porém não foi observado efeito de fonte de nitrogênio para o consumo de suplemento. Contudo, quando ureia e nitrato foram utilizados como as principais fontes de nitrogênio, ambos contribuíram igualmente para a síntese de proteína microbiana e sua eficiência. Os resultados mostraram que a suplementação de nitrato reduziu em 23,13% a liberação de energia no rúmen na forma de ácido acético durante o verão. As concentrações de ácido butírico foram maiores em animais sob sistemas de pastejo rotacionado nas estações de primavera e outono. O estudo também observou uma redução de 13,1% na produção de metano e uma diminuição subsequente de 15,7% na liberação de energia no rúmen de animais

suplementados com nitrato em comparação com a ureia. Os sistemas de pastejo rotacionado contribuíram para uma redução de 21,3% na produção de metano durante a estação de verão. Microrganismos da família *Methanobacteriaceae* apresentaram maior abundância relativa no rúmen de animais mantidos em pastejo diferido, e a suplementação de nitrato diminuiu significativamente sua abundância. A suplementação de nitrato influenciou em maior abundância de relativa da *Veillonellaceae-UCG*. A adoção do pastejo rotacionado em conjunto com a suplementação de nitrato de amônio foram uma excelente alternativa para a intensificação de sistema de produção de bovinos de corte a pasto, possibilitando melhorias quanto a digestibilidade da dieta, maior consumo de NNP, possibilitar maior aporte de energia ruminal e mitigar a emissão de metano entérico.

Palavras-chave: Bovinos de corte, forragem, metano, suplementação de nitrato.

ABSTRACT

ANDRADE, W. R. **Metabolism, and rumen microbiome of Nellore beef cattle submitted to intensified grazing systems during different season.** 2023. 208 f. Thesis (Ph.D in Science) – College of Veterinary Medicine and Science, University of São Paulo, Pirassununga, 2023.

In order to assess alternatives that can mitigate the environmental impact of beef cattle production, this study aimed to evaluate whether intensifying the grazing system in conjunction with ammonium nitrate supplementation could alter ruminal metabolism and reduce methane emissions from animals kept in grazing systems. The specific objectives were to measure the effects of grazing methods and supplements on dry matter intake, digestibility, degradability parameters, ruminal pH, concentration and production of fermentation metabolites, microbial protein synthesis, and ruminal microbiota. Eight cannulated Nellore cows were randomly allocated to eight paddocks in a randomized block design with a factorial arrangement formed by the nitrogen source (ammonium nitrate or urea), grazing method (rotational or deferred), and seasons. The treatments consisted of the combination of two grazing methods, deferred or rotational, with supplementation of either ammonium nitrate or urea. Variables such as dry matter intake, digestibility, dry matter, protein, and neutral detergent fiber degradability parameters, ruminal emptying, production and concentrations of short-chain fatty acids, methane, ammonia nitrogen, urinary parameters, and protozoa count were evaluated using the PROC MIXED procedure of SAS 9.4. However, the degradability parameters were first obtained using the linear procedure (PROC NLIN). Data related to ruminal microbiota were analyzed using the Mothur software, R, and SAS 9.4. The results showed that animals kept in rotational grazing had higher non-protein nitrogen intake, but no effect of nitrogen source was observed for supplement intake. However, when urea and nitrate were used as the main nitrogen sources, both equally contributed to microbial protein synthesis and its efficiency. Nitrate supplementation reduced ruminal energy release in the form of acetic acid by 23.13% during the Summer. Butyric acid concentrations were higher in animals under rotational grazing systems in the Spring and Autumn. The study also observed a 13.1% reduction in methane production and a subsequent 15.7% decrease in ruminal energy release in animals supplemented with nitrate compared to urea. Rotational grazing systems contributed to a 21.3% reduction in methane production during the Summer season. Microorganisms from the *Methanobacteriaceae* family showed higher relative abundance in the rumen of animals kept in deferred grazing, and nitrate supplementation significantly decreased their abundance. Nitrate supplementation had an impact on specific groups of bacteria and archaea, with bacteria from

the *Veillonellaceae* family showing an increase in relative abundance, while inhibiting archaea belonging to the *Methanobacteriaceae* family. Adoption of rotational grazing and nitrate supplementation proves to be a great alternative for the intensification of pasture-based beef cattle production, allowing slight improvements in diet digestibility, increased non-protein nitrogen intake, providing greater ruminal energy supply, and mitigating enteric methane emissions.

Key words: Beef cattle, forage, methane, nitrate supplementation

SUMMARY OF TABLES

SUMMARY OF FIGURES

SUMMARY OF CONTENTS

Introduction

Ranked as the second largest beef herd in the world with about 196 million heads, the Brazilian beef cattle has a prominent position when it comes to the global beef cattle industry (ABIEC, 2022). Despite of that, these ginormous cattle herd has a downside which has been extensively and deep discussed around the globe, which is its direct effect on greenhouse gases emission.

Ruminants contribute directly to increasing concentration of atmospheric CH_4 through two main routes: enteric fermentation and fermentation of organic waste (USEPA, 2021). To reduce greenhouse gases emissions, nutritional strategies can be developed to manipulate rumen fermentation. It must, however, be considered that CH_4 production is directly proportional to the concentration of dissolved H_2, which is influenced by factors in the rumen's ecosystem, such as the rates of acetate and propionate production. Research on the methanogenesis process intends to determine a method to move the dissolved H_2 to alternative routes in the rumen fermentation process in order to reduce the availability of substrate for methanogenesis (Hristov et al, 2022).

Recently, researchers on the mitigation of CH_4 produced by ruminants have received much attention (Palangani et al., 2022). Nutritional techniques, such as the use of ionophores (Beauchemin et al., 2022), tannins (Berça et al., 2023), saponins (Torres et al., 2023), essential oils (Benetel et al., 2022) and lipids (Castañeda-Rodríguez et al., 2023) have all been used to manipulate ruminal fermentation and to reduce enteric methane production, in addition to other techniques like pasture management strategies, genetic improvement, and more efficient systems of production (Arndt et al., 2022; Vargas et al., 2022; Magnani et al., 2023).

The majority of Brazilian beef cattle production occurs in extensive grazing system since Brazil's climate congregate ideal conditions for forage growth which might occurs in two well-defined periods of the year, during rainy and dry seasons. As forage prevails as the basis feed for beef cattle, its availability and quality play an important role in grazing systems production.

Despite of being economically attractive to grow beef cattle in grazing systems, it has some disadvantages regarding pastures vulnerability to climatic seasonality, which may lead to forages with low nutritional value and in some cases poor availability (Rufino et al., 2020). Alternatives have been studied for growing beef cattle in grazing systems as a tool to improve performance and mitigate negative effects of seasonality, such as deferred (Souza et al., 2022)

and rotation grazing systems (Kuinchtner et al., 2018). Management strategies have been suggested to mitigate negative effects of seasonality in grazing beef cattle production such as deferred pasture. Deferred is an option that plays an important role in the conservation of forage and its availability in critical periods, when temperature, rain and insolation are not enough to allow pasture growth. Usually, grazing on deferred pasture is ceased in the end of raining season and then pasture grows up until the beginning of dry period, moment when pasture with reasonable quality is most needed to avoid lower performance (Souza et al., 2022).

Other possibility is the adoption of rotational stocking, which is known as the grazing method that has recurring grazing and resting periods between the paddocks in a grazing management unit. This technique allows beef cattle production to be conducted with a higher stocking rate, ensuring ideal nutritional conditions, with more digestible fed available, and regrowth of pasture in resting (Boyer et al., 2022).

If well managed, both systems will provide reasonable pasture availability and that may play an important role with ruminal metabolites production, especially short chain fatty acids and methane. Nevertheless, to attain higher performance on both grazing systems, the intensification of beef production is one possible way for improving animal productivity indexes and reduce even more negative effects of enteric methane generation.

Intensification of grazing beef cattle production may be achieved by adopting supplementation. Significant improvement can be achieved when grazing beef cattle are supplemented allowing at least weight maintenance or even moderate gains. Nitrate into beef feeding, for instance, is known for being efficiently effective as a source of non-protein nitrogen and for reduction in methane emission (Palangani et al., 2022). Nitrate supplementation has been considered thermodynamically favorable 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 (Ungerfeld, 2020). According to Yang et al. (2016) nitrate is ultimately converted into ammonia and thus ruminal microbial protein synthesis can be favored since nitrate, just like urea supplementation, is used as a source of non-protein nitrogen.

In terms of inhibition of methanogenesis, when nitrate reaches ruminal environment, it is rapidly reduced from nitrate to ammonia and that significantly contributes to the reduction of enteric methane since by this pathway there is a consume of hydrogen generating ammonia (NH_3), an alternatively sink for enteric methane production (Almeida et al., 2022). This metabolic process is very well described on the literature, nevertheless, much of the effort and

work on this research field comes from the effect of feeding nitrate to animals raised in feedlot conditions with more energetic diets.

It is known that proportionally to all fermented products ruminant generates greater amount of enteric methane when fed forage as compared to concentrate diets. Therefore, it is important to assess whether utilization ammonium nitrate (NH_4NO_3) supplementation for beef cattle can mitigate the methane daily generated from animals raised in grazing systems. Positive results in terms of improving ruminant performance, favorable change in ruminal fermentation and lower enteric methane production might give indicative of a profitable system production for tropical areas that would not only be economically appealing but also environmentally.

There is evidence that inclusion of nitrate in ruminant diet selects nitrate reducing bacteria and shift microbial community (Alemu et al.; 2018; Granja-Salcedo et al., 2019; Natel et al., 2022). According to the same authors possible toxics effect of nitrate can inhibit cellulolytic bacteria and methanogens as well nevertheless, the effect on overall ruminal environment of grazing animals under intensified rotational and deferred pasture regarding concentration of short chain fatty acids profile, enteric methane production and metagenomics of the ruminal content during rainy and dry season is not well described.

CHAPTER 1 - Literature Review

Beef cattle production in the Tropics

Considering Earth's elliptic pathway and as well as local latitude, solar radiation over Brazil along the year is abundant. Since most of the country territory is in between the Tropics, Brazil is privileged in terms of solar radiation and pluviometry, and those favor high photosynthetic activity. According to Assunção & Schutze (2017) annual solar radiation from 2005 to 2015 was in average 5.03 $kWh.m^2$.day and annual precipitation ranging from 387 to 4003 mm. The climatic condition and its location make the Brazilian territory great to vegetal biomass production as well as for animal production.

Despite of increased attention to confined intensive beef production, Brazilian beef cattle production relay mostly on animals raised on grazing systems without supplementation (Malafaia & Filho, 2019) and, thus, forage production and its management play a pivotal role on that scenario. However, considering the existence of rainy and dry season on the tropics, the seasonality is an important factor that can greatly affect the forage mass production and its quality causing direct effect on animal production.

Seasonality plays a major role in the Brazilian beef cattle production as the biggest part (about 85%) of the commercial beef herd is raised on pastures (Malafaia & Filho, 2019) and the biggest slice (80%) of forage production occurs during the rainy season. Due to that fact, forage has great fluctuations on its mass production and its nutritional composition along the year, which makes difficult the adequation of animal's nutritional requirements specially in dry season when pastures decrease its digestibility.

As forage get mature, 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). 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. Based on that different grazing systems may be used in different locations to attend its own demands.

GHG emissions from agricultural and livestock production

Nowadays, anthropogenic activities have been considered by many one of the main causes of high pollutants emissions to the atmosphere and global warming. From the total global GHG emissions, considering not only anthropogenic activities but also emissions from natural systems, the anthropogenic emissions are estimated to account for 47.9 to 66.6% of the total GHG (Yue & Gao, 2018).

The livestock sector as a hole is gigantic and according to Herrero et al. (2016) more than 20 billion animals (with no distinction of class) are allocated in production systems, which covers about 30% of the terrestrial land. According to Van Dijk et al. (2021), based on projection of population growth, the demand for food is going to increase 35 to 56% between 2010 to 2050 and, at some point, human population would outrun the growth of food supplies.

It is evident that beef cattle production such as any other sector from livestock has a very dynamic path along the years with increasing demands. Evidently that this increase of demands in terms of livestock products comes from increasing human population alongside to the economic power, especially from developing countries, where lower classes are upgrading to upper middle class with consequent income increase. The best data to justify that is the constant increase of global per capita consumption of livestock products, which is projected to increase over the next 20 years (Herrero et al., 2016).

In developing economies from Africa, Asia and Latin America the increased production not always lays on intensification in terms of higher amount of product per unit area. In some countries this increase is direct related to extensive and poor land use, which certainly goes against polices of sustainable development as it can have negative effect on the environment with increase on greenhouse gas emissions to the atmosphere.

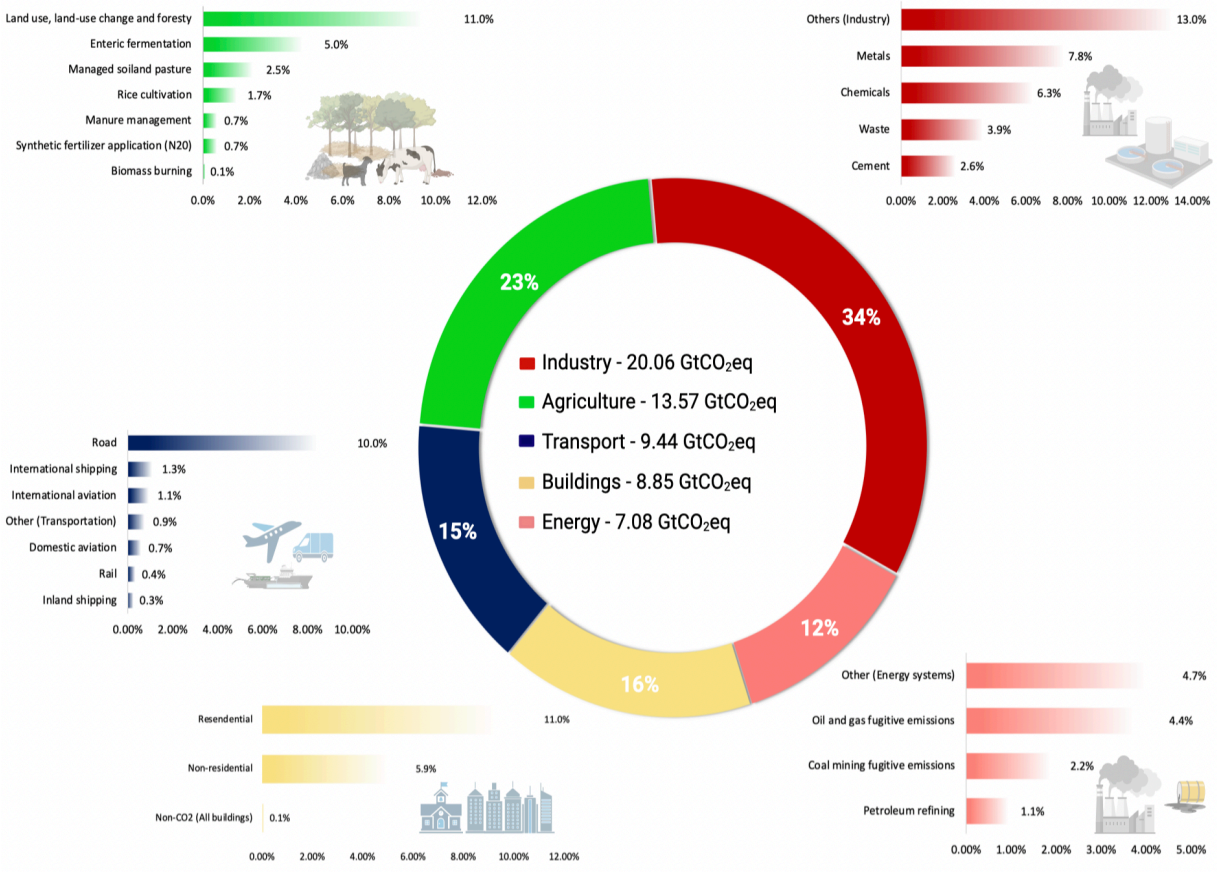
The greenhouse gases are harmful compounds to the environment when released in higher rate than that of natural system sink. The main gases involved on this novel are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and ozone (O_3), and they greatly contribute to the global warming (Patterson, 2020).

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, from the total global 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 each subsector we can see that enteric fermentation contributes only with 5.0% of the total GHG emissions, which roughly means that in terms of total anthropogenic GHG emissions, enteric fermentation released in 2019 to the atmosphere around 2,945 Gt of CO_2eq.

Figure 1. Total anthropogenic greenhouse gases emissions in 2019 expressed as GtCO2eq. Data retrieved from the sixth IPCC report of 2022. Biorender was used to add some feature to the figure.



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 some of the main reasons why cattle production occupies a great slice of the total livestock GHG emissions.

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 Gt CO_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 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 incentivizing 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.

Grazing methods

From all 196 million heads of the beef cattle commercial heard, the industry slaughtered 39 million animals in 2021 with 84% of that coming from forage-based systems and 16% 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-quality tend to change through the year, which particularly makes difficult to ensure that pastures will provide to the herd its full nutritional needs, especially during dry season when forage decrease its nutritional value. Minerals, proteins and energy for instance might be a limiting factor to ideal ruminal fermentation and overall animal performance under grazing systems (McAllister et al., 2019). 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 clear that beef cattle production under grazing systems is not as easy as it seems to be, especially for those that runs beef cattle systems in tropical regions.

Despite of difficulties, there is different strategies that may be adopted in grazing-based beef cattle farms to overcome and mitigate some negative effect that seasonality can cause in tropical regions. Specific grazing systems are usually adopted to improve forage quality and quantity to meet animal's nutritional needs (Rufino et al., 2020; Reis et al., 2020).

Forage stockpiling for instance is a technique used to exclude grazing from a certain area by the end of the Summer (not mandatory) and then allow animals grazing on it in the dry season. This can allow reasonable performance of animals during Winter and the beginning of Spring (in tropical countries) as opposed to the no adoption of this grazing system method (Aguilar et al., 2016).

Since pastures are deferred, there will be continuous stock of mass forage production that can be efficiently used during the dry season. Although it sems to be an interesting tool for pasture management, it is important to consider some aspects that can have direct influence over the mass forage availability and quality, for instance the canopy height of the beginning of the forage stockpiling period, the period adopted to the stockpiling, fertilization and supplementation of animals under grazing.

It is expected to have a deferred pasture system decreasing its quality (especially when pastures achieve its maturing-age) and quantity of forage due to accumulation of stems and low retention of green leaves (Aguilar et al., 2016). This can lead to limitations in terms of forage

structures and consequently it may play a key role in the ruminant metabolism, having direct consequences on animal performance.

In fact, a range of factors can affect forage growth in terms of mass and quality, but as seasonality usually plays an important role on it, partial absence of luminosity associated with lower temperatures and low pluviometry conditions can decrease nutrients solubility in the soil. Thus, pastures growth can be reduced, which implicates in lower availability of mass forage and lower nutritional quality (Sene et al., 2019). Therefore, deferred pastures technique can be used as tool to mitigate the ups and downs related to seasonality.

Another method that can be used is the rotational grazing, which is a grazing method that involves the resting of the forage up to a moment in which pasture is occupied over a period (Boyer et al., 2022). Usually a greater grazing area is divided into small grazing paddocks, where animals graze up to the ideal forage height, and then they are rotated to the following grazing area with more availability of forage mass while the previous grazing paddocks are kept in rest renewing its energy reserves up to the grazing moment (Sevov et al., 2018). Some aspects are very important regarding this grazing management such as, the control of stocking rate, which should be based on mass forage availability. Ideal stocking hate allows us to overcome forage losses due to trampling and, pastures can have better regrowing as a function of resting period, and the grazing can be more uniform as forage intake is limited to the availability in that pasture over that occupation period (Sevov et al., 2018).

One of the big problems of this system occurs during dry season, when pluviometry greatly decreases, and mass forage production is not vigorous as in rainy season. In more intensified systems stocking rate during rainy season can be higher (Sone et al., 2020). However, it is going to depend on the forage specie and type of fertilization adopted. Nonetheless, during dry season strategies to overcome seasonal negative effects must be taken, and one of the possible ways to overcome that is adopting supplementation and, if possible, decreasing stocking rate.

An alternative to improve feed efficiency in grazing-based beef cattle systems is by means adoption of supplementation. This 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.

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 or 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 *Urochloa 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 *Urochloa brizantha* cv Marandu pastures had lower CP (-8.45%) and higher lignin (+80.9%) concentrations on dry season as compared to the rainy season. 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 *Urochloa 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.

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.

Nitrate

Ammonium nitrate is a chemical product usually white or even colorless, which has 80.06 g of molecular weight and specific gravity of 1.73 $g.cm^{-1}$. It is used in agriculture sector as fertilizers and it can be also used in ruminants supplementation.

Nitrate tastes biter and it might reduce palatability (Araujo et al., 2022; Almeida et al., 2022); however its effect on supplement intake varies . When it is administered in ruminants' diet is initially converted to nitrite and later to ammonia within the rumen. Depending on its conversion rate to nitrite, it can be partially accumulated in the rumen and part of it is absorbed into the bloodstream (Khalil et al., 2023) which may lead to oxidation hemoglobin to methemoglobin. High levels of methemoglobin prevent oxygen transport leading the animal to death (Zurak et al., 2023). The main sings of ammonium nitrate poisoning are brown mucous membrane, increased heart rate and respiratory distress, muscle tremors, weakness, excess of saliva and tear production, frequent urination, low body temperature, disorientation, and an inability to get up (Zurak et al., 2023).

When chronic nitrate toxicity happens usually clinical signs of it are not noticed. However, some variables may indicate that such as weight loss or gain bellow the ideal, depressed appetite, and susceptibility to infections.

Urea and nitrate 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 DM diet (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 and thus DMI as 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 or phosphorus concentration) did not display statistical effect for any of the nitrate level 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 1. 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 equivalent. 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 equivalent. 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 1, 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[1]	**Diet**	**Inclusion**[**]	**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[*]	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	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)

[1] Bos indicus: *Bos taurus indicus*; Bos taurus: *Bos taurus taurus*; [*] Forage: concentrate ratio; [**]inclusion is based on % of the DM; [***] total feed intake kg.day^{-1}. Diff: difference in %.

In spite of no major effect on feed intake, nitrate supplementation has been considered thermodynamically favorable 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. Nitrate is ultimately converted into ammonia and thus ruminal microbial protein synthesis can be favored.

Ruminal Environment and Fermentation

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. This organ 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 in abscess of oxygen, which provides ideal condition to the presence, activity and growth of anaerobic microorganisms that are 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.

On its own, the rumen has a diverse-opened ecosystem that favour all the symbiotic activity and for that requires frequent ideal maintenance conditions that favor all the symbiotic activity such as pH, temperature, oxygen concentration, ruminal motility, and microbial diversity (Nagaraja et al., 2016).

To have a cadenced metabolism in the ruminal environment, stable conditions should be kept under certain range such as pH, around 5.5 to 7.0, and temperature around 39°C (Valadares Filho & Pina, 2011). The rumen has a diverse community of microorganism that for ideal growth and activity requires a specific pH range. Its fluctuations around the previous values range are considered ideal and when it is not in the desirable limit edge, ruminal pH tend to be kept in the range by means daily salivation, which incorporates inorganic components into the ruminal fluid, or even by ruminal motility (Furlan, Macari & Faria Filho, 2011).

As a reductive oxygen environment, the rumen must be kept as oxygen free as possible to provides ideal conditions to the fermentative process. Nevertheless, some minor concentration of oxygen might be attached to the feed but in that regard facultative anaerobic bacteria rapidly depletes it. Combined with all the previous mentioned traits, ruminal motility plays an important role in the ruminal environment maintenance as well. It mainly allows ruminal microorganisms to be in constant contact with the ingested substrate, it is responsible for moving, mixing, and boosting rumen content, facilitating the eructation of gases from

fermentation and the regurgitation of the more fibrous particles to be subjected to rumination. Since ruminal wall and pillars have chemoreceptors that understands the ruminal volume size and its chemical composition, motility can alter its activity influencing in lower or faster motility, which will provide conditions to keep the ruminal environment under controlled and ideal state to microorganism activities (Furlan, Macari & Faria Filho, 2011).

Among the microorganisms that inhabits the rumen of a cattle there are bacteria, protozoa, fungi, and archaea methanogenic. Their activity of growth, and multiplication are orchestrated not only by the previous factors that keep rumen under steady state, but also by the presence of energy and protein, which determines the extension of short chain fatty acids production, used as the main energetic source by the ruminants.

Ruminant microorganisms can be simply classified according to the substrate that they predominantly use; therefore, bacteria responsible to the breakdown of cellulose, hemicellulose, pectin, lipids, or starch are cellulolytic, hemicellulolytic, pectinolytic, lipolytic, amylolytic, respectively.

Ruminal fermentative activity allows polysaccharides, such as cellulose, hemicellulose and pectin, be broken down by means of their specific enzymes (cellulolytic, hemicellulolytic and pectinolytic) up to the conversion of glucose, an intermediate substrate that is readily available and used by ruminal microorganisms as their main source of energy. Glucose is then uptake by the microorganisms and oxidized into pyruvate. This last is extensively used as precursor to the production of the main short chain fatty acids (SCFAs), which are acetic, butyric, and propionic acid, with concomitant production of other components as H_2, CO_2 and CH_4. Despite of the generation of H_2, CO_2 and CH_4, short chain fatty acids are the main fermentative products produced in the rumen, and it corresponds to about 50 to 70% of ruminant's energy (Nagaraja et al., 2016). The proportion and magnitude in which each SCFAs is produced varies and depends on the bacterial specie, and by the concentration of Nicotinamide adenine dinucleotide (NADH) and H_2 in the cell, and these both conditions depend on the diet regime cattle is submitted (Kozloski, 2009). Besides SCFAs, H_2, CO_2 and CH_4 production, there is also the generation of microbial protein, which is a valuable protein source that ruminants use to attain its nutritional requirements.

Methanogens and Methanogenesis in Ruminants

In the anoxic habitat of the rumen a certain diversity of microorganisms symbiotically interacts degrading complex organic matter generating short chain fat acids (SCFAs), CO_2 and CH_4. It is noteworthy mention that up to the generation of SCFAs methanogens have fewer actions, and they could be positioned at the very bottom of this trophic chain since they only use the end products generated along the fermentation process.

The methanogens are prokaryotic organisms representants of the *Archaea* domain and the phylum Euryarchaeota. Structurally *Archaea* are quite distinct of bacteria as its cell wall lacks in peptidoglycan and it is constituted by pseudomurein (*Methanobrevibacter* and *Methanobacterium*), heteropolysaccharide (*Methanosarcina*) and protein (*Methanomicrobium*) (Cena et al. 2023). One other specificity of the methanogens is that all of them possess coenzyme factors enrolled to the ultimate methane production such as coenzyme F_{420}, which plays a role in the electron transport F_{420}, the coenzyme M, which is important for the methyl group transfer reactions (and within coenzyme M there is F_{430}, which plays a key role in the catalyzation and reductive cleavage of methyl – coenzyme M (Nakagaki et al., 2006).

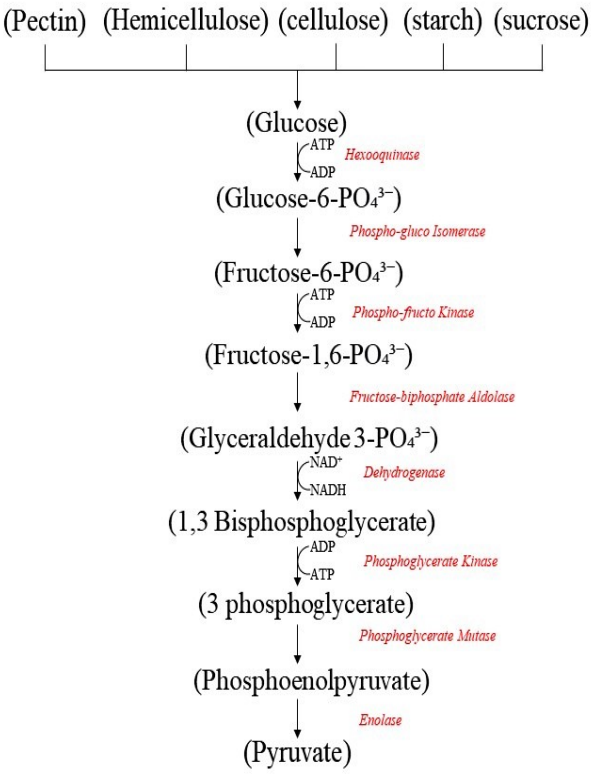
The methanogens that inhabit cattle rumen have been studied and yet it has great controversy among the prevalence of species, which are usually attributed to diet, breed, season, feeding strategies, intake, growth stages and physiological conditions of the host (Lan & Yang, 2019). However, according to Chellapandi et al. (2018) the culturable rumen *Archaea* group present and studied in the rumen known up to date are *Methanobrevibacter ruminantium, Methanobrevibacter smithi, Methanomicrobium mobile, Methanobacterium lacus, Methanobacterium formicicum, Methanomicrobium bryantii, Methanosarcina barkeri* and *Methanosarcina mazei.* Despite that, literature cites *Methanobrevibacter spp* as the most prevalent genera present in cattle's rumen. According to Janssen & Kirs (2008) great slice of the methanogen is assigned to three main genus groups: *Methanobrevibacter* (61.6%) *Methanomicrobium* (14.9%) and some unculture ruminal *archaea* known as cluster C (RCC) (15.8%).

They are a very specialized group of microorganisms that, even living in the anoxic habitat of the rumen, smartly interact with other ruminal microorganisms that works directly subsiding ruminant's digestion of plant biomass polymers through a trophic food chain up to the generation of valuable products, such as short chain fatty acids (Lan & Yang, 2019).

Independently of which source of carbohydrate is going under fermentation, glucose is going to be the universal yield at end, with differences regarding the rate it is generated as seen

on figure 1. The following step after getting the glucose molecule is the phosphorylation of it up to the generation of two molecules of pyruvate, with no carbon loss during the oxidative phosphorylation (Figure 1). During this action, there is a release of hydrogen in the ruminal environment while NAD^+ is reduced to NADH bringing to the ruminal environment hydrogen (Ungerfeld, 2020).

Figure 2 - Simplified schematic representation of Glycolytic pathway up to the generation of pyruvate. (Adapted from: Kozloski, 2009)



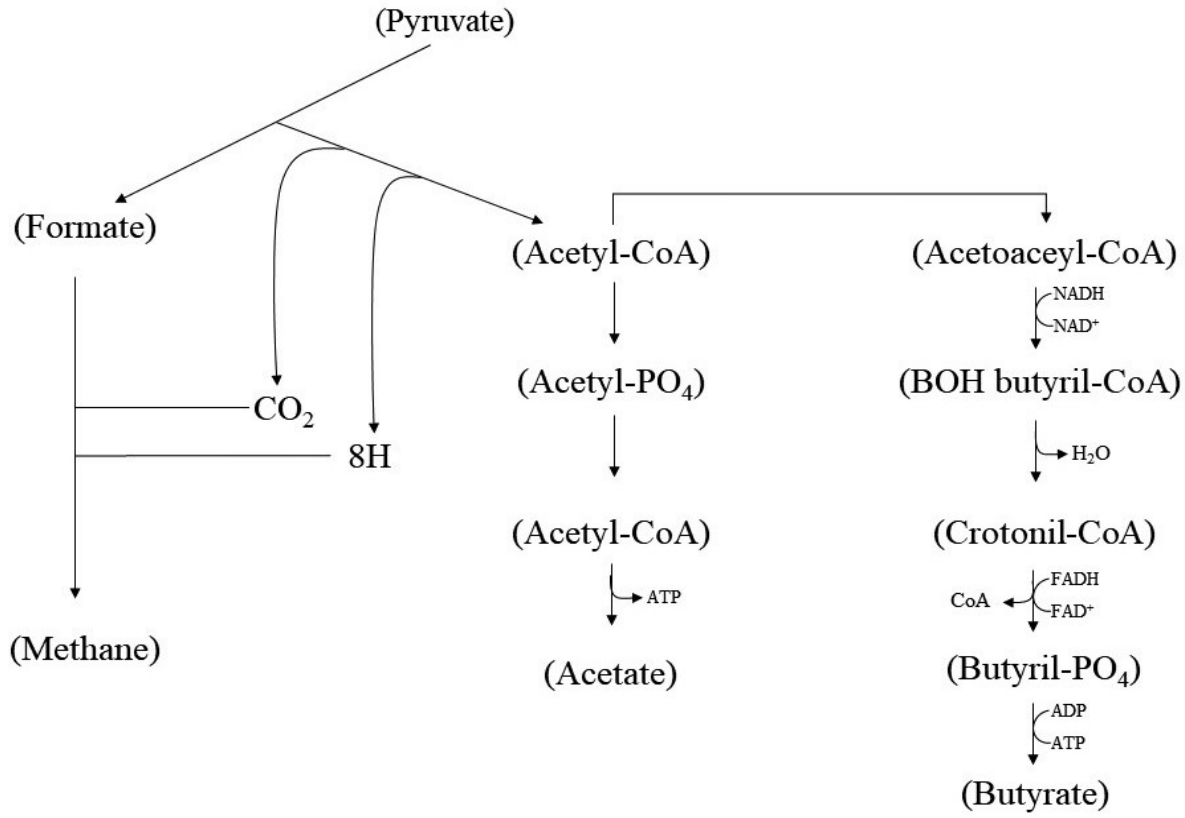
It is quite difficult to have a rumen with pH on cattle under grazing systems, mainly because the generation of glucose production runs at a slower rate as opposed to animals fed high concentrated diet. The hydrogen produced by the phosphorylation process can also be used by propionate pathway since its production is lower than its uptake (Ungerfeld, 2020).

Considering the pathways for short chain fatty acids production, the main precursors to methane production will come from two pathways involved in the generation of SCFAs, which is by generation of acetate and butyrate.

Acetate is a two-carbons molecule that to be build up requires an uptake of carbon from pyruvate (oxidative decarboxylation). Firstly, pyruvate is converted to acetyl-CoA (a two-carbon molecule) and on this first biochemical step there is loss of carbon as CO_2 or as formate production, which may directly subside CH_4 generation, as seen on figure 3.

The degradation of pyruvate to CO_2 and acetyl-CoA occurs by means catalyzation of oxidoreductase and electron transfer mediated by the ferredoxin. The Coenzyme-A is then replaced by a phosphate group generating acetyl-phosphate. The ultimate oxidation will result in the generation of the acetate with yield of ATP to the bacteria cell (Kozloski, 2009). Complete oxidation of glucose to acetate yields two molecules of acetate and four of ATP.

Figure 3 - Schematic representation of short chain fatty acids production, whit emphasis on the acetate and butyrate metabolic pathways (Adapted from: Kozloski, 2009)



In the first step where pyruvate is decarboxylated to acetyl-CoA molecular hydrogen(H_2) is released (Ungerfeld, 2020) and the bacteria that mediate the process via enzymatic action of pyruvate:ferredoxin oxidoreductase are usually from *Selenomonas* and *Clostridium* genera (Desvaux, 2005). However, when the pathway goes by pyruvate reduced to acetyl-CoA and formate production by means enzymatic action of pyruvate:formate lyase, *Streptococcus bovis* and *Butyrivibrios* are the two main bacteria that mediate this process (Xue et al., 2018).

As already mentioned, generation of acetyl-CoA can also subside the formation not only of acetate but also butyrate, and the production of one or another will depend on the bacteria

(specie) that will be acting on the substrate. When it comes to the production of butyrate, it is important to remember that it is a four-carbons molecule, so that it is required two pyruvates losing one carbon each as CO_2 or formate. Following the schematic representation of figure 3, from two molecules of acetyl-CoA, Acetoacetil-CoA is generated, which is reduced by means a dehydrogenase-enzyme, generating beta-hydroxybutyril-CoA, this molecule is then converter to Crotonil-CoA by crotonase, which it is then dehydrated and reduced, eliciting butyril-CoA. This this last loses the CoA group and gains a phosphate (Butyril-phosphate), which is then dephosphorylated, releasing butyrate and producing ATP (Kozloski, 2009).

Molecular hydrogen is one of the main intermediate products used (by methanogens) in the methane production, which allows rumen to keep efficient fermentation with no decrease of its pH (Greening et al., 2019). To make better use of H_2 available, some methanogens have close association with protozoa, which produces a reasonable amount of hydrogen as fermentative end-product by means the hydrogenosome, a membrane-bounded organelle that generates H_2 by means of oxidation of malate (Patra et al., 2017). According to the same authors up to 25% of the ruminal methanogens are found inside or aside the protozoa cell making this H_2 transfer interspecies interactions that can favor not only the methanogens but also the protozoa. *Methanobrevibater* and *Methanomicrobium* are the ones assigned as protozoa associated methanogens (PAM) and they account for 56% of all PAM sequences.

The idea of incorporating H_2 into electron sinks that are nutritionally beneficial to beef cattle can be an important path that may reduce digestible energy losses from gas production (Lan & Yang, 2019). 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.

Fermentative process that occurs into the rumen not only supply ruminants with SCFAs but also part of the negative Gibbs energy change in association with the fermentation process elicit ATP, which is used for microorganism's growth (microbial protein synthesis), transport of substrate and mobility (Ungerfeld, 2020).

Metabolism of Nitrate in the Rumen

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 nitrite (NO_2^-) and that significantly contributes to the reduction of enteric methane since by this pathway there is a consume of hydrogen generating ammonia nitrogen (NH_3), disposing hydrogen by a path that is considered to be thermodynamically more favorable 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 2e- electrons as showed in the following equation:

$$NO_3^- + [H_2] \rightarrow NO_2^- + H_2O \rightarrow (\Delta G = -130 \text{ kJ.mol}) \quad \text{Eq.1}$$

$$NO_3^- + 2H + \mathbf{2e^-} \rightarrow NO_2^- + H_2O \rightarrow (2e^- \text{ electrons not available to methanogenesis}) \quad \text{Eq.2}$$

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

$$NO_2^- + [3H_2] + 2H^+ \rightarrow NH_4^+ + 2H_2O \rightarrow (\Delta G = -371 \text{ kJ.mol}) \quad \text{Eq.3}$$

$$NO_2^- + 8H^+ + \mathbf{6e^-} \rightarrow NH_4^+ + 2H_2O \rightarrow (6e^- \text{ electrons not available to methanogenesis}) \quad \text{Eq.4}$$

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.

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

Van Zijderveldet et al. (2010) explain that through this path there is an efficient sink for hydrogen since for each mol of reduced nitrate 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).

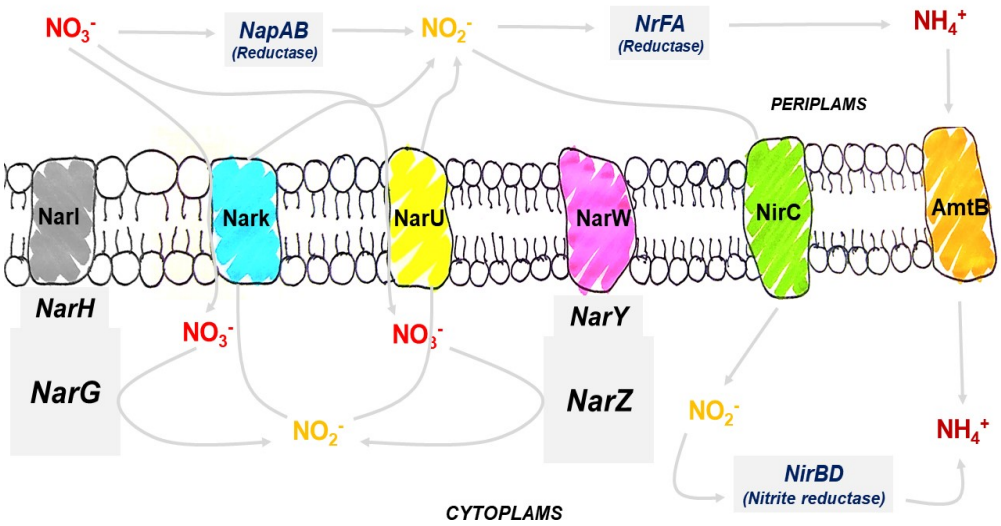
It is known that quite high concentrations of nitrate in ruminants' diet can lead to nitrite accumulation; however, that can and only happen when kinetics of nitrite removal is running lower than that of nitrate first step reduction, as seen on equations 2 and 4. Intense accumulation

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 nitrate use (Besson et al., 2022). Nitrate metabolism in a cell of a ruminant bacteria is not well described yet, as 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 4.

Since inside of membrane has a negative potential, nitrate uptake by ruminal bacteria should be by means of and active transport mechanisms to allows nitrate goes through the cytoplasmatic membrane with no harm to the bacterial (Andrade & Einsle, 2013) as seen on figure 4. Through a simplified view, nitrate reduction is catalyzed 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 center.

Figure 4 - Schematic representation of Nitrate uptake and metabolization of it by bacterial cell. Adapted from Andrade & Einsle (2013).



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 members of nitrate or nitrite porter, respectively, playing an important role in the facilitation of transportation, while NirC channel allows transportation of nitrate, whit 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. In this complex there is biding site for oxidation of the electron donor. There, one of the NarG subunit catalyzes 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 members 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 metabolize 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 of the available H_2. No significant effect was detected on protozoa population when nitrate is added into the diet. It is consistently found on the literature reduction ranging from 4 to 25% of CH_4 per amount of DMI when nitrate is added in the feed.

Nitrate into cattle feeding, for instance, is known for being efficiently effective as a source of non-protein nitrogen and for reduction in methane emission (Latham et al., 2016). Nitrate supplementation has been considered thermodynamically favorable 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 by means a source of non-protein nitrogen.

Effect of Nitrate in the Ruminal population

As covered by Yang et al. (2016) there are many species of bacteria but the most predominant in the rumen slides into a narrow set of the domain bacteria with Bacteroidetes, Firmicutes and Protobacteria. Some of these bacteria may possess a reduction complex which allows them to work in the nitrate reductase activity.

Selenomonas ruminatium, Veillonella parvula, Campylobacter fetus, and *Wolinella succiogenes* are one of the species that appears more frequently when nitrate is added into ruminants' diet (Iwamoto et al., 2002; Lin et al., 2013; Zhao et al., 2015). According to Iwamoto et al. (2002), these microorganisms are actively acting on nitrate reduction.

Researchers have observed that when nitrate is added into the diet there is an increase of cellulolytic bacteria, and that is positively associated to the increase of fiber digestibility (Patra & Yu, 2015). In consonance with that, Zhao et al. (2015) showed that some important cellulolytic bacteria from the genera were favored by nitrate such as *R. albus, R flavefaciens*, and *F. succinogenes.* According to them, *R. albus* and *F. succinogenes* linearly increased to nitrate addition, while *R. flavefaciens* abundance showed a quadratic increase. On the other hand, Wang et al. (2018) did not detect effect on fiber digestibility whit Holstein cows fed 14.6 g of nitrate per amount of DM. According to the authors that result was in consonance with no major change in 16S rRNA genes copies of ruminal fiber-degrading bacteria previously cited. In contrast to that, Klop et al. (2016) mentioned a negative effect of nitrate over neutral detergent fiber (NDF) digestibility of Holstein cows fed 49% corn silage, 21% of grass silage and 30% of concentrate. According to the authors, the negative effect over the digestibility might be due to natural increase of H_2 in the ruminal environment after nitrate feeding. McAllister & Newbold (2008) mentioned that high concentration of H_2 may disturb the regeneration of NAD^+ from NADH, and that causes direct effect on cell wall degradation.

As it is possible to notice, information regarding bacteria-population change in rumen of cattle nitrate-fed is quite sparce since many factors such as nitrate dosage, administration period, adaptation and diet composition, can play a key role changing the ruminal environment. Some ruminal bacteria are equipped with nitrate reductase enzymes and use NO_3^- for respiration or as substrate for incorporation of Nitrogen into biomass (Besson et al., 2022). *Veillonella* and *Wolinella* were persistently detected in reasonable amount by Iwamoto et al. (2002) in an *in vitro* mixed culture with nitrate addition. The authors noticed that when nitrate was removed from media there was a sharp decrease on their presence. Besides that, the same group of authors showed that *Selenomonas ruminatium* seems to be tolerant to nitrate and nitrite toxicity as they mainly acquire energy from the nitrate reduction, a fact that similarly happens to *Veillonella*.

Wolinella succinogenes is recognized as the fastest nitrate-reducing bacteria. They acquire energy from ATP through ETP systems with nitrate, in which H_2 and nitrate are used as electron doner and acceptor, respectively (Guo et al., 2009). However, their activity rapidly decreases when environment has high amount of ruminal fermented end-products and sugar. Some researchers also point that *C.fetus* and *M. succiniciproducens* tend to increase its activity when nitrate is added in ruminants diet (Lin et al., 2013).

Changes on ruminal environment is expected when inclusion of nitrate is done, especially in terms of methanogenic microorganism population. Most of the changes regard the decrease of some specific species that might be very sensitive to nitrate and nitrite.

Protozoa activity plays an important role on methanogenesis and its absence can significantly coordinate a lowered methane production (Qin et al., 2012) since protozoa can contribute to about 37% of total methane ruminal production (Finlay et al., 1994).

In investigation conducted by Popova et al. (2018) it was not found significant effect of nitrate on protozoa counting. In consonance with them, Li et al. (2012) and Van Zijderveld et al. (2010) did not find any effect of nitrate on protozoa population counting. Despite of that, in a recent work developed by El-Zaiat (2017) with sheep receiving encapsulated nitrate in diet, it was identified reduction of protozoa number when animals were fed nitrate and the extent of that reduction was about 12% as compared to control group. However, it is not consistent on literature the nitrate effect on protozoa population.

As exhausted stated in this review, inclusion of nitrate into ruminants' diet results occurs a shift of path related to the use of hydrogen. Basically, nitrate sinks the energy that could be provided to the methanogen's growth and that is probably one of the reasons to some changes in the rumen. Nitrate in ruminants diet changes the population of microorganisms in the rumen

by means serving as substrate to the development of specific species. However, direct inhibition of methanogens may occur as well (Zhao et al., 2018).

Zhao et al. (2018), working with steers receiving three different doses of nitrate in diet, observed some changes on the rumen with higher presence of methanogens classes such as *Methanobacteria* and *RCC (Thermoplasmata*). At genera level, *Methanobrevibacter* (*Methanobacteria)* and vadin CA11 from RCC accounted for more than 90% of the total sequences. Nevertheless, they also detected prevalent genera in a very less intensity, accounting for 1.25%. On that can be included, *Methanosphera, Methanimicrococcus, Methanosarcina, Methanobacterium, Methanoplanus and Methanoculleus*. According to the authors, *Methanoplanus* showed a suppressed effect when nitrate was added. However, *Methanoplanus* and *Methanorevibacter* showed a positive correlation to *in vitro* methane production.

Effect of nitrate on enteric methane emission

Despite of many 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 Van Zijderveld et al. (2010), stoichiometrically, 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 died 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 2).

Lee et al. (2014) assessing encapsulated nitrate on ruminal-cannulated beef heifers (451 kg BW) obtained maximum CH_4 reduction of 18%, as well as 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 contained 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 fistulated Bos indicus steers under grazing were supplemented with 4.6 or 7.9 g of nitrate/kg of DM.

Table 2 - Description of conducted experiments in which nitrate encapsulated or not (from different sources) was used in the supplementation as a H2 sinker. In each case inclusion of nitrate, days or experimental run, methane reduction and sources are described.

Nitrate (g/kg.DM)	Source	Days*	CH_4 reduction (%)	Technique	Sources
22		46	27	*SF_6*[1]	(Hulshof et al., 2012)
6 to 30		33	12 to 29	*SF_6*	(Newbold et al., 2014)
10 to 30		112	4.2 to 18	*R.Chamber*[2]	(Lee et al., 2015)
21		56	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		84	17	*R.Chamber*	(Alemu et al., 2019)
12.5		76	16.1	-	-

1 SF6 - Sulphur-hexafluoride; 2 - R. Chamber – Respiratory chamber. *Days- represents the amount of day animals were submitted to the treatment.

As 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*) and they achieved a much higher reduction for methane emission of 22.6% as compared to control group.

Capelari (2018), working with angus crossed steers achieved reduction in methane of 8.6% with inclusion of 15g of nitrate/kg of DM for 64 days. 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 2, methane generation from beef cattle fed nitrate diet have positive effect on methane emission reduction. Certainly, the extent of reduction results from the nitrate 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 21.0%.

Effect of Nitrate on Short Chain Fatty Acids Production

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 by re-oxidation of reduced cofactors (NADH, NADPH and FADH), as well as 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.

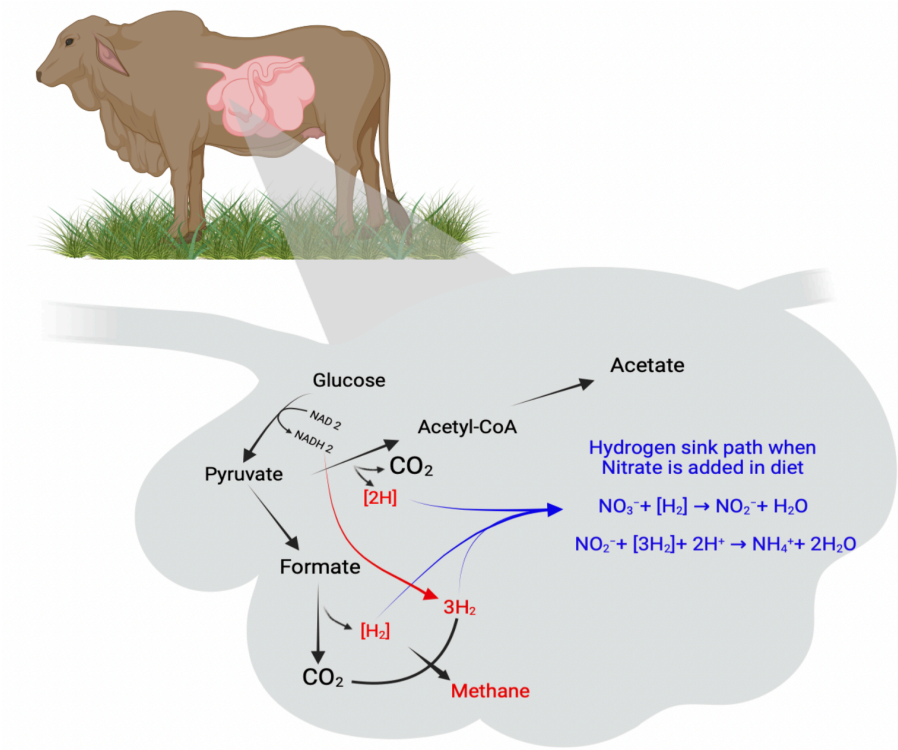
Some papers on literature associate nitrate inclusion on ruminants' diet with increased propionate production as some H_2 could be uptake into propiogenesis pathway competing H_2 with nitrate (Ungerfeld, 2020). In fact, that can be corroborated by some work on literature; however, reduction on propionate 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 5, that draws a possible metabolic path that involves the methane reduction and a redirection of hydrogen for nitrate reduction to ammonia.

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 are detected and thus addition of nitrate may have a direct effect on mitigation of methane production via this metabolic path.

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.

Figure 5 - 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 of formate path (highlighted in red). Schematic design created with Biorender. (Own authorship)



The most recent works on literature brings divergences regarding fermented end products. Capelari (2018), working with beef cattle with average body weight of 335kg fed mixed ratio with inclusion of 15g of nitrate/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 (V: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 nitrate reduction to NH_3.

Differently from the previous authors, Henry (2017), working with beef cattle under grazing, did not 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 taurus 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 nitrate 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, this is simply explained by the fact that nitrate is efficiently acting as H_2 sinker, redirecting them to nitrate 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 production was also detected by Villar et al. (2020) of 23.5% 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 CHO's sources as the sole source of energy, converting them (for instance: polymers starch, xylan, glucose, arabinose, xylose and cellobiose) into butyric acid (Miguel et al., 2019).

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CHAPTER 2 –Can intensified grazing systems with adoption of ammonium nitrate as a nitrogen supplementation source be a strategic tool to replace urea?

Abstract

It was aimed to investigate if intensified grazing systems (deferred or rotational grazing methods) with adoption of ammonium nitrate nitrogen can be a strategic approach to replace urea supplementation for beef cattle during different seasons. It was assessed dry matter intake, total apparent digestibility, ruminal degradability, synthesis, and efficiency of microbial protein synthesis. To conduct the experiment, it was used eight Nellore female cows were used as experimental animals, and randomly allotted to 8 paddocks. Each treatment was allotted to a paddock in a randomized block design, composed by two different grazing methods with supplementation of ammonium nitrate or urea making up a factorial design, in which seasons of the year was also included. The experimental treatments were as follows: Rotational grazing with urea supplementation, rotational grazing with nitrate supplementation, deferred grazing with urea supplementation and deferred grazing with ammonium nitrate supplementation. All variables were collected during four seasons over two years. Animals supplemented with ammonium nitrate had similar dry matter intake compared to those supplemented with urea. However, there was effect of grazing and nitrogen source for non-protein nitrogen intake, in which animals in rotated grazing had greater NPN intake. Improved apparent digestibility of neutral detergent fiber, acid detergent fiber and ether extract were detected when animals were in rotated pastures grazing systems. Potential degradability of dry matter and crude protein was influenced by nitrogen source within seasons. Overall, the nitrogen sources had similar effects on most of the degradability parameters. Nitrate supplementation did not affect microbial nitrogen compounds synthesis, or efficiency of microbial protein synthesis. When urea and nitrate were used as the main nitrogen sources, both contributed equally to microbial protein synthesis and its efficiency. This finding suggests that ammonium nitrate can serve as a valuable non-protein nitrogen supplementation source that does not negatively affect feed consumption for beef cattle in intensified grazing systems as feed intake expressed as NPN (kg.day) was lower for animals supplemented with nitrate. Thus, on this study we highlight the positive benefits of the rotated grazing method and the use of ammonium nitrate as a strategy key to intensify the production system in grassland.

Keywords: beef cattle, deferred grazing, non-protein nitrogen, rotated grazing,

Introduction

In tropical regions the Beef industry can rely on grazing systems as it is economically more attractive; however, it has some disadvantages regarding pastures vulnerability to climatic seasonality. Alternatives have been studied for growing beef cattle in tropical grazing systems as a tool to improve performance and mitigate negative effects of seasonality (Sene et al., 2019; Rodrigues et al., 2021; Andrade et al., 2022; Black et al., 2022). The intensification of the grazing systems, for instance, by pasture management (stockpiling or rotation) and adoption of supplementation are the most palatable tools to overcome the seasonality of the tropics and ensure ideal beef cattle production (Lelis, 2021).

Non-protein nitrogen (NPN) supplementation is used as a strategy to increase the apport of protein into the diet and meet the requirement of nitrogen 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. The use of alternative sources such as nitrate it has also been done (Duthie et al., 2018; Granja-Salcedo et al., 2019; Alemu et al., 2019); however, to our understand most of the research have been performed with cattle in feedlot systems, and fewer results are found on literature with cattle in grazing systems being supplemented with nitrate. NPN supplementation source can be nutritionally favorable, especially in tropical regions, where forage quality and availability have a great variation through the year (Rufino et al., 2020), displaying lower protein content and increased lignification during dry season, which are enough to directly affect feed intake and the digestion of structural carbohydrates in the rumen (Reis et al., 2020).

Theoretically, among the NPNs, nitrate stands out for being an efficient source of non-protein nitrogen, since by its metabolic path, nitrate is reduced to ammonia and compared to other sources of NPN, it leads to a higher flow of negative Gibbs free energy that is incorporated into the rumen and furnish energy for microorganism's growth (microbial protein synthesis), transport of substrate and mobility (Ungerfeld, 2020). Coupled with fomenting bacteria growth, nitrate has a fantastic capability to lower methane production, and thus playing an important role on mitigation of GHG emissions to the atmosphere (Natel et al., 2022; Black et al., 2022) while preserving the energy the energy that can be used by the animal.

Nonetheless, nitrate bitterness can be a limiting factor affecting feed intake (Araujo et al., 2022; Almeida et al., 2022), which might elicit lower digestibility of diet's nutrients of cattle in tropical grazing systems and thus negatively compromising the synthesis and efficiency of

microbial protein in the rumen. The previous effect can be even potentialized through the seasons of the year if any supplementation is adopted, as forage quality and its availability changes through the year (Rufino et al., 2020).

Therefore, the hypothesis of this study is that the intensification of grazing systems by adoption of rotated and deferred grazing methods associated with ammonium nitrate supplementation have positive effect on Nellore beef cattle ruminal metabolism, and thus, improve nutrient utilization and microbial synthesis and efficiency in Nellore beef cattle when compared to the adoption or urea as the main non-protein nitrogen.

Objective

The objective of this experiment was to investigate the adoption of ammonium nitrate over a urea supplementation, for beef cattle kept in intensified grazing systems (rotated and deferred grazing associated with nitrate or urea supplementation) during different seasons and assess the effect of them on dry matter feed intake, total apparent digestibility, ruminal kinetics parameters, synthesis, and efficiency of microbial protein.

Material and Methods

Location

The experiment was carried out at College of Veterinary Medicine and Animal Science (FMVZ/USP), Pirassununga, Sao Paulo State, Brazil, for two years, in between June of 2019 and April of 2021. The experimental animals were handled and managed according to the Ethic Committee on Animal Use on Research (FMVZ/USP). A total of 8 Nellore female cows, of approximately 551 ± 7.01 kg of BW were used as experimental animals for rumen fermentation data (cannulated animals).

Experimental design, pasture system and treatments

The experimental animals were randomly allotted to 8 paddocks. Each treatment was allotted to a paddock in a randomized block design (blocks were formed as a function of terrain location) for two years (total of four replicates). Treatments is composed by combination of two different grazing systems with supplementation of ammonium nitrate (NH_4NO_3) or urea within each season, they are as follows:

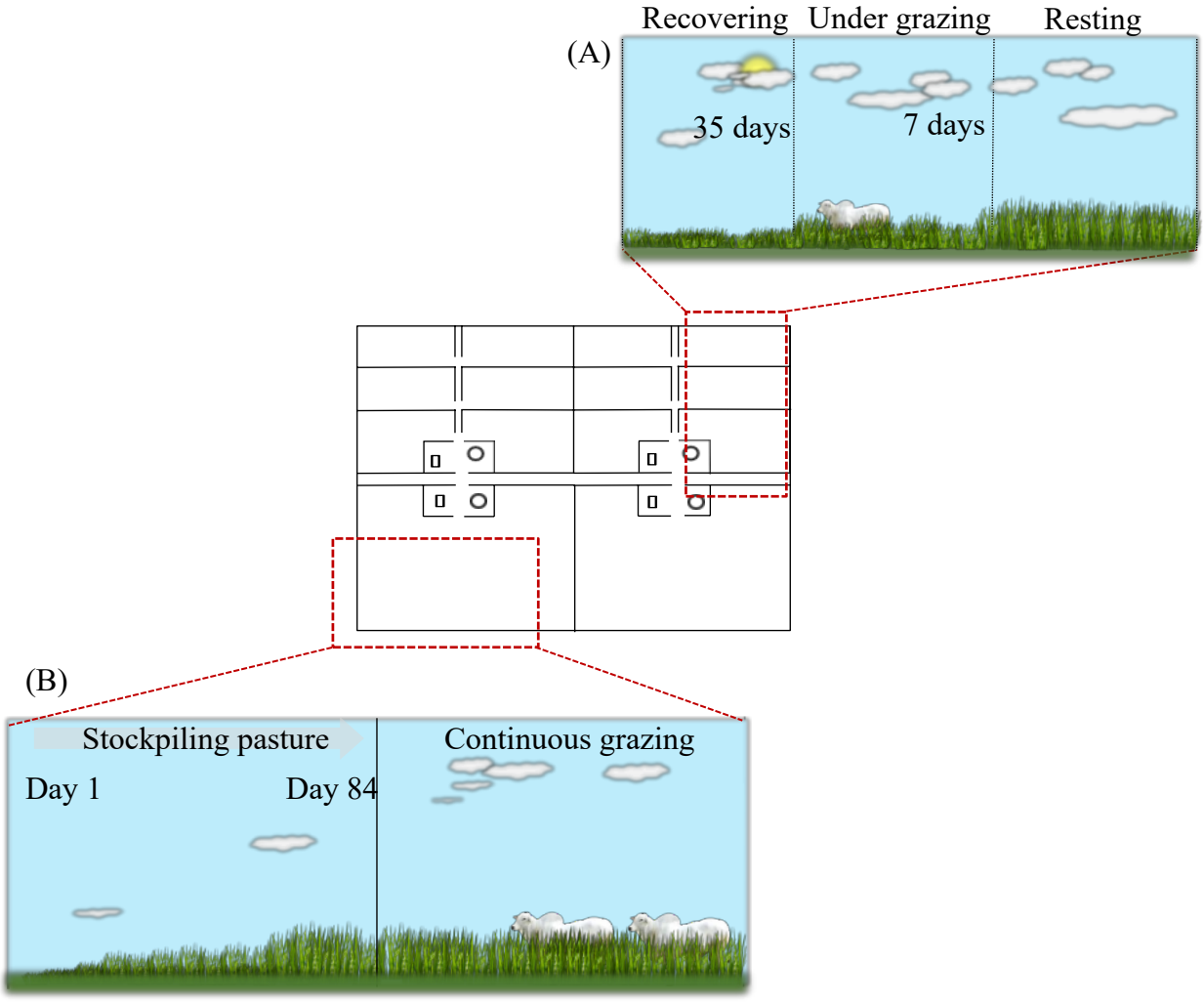
1) Deferred grazing system with NH_4NO_3 supplementation (DGN),
2) Deferred grazing system with urea supplementation (DGU),
3) Rotational grazing system with NH_4NO_3 supplementation (RGN), and
4) Rotational grazing system with urea supplementation (RGU).

The experimental area as seen on Figure 6, has 26.5 ha^{-1} divided by management corridors. The area was established in 1999 with Urochloa (syn. Urochloa brizantha) brizantha. Additionally, 13.1 ha^{-1} was used for allocation of extra animals used to adjust stocking rate. Fertilization procedures were adopted along the experimental period following soil's recommendation.

In January of 2019, previously to the beginning, experimental area was fertilized with 50 kg ha^{-1} of nitrogen and 50 kg ha^{-1} of potassium. In November of 2019, the pastures were fertilized with 53 kg ha^{-1} of nitrogen and 57.5 kg ha^{-1} of sulfur, using ammonium sulfate. In January and March of 2020, ammonium nitrate fertilizer was applied in the amount of 56.7 kg ha^{-1} of nitrogen in each post-grazing rotated paddocks, and in the deferred pastures it was carried at once, on the same day when the last paddock of the rotated pastures was fertilized.

The deferred pastures, where stockpiling was adopted, were locked for 84 days at the end of rainy season in the first and second year (at the end of March). The management of grazing in the deferred systems was simple and animals were introduced into the systems when stockpiling was ceased, then animals were left to continuously grazing. Each rotational system was comprised by 6 even paddocks, where animals were left under grazing in each paddock for seven defoliation days with a resting period of 35 days.

Figure 6 - Schematic representation of the rotational and deferred grazing systems with indication of recovering period, under grazing, resting period and deferred period.



Independently of the systems, animals had easy access to fresh water and to the formulated supplement, which has its composition described on table 3.

Table 3 - Composition and proportion of each ingredient used to prepare supplement for the adaptation, rainy and dry season using urea or nitrate as nitrogen source.

Ingredients		Adaptation		(Dry season)		Rainy (Season)	
		Urea (%)	Nitrate (%)	Urea (%)	Nitrate (%)	Urea (%)	Nitrate (%)
Ground corn		55	55	48	45	72	69
Urea		10		22		13	
Salt		20	15	15	10	7	5
Mineral mixture[1]		15	15	15	15	8	8
Ammonium nitrate			15		30		18
Nutritional composition							
CP	(%)	33.14	33.49	66.34	61.13	43.01	43.34
TDN	(%)	48.22	48.22	42.02	39.46	63.13	60.5
EE	(%)	1.60	1.60	1.39	1.31	2.09	2.00
NDF	(%)	4.35	4.35	3.79	3.56	5.69	5.45
ADF	(%)	1.43	1.43	1.25	1.17	1.87	1.79
Ca	(%)	2.70	2.70	2.69	2.69	1.45	1.45
P	(%)	2.54	2.54	2.52	2.52	1.47	1.46
Na	(%)	7.81	5.86	5.86	3.91	2.74	1.96

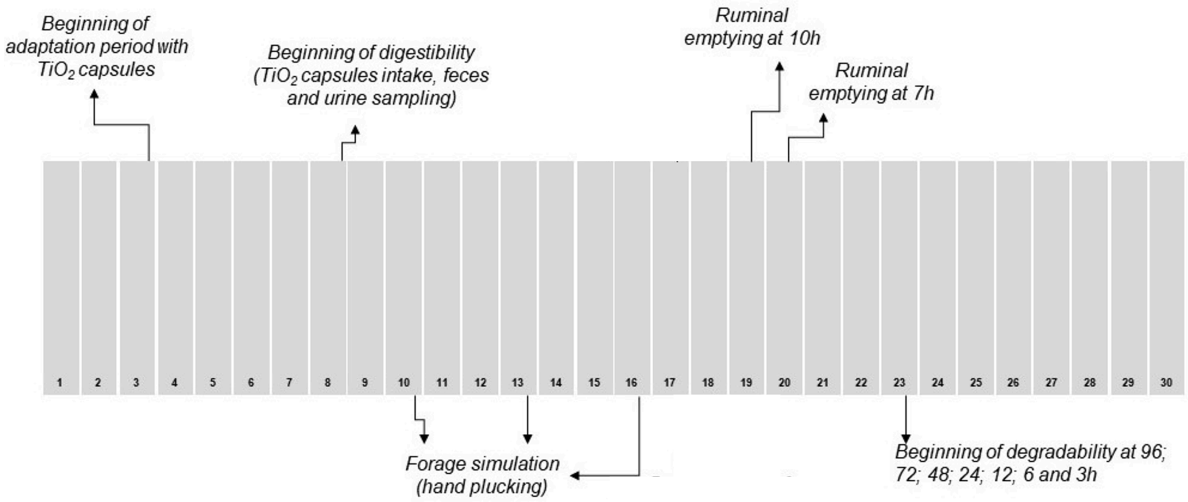
[1]Minerthal®**Estimated Macro and micromineral composition for the urea and nitrate supplement adopted in adaptation period and dry season:** 1.93 g/kg of potassium, 0.77 g/kg of magnesium, 3.29 g/kg of sulfur, 12.30 mg/kg of cobalt, 342.45 mg/kg of copper, 16.79 mg/kg of iodine, 402.90 mg/kg of Iron, 291.00 mg/kg of molybdenum, 3.36 mg/kg of selenium, 812.70 mg/kg of zinc. **Estimated Macro and micromineral composition for the urea supplement adopted in rainy season:** 2.52 g/kg of potassium, 1.01 g/kg of magnesium, 2.22 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. **Estimated Macro and micromineral composition for the ammonium nitrate supplement adopted in rainy season**: 2.42 g/kg of potassium, 0.97 g/kg of magnesium, 2.19 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. CP: crude protein; TDN: total digestible energy, EE: ether extract, NDF: neutral detergent fiber; ADF: acid detergent fiber; Lig: lignin; EE: ether extract; Ca: Calcium, P: phosphorous, Na: sodium.

Animals were adapted to the supplementation of ammonium nitrate and previously to this experiment other studies were carried out evaluating the inclusion of different dosages of nitrate into the diet and no intoxication was detected. Despite of that, Methylene blue antidote was readily available in any case of intoxication sign.

Experimental period

All variables were collected during four periods of two years (Winter, Spring, Summer and Autumn). In the following schematic representation, it is shown all the activities scheduled in each month (the second month of the season) of each season.

Figure 7 - Schematic representation of the activities scheduled in each sampling month of each season.



In the first month of the season all animals were under adaptation on its respective experimental units.

Hand-plucking technique

Hand-plucking technique was adapted from Sollenberger and Sherney (1995) and performed to simulate what the animal's graze to get an accurate estimate of diet nutritive value. In a rotated stocked pasture, samples of the pasture were taken while animals were under grazing. It was considered approximately 10 meters distance from where animal's grazing took place, and then, by clipping a hand full of forage at the locations where animals were grazing the samples were taken to attain approximately 500g of material. This procedure was also adopted in the continuous stocked pasture, and, to better represent the quality of the forage that animals were grazing at the week, in which the other parameters were also being taken, hand plucking method was performed in the day 1, 4 and 7 of the rotational periods. After that, material was dried at 65°C for 72 hours and milled at 2 mm. Processed material was then analyzed for Chemical composition of forage canopy (table 4) and used for the determination of parameters such as feed intake of nutrients and digestibility.

Dry matter intake of forage and supplement

The feed intake was determined by using the external, Titanium dioxide and Chromium oxide, and as internal marker, iNDF. Therefore, to assess forage intake, during 10 days of each experimental period, TiO_2 was administered (15 g/cow.day) directly into the rumen through the cannula twice a day at 8 a.m. (7.5 g) and at 4 p.m. (7.5 g), the first five days for adaptation and the last five ones for feces collection, twice a day (8 a.m. and 4:00 p.m.). The collected feces, directly from animal's rectum, were pooled and stored in a freezer (at - 20ºC) up to the time of analysis that followed methodology described by Myers et al. (2004). After laboratorial analysis, marker concentrations were obtained in ppm and subsequently converted to kilograms (kg) to the determination of fecal excretion by means a known amount of external marker administered (kg/day) and that found on the feces as follows:

$$\text{TDFE} = \left(\frac{\text{Marker in the diet}}{\text{Marker in the feces}}\right)$$

In which: TDFE: Total daily fecal excretion (kg); Marker in the diet = (kg); Marker in the feces = (kg)

Having fecal excretion data, it was then calculated de forage dry matter intake (DMI) by means the iNDF as internal marker concentration (%) from pastures and feces.

To obtain the Internal marker (iNDF) concentration, samples of dried (65°C) feces and diet were placed in 100 $g.m^2$ TNT filter bags and incubated for 288 hours in rumen of cannulated animals kept in grazing pastures. After removing the TNT bags from rumen, they were washed to remove all the impurities. Then it was subsequently dried in forced air circulation at 65°C for 72 hours, to determine the NDF, according to the method described by Van Soest et al. (1991). The remaining residue was considered as iNDF content. Therefore, the forage intake was estimated then by using the following equation:

$$\text{DMIF} = \left(\frac{\text{TDFE} * \text{Indigestibility of feces}}{\text{Marker Indigestibility of the diet}}\right)$$

In which: DMIF: Dry matter intake of forage (kg/day); Indigestibility of feces = (%); Indigestibility of diet = (%)

We also used a second external marker (Chromium oxide) to assess the supplement intake. The marker was manually added and mixed within the formulated supplement at an inclusion of 7.5%. The determination of Chromium concentration followed methodology described by Almeida et al., (2007), which used energy dispersive X-ray fluorescence technique. Thus, to the determination of supplement dry matter intake, we used the following equation:

$$\text{DMIS} = \left(\frac{\text{TDFE} * \text{Indigestibility of feces}}{\text{Marker Indigestibility of the diet}}\right)$$

In which: DMIS: Dry matter intake of supplement (kg/day); Indigestibility of feces = (%); Indigestibility of diet = (%)

Since a different source of non-protein nitrogen (NPN) was used in both supplement treatments (Urea and Ammonium nitrate), we took the amount of supplement feed intake and expressed it as protein equivalent using the following equation:

$$\text{NPN} = \left(\frac{\text{DMIS} * \text{NPN inclusion} * \text{N}(\%)}{100}\right)$$

Where:
NPN: total NPN (kg/day) feed intake
DMIS; Dry matter intake of supplement (kg/day)
NPN inclusion: Amount in % of NPN source inclusion in the formula.
N%: concentration of molecular nitrogen of the NPN source.

Total apparent digestibility of DM and its fractions

The apparent digestibility coefficients (ADC) were calculated based on the TiO_2 content of the diet and feces using the following equations:

$$\mathbf{ADC_{DM}} = 100 - (100 \text{ x } (\frac{\text{TiO2 (\%) in diet}}{\text{TiO2 (\%)in feces}}))$$

$$\mathbf{ADC_N} = 100 - 100 \text{ x} \frac{(\%\text{TiO2d})}{(\%\text{TiO2f})} \text{ x } \frac{(\%\text{ Nf})}{(\%\text{Nd})}$$

Where: ADC_{DM} = DM apparent digestibility coefficient; ADC_N = Nutrient apparent digestibility coefficient; % TiO_2d = Titanium dioxide content in diet; % TiO_2f = Titanium dioxide content in feces; % Nd = Nutrient content in the diet; % Nf = Nutrient content in feces.

Paraments such as DM, CP, NDF, ADF, CF, Lig, EE, MM and DIVMS of the forage and supplement had the concentration determined by near infrared spectrophotometer (NIRS) technique, model NIRFlex N-500 Solids (BÜCHI, Flawil, São Galo, Suíça, SWI) with a validated calibration.

Feces had its water content removed by drying them in a forced air oven at 65ºC for 72 hours according to AOAC (1995). After drying, samples were milled in willy-type knives mill of 1 mm sieves and assessed for: CP concentration, which was determined by the total N content (N x 6.25) using the micro-Kjeldahl technique (method 920.87; AOAC, 1990); the EE was determined by means the ANKOM XT15 Extractor® equipment (method Am 5- 04; AOCS, 2005); MM that was obtained by calcination in a muffle furnace at 550ºC for 4 hours; the organic matter (OM) was obtained by calculating the difference between 100 and MM. The Gross energy (GE) of feces, supplement and forage were determined by means complete oxidation of samples in a calorimetric bomb (C5000 control, IKA®, Staufen, Germany).

The NDF and ADF analysis were determined by the method described by Van Soest et al. (1991), and calculated using the following equation:

$$\text{NDF or ADF}(\%) = \left(\frac{\left(\frac{\text{PSE} - \text{PSV}}{\text{PAM}} \right)}{DM} \right) * 100$$

Where:

NDF or ADF: total concentration of neutral detergent fiber or acid detergent fiber (%);

PAM: samples weight (%).

PSE: weight of sample after dried at 65 (%).

PSV: weight of empty bag (%).

DM: dry matter concentration of feces (%).

The non-fibrous carbohydrate (NFC) content of feed and feces were obtained by subtracting the amounts expressed in percentage of DM of CP, EE, MM and NDF from 100 as follows:

$$NFC_{(\%DM)} = (100_{(\%DM)} - (EE_{(\%)} + NDF_{(\%)} + MM_{(\%)} + CP_{(\%)})$$

Where:

NFC: total concentration of non-fiber carbohydrates (%).

EE: concentration of Eter extract (%).

NDF: concentration of neutral detergent fiber (%).

MM: concentration of mineral mater (%).

CP: concentration of crude protein (%).

The concentration of total digestible nutrients (TDN) of feed and feces we obtained using the following equation:

$$\text{TDN (\%)} = \left(\frac{(\text{ADcp} * \text{CP (\%)}) + (\text{ADndf} * \text{NDF (\%)}) + (\text{ADee} * (\text{EE (\%)} * 2.24)) + (\text{ADnfc} * \text{NFC (\%)})}{100}\right)$$

Where:

TDN: Concentration of total digestive nutrients in the feed.

ADcp: Apparent digestibility of crude protein (%).

ADndf: Apparent digestibility of neutral detergent fiber (NFD) (%).

ADee: Apparent digestibility of ether extract (%).

ADnfc: Apparent digestibility of non-fiber carbohydrates (%).

The concentration of the nutrient in the feed concentration of the nutrient (CP, NDF, EE and NFC) in the feed were expressed as percentage of the DM.

Rumen kinetics

Determination of disappearance rate of rumen solid mass

Ruminal digesta was manually removed through rumen cannula and samples (liquid and solid phases separately) taken at 10 a.m and 7 a.m on days 19 and 20, respectively. Immediately after that, ruminal contents were placed back in the rumen. The solid and liquid samples were dried at 60°C (forced-air oven) for 72 hours to attain dry matter content, and the solid and liquid mass were calculated using solid and liquid content weighted and adjusted by dry matter content.

Ruminal solid and liquid mass used to calculate solid disappearance rate uses the following equation suggested by Robinson et al. (1987):

In which:

$$\textbf{kt (\%/h)} = 100 \times [\text{DMI (kg)} / \text{Rumen DM (kg)}] / 24$$

$$\textbf{kt (kg/h)} = \text{Rumen content DM (kg)} \times [\text{kt (\%/h)} / 100]$$

where:

kt: percentage of disappearance rate per hour.

DMI: Average dry matter feed intake in kg.

Rumen DM: dry matter content in kg of rumen material.

Ruminal degradability of DM and nutrients

Ruminal digestion rate was conducted and evaluated by ruminal degradability using nylon bags technique (Ørskov et al., 1980) to assess the dry mater' disappearance and its fractions. Samples collected from each experimental unit by means hand plucking method, as described by Cook (1964), were dried at 65°C for 72 hours and milled at 2 mm.

Following Oskov et al. (1980) methodology, approximately 5 g of dry matter from each sample is placed in identified nylon bags and then incubated in the rumen via ruminal cannula in the ventral region for 0, 3, 6, 12, 24, 48, 72 and 96 hours. After the incubation period, nylon bags is washed to remove the soluble material and then dried at 65°C for 72 hours. Dry matter disappearance is obtained by the difference of initial and final weight of incubation, calculating forage fraction and its degradable fraction in the rumen. Remaining dried material in the bags were assessed for DM, CP, and NDF (AOAC, 2005) to determine the disappearance of these

fractions as well. The curve for ruminal degradability of DM and CP are adjusted to a non-linear regression as equation suggested by Ørskov and McDonald (1979) as follows:

$$\mathbf{P} = a + b\,(1 - e^{-ct})$$

In which:

P = disappearance of the nutritive component at time "t";
a = intercept of the curve when t = 0, that correspond to the soluble fraction in water.
b = degradable potential of the insoluble fraction in water.
c = degradability rate by fermentative action of b.
t = incubation time.

Potential degradability given by a+b represents the amount of the assessed nutritive component that might be dissolved and degraded in the rumen when time is not a limiting factor. Non-degradable ruminal fraction is calculated by the following equation: Ind% = 100 - (a + b), as indicated by Ørskov et al. (1980).

After determination of the coefficient a, b and c, they were applied in the equation proposed by Ørskov and McDonald (1979), to calculate the real degradability using the following equation:

$$\mathbf{De} = a + (b \times c)/c + k$$

In which:
De = Real degradability of the nutritive component.
k = degradability rate at 2, 6 and 8% per hour

The effective degradability represents the amount of each nutrient (DM, CP and NDF) analyzed that will be degraded in the rumen. Using the previous equation, it was possible to determine the degradation rate of nutrients, which is the rate that DM or CP are potentially degradable in the rumen considering the coefficient "c" previously calculated.

Determination of Urinary Parameters

For the calculation of the production of microbial protein, the urinary volume was determined through creatinine in the urine, according to the methodology described by Valadares et al. (1999). Urine was collected once a day during five days at 8 a.m and 4 p.m by means spontaneous urination or stimulation by vulva massage. At each collection time, 10 mL of urine were taken and preserved in 40 mL of 0.036 N sulfuric acid. Samples were stored at -20ºC for analysis of allantoin, uric acid, urea, and creatinine.

Allantoin was determined according to the colorimetric method described by Chen and Gomes (1992). The uric acid was determined by colorimetric enzymatic reaction with Uricase and Peroxidase, through commercial kit (Bioclin® Ref K139). The concentrations of urea and creatinine were determined by using commercial kits (Bioclin® Ref K047 and Bioclin® Ref K067, respectively), through the colorimetric enzymatic reaction and reaction with Alkaline Picrate in buffered medium, respectively. The daily urinary creatinine excretion (CE) was estimated in relation to animal body weight (BW) in kg using the equation proposed by Chizzotti et al. (2004):

$$\textbf{CE (mg/kg BW/d)} = 0.0345 * EBW^{0.9491}$$

The daily total urinary volume (L/cow) was determined by dividing the daily urinary creatinine excretion by the observed values of urinary creatinine concentration (mg/dL) of the spot samples. This volume was used to calculate the estimated daily excretions of urea, allantoin and uric acid from each cow. The excretion of purine derivatives (PuD) was calculated by multiplying the daily urine volume by the concentration of PuD in the urine sample. The absorbed microbial purines (AP, mmol/day) were calculated from the excretion of purine derivatives in urine (PuD, mmol/day) using the following equation:

$$\textbf{PuD} = (0.85 * AP) + (0.385 * BW)$$

Where:

PuD = purine derivatives;

AP = absorbed microbial purines;

0.85 = recovery of purines absorbed as urinary derivatives of purines;

0.385*BW = excretion of purines of endogenous origin per kg of weight per day.

The intestinal flow of microbial nitrogen compounds (micN, g N/day) was calculated in relation to absorbed microbial purines (AP, mmol/day) using the equation described by Chen and Gomes (1992):

$$\textbf{micN} = (70 * AP)/(0.83 * 0.116 * 1000)$$

Where:

micN = microbial nitrogen.

AP = Absorbed microbial purines.

70 = N content in the purines (mg N/mmol).

0.83 = digestibility of microbial purines.

0.116 = ratio of purine N and total N of rumen microorganisms.

The efficiency of microbial N synthesis (EMNS) was calculated by the ratio between the production of microbial N (g) and the amount of digested organic matter (OM).

Statistical analysis

Data was statistically analyzed using the online version of the software Statistical Analysis Systems – OnDemand for Academics SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Previously to the statistical analysis, the data was assessed for the presence of disparate information ("outliers") and the normality assumption of the residuals was assessed by means the Shapiro-Wilk test. When the normality assumption was not accepted, the logarithmic or the square root transformation was applied.

Data was analyzed according to the mixed procedure (PROC MIXED), in which season was considered as repeated variable (split-plot in time). A total of 15 different covariance structures were tested, and the chosen one was based on the lower value of Corrected Akaike Information Criterion (AICC) (Wang and Goonewardene, 2004).

The model includes the effect of grazing method, nitrogen source, period of the year (Winter, Spring, Summer and Autumn) and the interaction between grazing method, nitrogen source and season of the year. The effects of block were considered as random factor.

$$Y_{ijkl} = u + b_i + g_j + n_k + (gn)_{jk}\, e_{(1)ijk} + s_l + (sg)_{lj} + (sn)_{lk} + (sgn)_{ljk}\, e_{(2)ljk}$$

Where:

Y_{ijkl}: experimental answer

u: Constant

b_i: Effect of the block

g_j: Effect of grazing

n_k: Effect of nitrogen source

$(gn)_{jk}$: Interaction effect of grazing and nitrogen source

$e(1)_{ijk}$: Random error

s_l: Effect of season

$(sg)_{lj}$: Interaction effect of season and grazing

$(sn)_{lk}$: Interaction effect of season and nitrogen source

$(sgn)_{ljk}$: Interaction effect of season, grazing and nitrogen source.

$e(2)_{ljk}$: Random error

In the presence of interaction, effects of one factor inside the other were evaluated using the SLICE command of Mixed Procedure. All means were presented as least squares means and statistical differences by treatment effects were obtained by pairwise difference test (PDIFF) using the Fisher test considering a significance of $P \leq 0.05$.

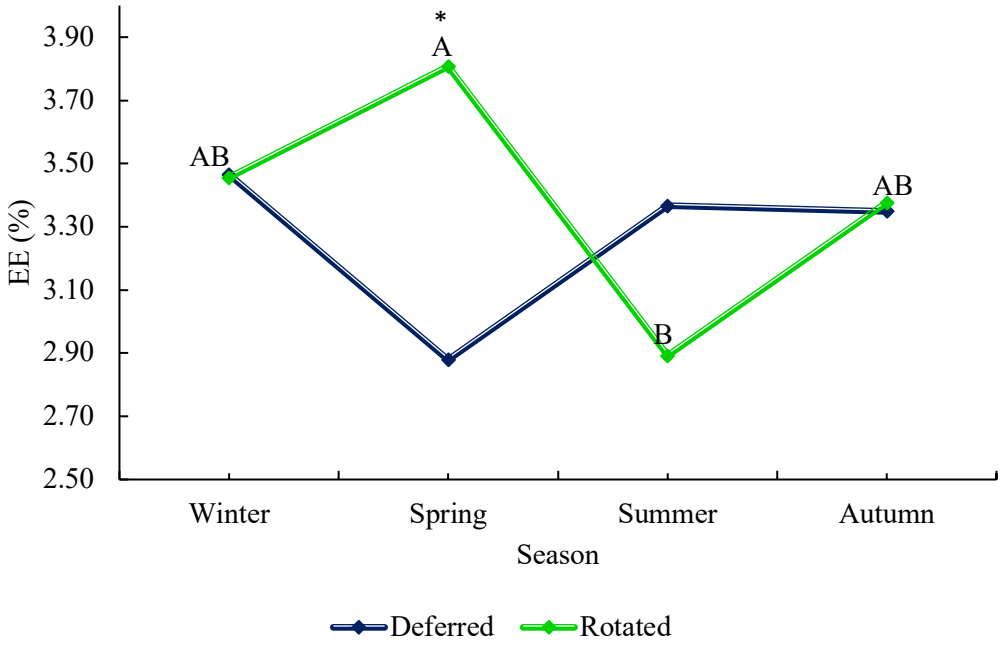
Results

Dry matter intake and apparent digestibility coefficients

Using the simulation technique to perform the chemical characterization of forage we were able to observe that TDN (%) was the only variable affected by treatment while EE (%) had an interaction effect which is shown in the Figure 8. Pastures from rotational grazing method has on average 3.29% more TDN (%), while in the interaction effect of grazing and season for the EE (%) concentration, higher values (+24%) was detected in rotated pastures within Spring season. No major effect was observed within the other seasons. Overall, as observed in the Table 4, the major effect upon most of the variable were from season.

Crude protein (CP) and Total digestible nutrients (%) concentrations were higher in Summer coupled with lower concentrations of ADF and Lignin (%) when compared to the dry season, Winter. The in vitro digestibility of the dry mater (IVDM) follows the pattern of the main chemical characteristics of the forage and displayed greater digestibility in Summer and lower in Winter as seen in Table 4.

Figure 8 - Interaction effect of grazing systems and season on EE (%) pasture composition by means simulation grazing during different seasons over two years.



Capital letters within grazing methods differs at P<0.05; * indicates statistical difference within season at P<0.05

Table 4 - Forage chemical composition of forage canopy during the experimental period by season and grazing methods

Fixed Effects			Variables									
Grazing	**N source**	**Season**	**CP (%)**	**NDF (%)**	**ADF (%)**	**CF (%)**	**LIG (%)**	**EE (%)**	**MM (%)**	**IVDM (%)**	**NFC (%)**	**CE (%)**	**TDN (%)**
Deferred			10.97	65.45	33.06	27.96	2.51	3.32	10.10	76.05	10.50	18.16	68.11
Rotated			11.09	64.41	34.16	28.62	3.08	3.28	9.99	74.55	10.46	18.22	70.43
	Nitrate		10.98	65.01	33.33	28.18	2.70	3.33	10.21	75.90	10.50	18.15	69.82
	Urea		11.08	64.86	33.89	28.41	2.89	3.27	9.88	74.70	10.46	18.23	68.72
		Winter	9.54^{c}	66.56^{a}	36.39^{a}	30.06^{a}	3.99^{a}	3.43	9.83^{bc}	70.65^{c}	10.47^{b}	17.95^{b}	65.39^{b}
		Spring	10.69^{b}	66.81^{a}	34.21^{b}	28.81^{b}	3.54^{a}	3.32	9.60^{c}	66.59^{d}	9.43^{c}	18.17^{a}	67.55^{b}
		Summer	10.78^{b}	64.20^{b}	31.81^{c}	27.76^{c}	1.25^{c}	3.10	10.62^{a}	84.41^{a}	11.14^{a}	18.27^{a}	71.82^{a}
		Autumn	13.10^{a}	62.15^{c}	32.03^{c}	26.54^{d}	2.40^{b}	3.35	10.13^{b}	79.56^{b}	10.87^{a}	18.37^{a}	72.33^{a}
Average data													
	Average		10.89	65.36	33.61	28.29	2.80	3.33	10.05	75.30	10.48	18.18	69.27
	SEM		0.24	0.33	0.35	0.24	0.18	0.10	0.09	1.07	0.13	0.05	0.73
Statistic Probabilities (p-value)													
Grazing			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.0097
N source			NS	NS	NS	NS	NS	NS	0.0283	NS	NS	NS	NS
Season			<.0001	<.0001	0.0001	<.0001	<.0001	NS	<.0001	<.0001	0.0003	0.0007	<.0001
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	0.0447	NS	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source, CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; CF: crude fiber; EE: ether extract, MM: mineral mater, IVDM: In vitro digestibility of dry mater, NFC: non-fiber carbohydrates, GE: gross energy, TDN: total digestible energy. SEM: standard error of the mean, NS: not significant.

No effect was detected for grazing method or nitrogen source for all the feed intake variables, as seen in the Table 5, except for NPN intake.

Animals kept in rotated grazing pastures had supplement NPN intake (kg/day) of 32.5% higher than those kept in deferred grazing (Table 5). We also identified that animals fed nitrate had NPN intake of 54 (g/day) while animals were supplemented with to urea the NPN intake was of 80 (g/day).

DMI of forage (kg/day) had only effect of season in which higher intake was observed in Summer (Table 5), moment which forage had lower concentration of ADF (-19.3%), lignin (-68.6%), higher concentration of NFC (+ 6%) and TDN (+ 9.05%) when compared to the dry season (Table 4). Coupled with the botanical composition of the forage over Summer, the digestibility of it was also higher for the variable NDF and OM (Table 6), which certainly influence in higher nutrient intake of NDF, NFC, TDN, OM and GE in the Summer season, as observed in the Table 7.

Dry matter intake of the forage showed to be statically higher over Summer (9.26 kg/day), as already expected, and it decreased 5.18% in the following season (Autumn) despite of no statistical difference among both seasons. Same trend was noticed for DMI of forage by LBW. Lowest DMI of the supplement by kg/day and LBW was revealed for Summer (0.35 and 0.06), while no major effect was detected among the other seasons.

Total dry matter intake also displayed effect of season and it was possible to see that Winter and Spring had the lowest total DMI (kg/day), being 8.41 and 10.97% lower than the group average data.

Table 5 - Forage, supplement, and total dry matter intake from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed effects			Variables							
Grazing	N Source	Season	DMI_F		DMI_S		DMI_T		NPN	
			(kg/day)	(%LBW)	(kg/day)	(%LBW)	(kg/day)	(%LBW)	(kg/day)	(%LBW)
Deferred			7.46	1.22	0.39	0.07	8.06	1.28	0.054	0.009
Rotated			7.43	1.25	0.63	0.10	8.03	1.42	0.080	0.013
	Nitrate		7.64	1.27	0.41	0.07	8.14	1.34	0.045	0.007
	Urea		7.25	1.19	0.60	0.10	7.95	1.37	0.089	0.014
		Winter	5.98[b]	1.06[b]	0.52[a]	0.09[a]	6.84[b]	1.21[b]	0.071	0.012
		Spring	5.76[b]	0.98[b]	0.55[a]	0.09[a]	6.28[b]	1.12[b]	0.074	0.012
		Summer	9.26[a]	1.53[a]	0.34[b]	0.05[b]	9.59[a]	1.61[a]	0.045	0.007
		Autumn	8.78[a]	1.35[a]	0.62[a]	0.10[a]	9.47[a]	1.48[a]	0.078	0.013
Average data										
	Average		7.68	1.29	0.50	0.08	8.20	1.34	0.232	0.038
	SEM		0.312	0.052	0.050	0.008	0.317	0.049	0.028	0.004
Statistics Probabilities (p-value)										
Grazing			NS	NS	NS	NS	NS	NS	0.0429	0.0425
N source			NS	NS	NS	NS	NS	NS	0.0008	0.0005
Season			<.0001	<.0001	0.0163	0.0179	<.0001	<.0001	NS	NS
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	NS
N source x Season			0.0299	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

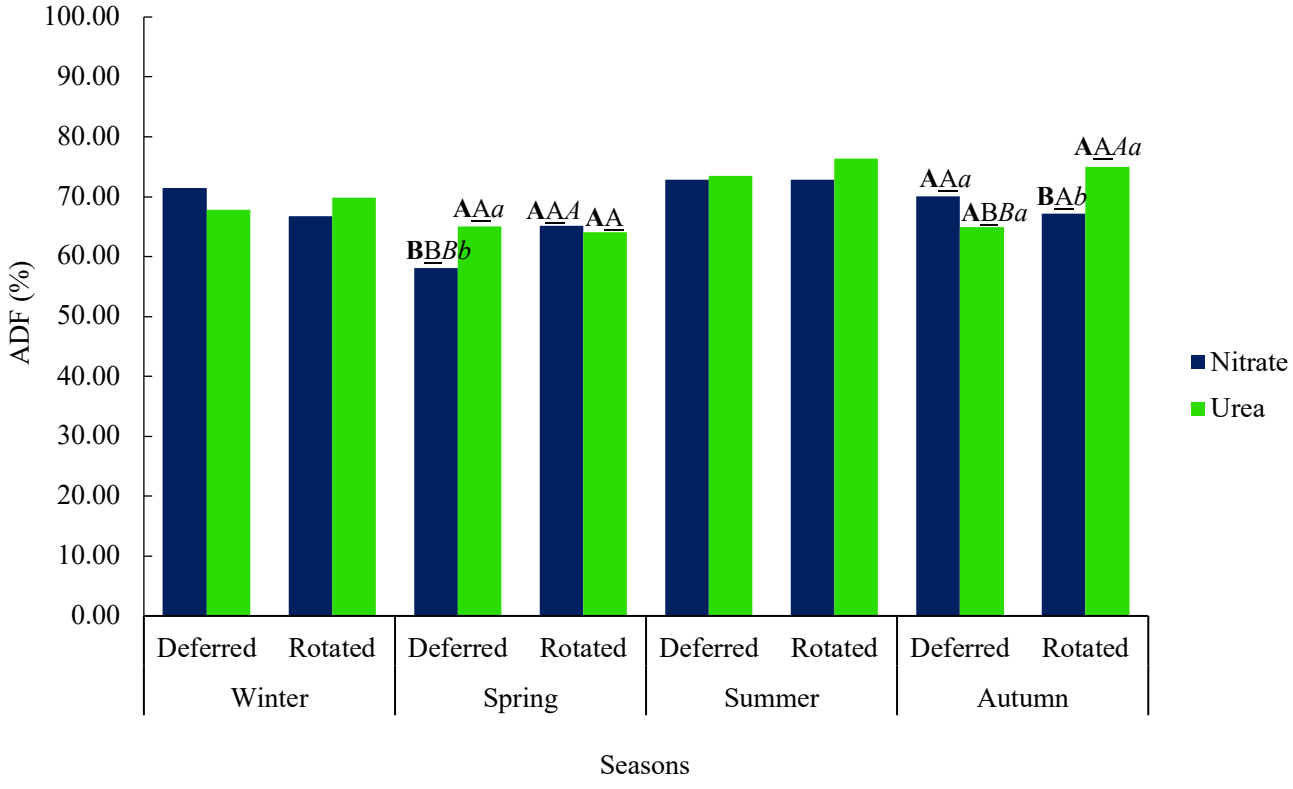
[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; DMIF: dry mater intake of forage, DMIS: dry mater intake of supplement, DIMT: total dry mater intake, NPN: equivalent non-protein nitrogen, LBW: live body weight, NS: not significant.

Digestibility, intake, and excretion of nutrients

When it comes to the total apparent digestibility, on Table 6, it can be observed that digestibility coefficients were heavily influenced by season and few variables had treatment or interaction effects. Diet DM apparent digestibility (%) was statistically higher during Summer and Autumn when compared to Winter and Spring. For the variable CP (%), no difference among Winter, Spring and Summer was detected, but higher values were noticed for the apparent digestibility of CP over Autumn, as seen on Table 6.

The apparent digestibility of NDF (%) was influence by the grazing method as it shows on Table 6. Rotated grazing method increased the digestibility of the NDF (%) when contrasting it to deferred grazing. Same trend was noticed for the variable EE (%), in which higher digestibility coefficient was detected for rotated grazing method. Apparent digestibility of ADF (%) had interaction effect for nitrogen source, grazing and season, and, as it is shown in the figure 9, the main effects among treatments were concentrated on Spring and Autumn.

Figure 9 - Interaction effect of nitrogen source, grazing method, and season for ADF digestibility (%) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Different capital bold letters (**A**) within season indicate difference between nitrogen source at P<0.05; Different underlined capital letters (A) within season indicate difference between grazing method at P<0.05; Different Italic capital letter (*A*) within nitrogen source indicate difference between grazing method within season at P<0.05;

Different italic lowercase letter (*a*) within grazing method indicates difference between nitrogen source within season at P<0.05.

Urea influenced in higher ADF (%) digestibility withing deferred grazing during Spring when compared to the use of nitrate; however, in the same season when unfolding the interaction within nitrate, higher digestibility was noticed when animals were in rotated grazing method as compared to deferred.

On the other hand, when unfolding the interaction within grazing method contrasting both nitrogen sources during Autumn, it was found significative effect for rotated grazing, which had higher ADF (%) digestibility coefficient when animals were supplemented with urea. No major effect was found within deferred grazing as seen on the Figure 9.

Analyzing the data within supplementation of nitrate, it was identified a significant effect for grazing during Spring over the variable ADF (%) with higher digestibility from animals under rotated grazing. Rotated grazing also influenced in higher ADF (%) digestibility during Autumn when animals were supplemented with urea. If compared the nitrogen sources within the deferred and rotated grazing method, nitrate influenced in lower ADF (%) digestibility during Spring and Autumn.

Ether extract (%) digestibility displayed and interaction effect for grazing and season, as depicted in the Figure 10. Higher digestibility of EE (%) was observed during Spring (75.80%) and Autumn (66.28%) in rotated grazing system, with average values being 12.6 and 5.26% higher in rotated grazing systems, respectively, when contrasted to deferred grazing.

NFC, TDN and OM (%) displayed season effect and it was clear that higher availability of rain associated with luminosity influence the digestibility of the diet since higher values were detected in Summer and Autumn when compared to Winter and Spring. gross energy digestibility (%), despite of the season effect, also had grazing method effect and as seen on Table 6, rotated grazing method had higher apparent digestibility coefficient (73.05%) of the diet when compared to deferred (70.91%).

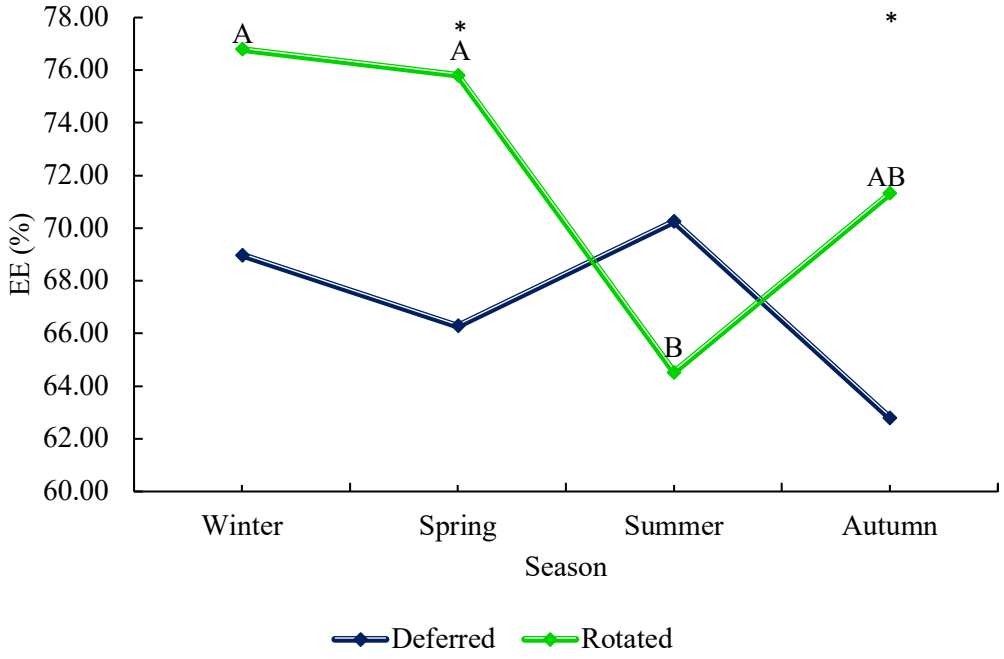
No treatment effect was detected for nutrient intake (kg) as seen in the table 6; however, season heavily influenced the intake of all nutrients with higher intake on Autumn and Summer, and lower during Winter and Spring. When it comes to nutrient excretion, the variable ADF (kg) had interaction effect for grazing method and season, and its decomposition is depicted on the Figure 11. Higher ADF excretion was observed for animals under deferred grazing systems within Spring, while no effect was detected contrasting treatments within the other seasons.

Table 6 - Digestibility coefficients (%) of DM and nutrients from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed effects			Digestibility Coefficient								
Grazing	**N Source**	**Season**	**DM (%)**	**CP (%)**	**NDF (%)**	**ADF (%)**	**EE (%)**	**NFC (%)**	**TDN (%)**	**MO (%)**	**GE (%)**
Deferred			70.96	77.35	71.34	67.98	67.08	76.70	74.62	74.50	70.91
Rotated			72.77	78.1	73.94	69.64	72.11	76.92	76.35	75.87	73.05
	Nitrate		72.43	77.88	72.95	68.04	70.62	78.28	76.19	75.71	72.65
	Urea		71.30	77.57	72.33	69.57	68.56	75.35	74.78	74.67	71.31
		Winter	67.33^{b}	74.03^{b}	67.67	68.98	72.88	74.16^{b}	71.82^{b}	71.24^{b}	67.02^{b}
		Spring	69.24^{b}	77.04^{b}	71.26	63.07	71.04	65.53^{c}	73.21^{b}	72.97^{b}	69.25^{b}
		Summer	75.55^{a}	77.09^{b}	77.34	73.89	67.39	81.37^{a}	78.26^{a}	78.33^{a}	75.90^{a}
		Autumn	75.34^{a}	82.74^{a}	74.30	69.28	67.06	86.19^{a}	78.65^{a}	78.21^{a}	75.74^{a}
Average Data											
	Average		71.6	77.55	72.42	68.73	69.38	76.30	75.33	74.98	71.65
	SEM		0.79	0.75	0.84	0.96	1.30	1.58	0.66	0.68	0.88
Statistics Probabilities (*p*-value)											
Grazing			NS	NS	0.0099	NS	0.0171	NS	NS	NS	0.0498
N source			NS	NS	NS	NS	NS	NS	NS	NS	NS
Season			<.0001	<.0001	<.0001	<0.001	NS	<.0001	<.0001	<.0001	<.0001
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	0.0029	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	0.0249	NS	NS	NS	NS	NS

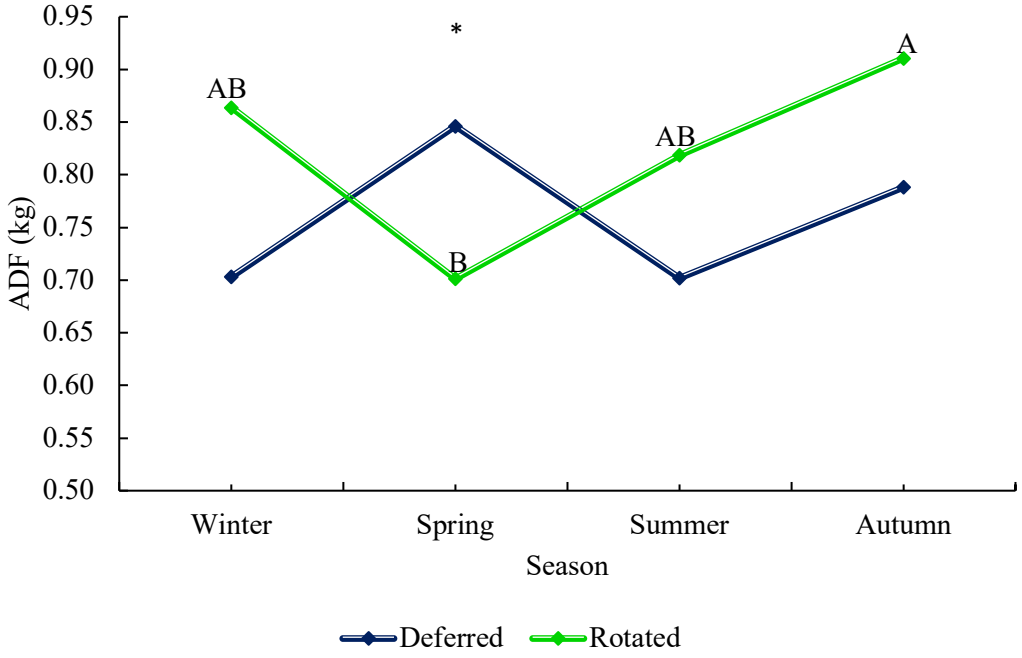
[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber, ADF: acid detergent fiber; EE: ether extract; NFC: non-fibrous carbohydrate; TDN: total digestible nutrients, OM: organic matter; GE: gross energy, NS: not significant.

Figure 10 - Interaction effect of grazing method and season for EE digestibility (%) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing methods differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 11 - Interaction effect of nitrogen source, grazing method and season for ADF excretion (kg) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing methods differs at P<0.05; * indicates statistical difference within season at P<0.05

Table 7 - Nutrients intake (kg) of diet from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed effects			Nutrient intake								
Grazing	**N Source**	**Season**	**DM (kg)**	**CP (kg)**	**NDF (kg)**	**ADF (kg)**	**EE (kg)**	**NFC (kg)**	**TDN (kg)**	**OM (kg)**	**GE (kg)**
Deferred			8.04	1.04	5.00	2.52	0.26	0.92	5.24	7.19	1.43
Rotated			8.04	1.07	4.91	2.52	0.26	0.95	5.52	7.19	1.42
	Nitrate		8.14	1.03	5.04	2.58	0.26	0.94	5.39	7.28	1.45
	Urea		7.94	1.07	4.86	2.46	0.25	0.93	5.37	7.10	1.40
		Winter	6.85[b]	0.83[c]	4.29[b]	2.35[b]	0.23[b]	0.79[b]	4.23[b]	6.15[b]	1.19[b]
		Spring	6.23[b]	0.82[c]	3.93[b]	1.95[b]	0.20[b]	0.69[b]	3.85[b]	5.59[b]	1.13[b]
		Summer	9.60[a]	1.13[b]	6.01[a]	2.95[a]	0.29[a]	1.12[a]	6.76[a]	8.55[a]	1.74[a]
		Autumn	9.47[a]	1.43[a]	5.58[a]	2.83[a]	0.31[a]	1.14[a]	6.69[a]	8.46[a]	1.64[a]
Average data											
	Average		8.28	1.06	4.99	2.54	0.26	0.95	5.45	7.26	1.43
	SEM		0.32	0.05	0.18	0.09	0.01	0.04	0.26	0.27	0.06
Statistics Probabilities (*p*-value)											
Grazing			NS	NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS	NS
Season			<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber, ADF: acid detergent fiber; EE: ether extract; NFC: non-fibrous carbohydrate; TDN: total digestible nutrients, OM: organic matter; GE: gross energy, NS: not significant.

Table 8 - Excretion of nutrients (kg) from Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed effects			Nutrient excretion							
Grazing	**N Source**	**Season**	**DM (kg)**	**CP (kg)**	**NDF (kg)**	**ADF (kg)**	**EE (kg)**	**NFC (kg)**	**OM (kg)**	**GE (kg)**
Deferred			2.15	0.22	1.29	0.76	0.08	0.20	1.8	0.39
Rotated			2.39	0.24	1.41	0.82	0.07	0.20	1.65	0.39
	Nitrate		2.13	0.21	1.29	0.78	0.07	0.19	1.68	0.37
	Urea		2.40	0.25	1.41	0.80	0.08	0.22	1.77	0.40
		Winter	2.39^{a}	0.23^{b}	1.47^{a}	0.78	0.05^{b}	0.21^{a}	1.83^{a}	0.41^{b}
		Spring	2.06^{b}	0.20^{b}	1.20^{b}	0.77	0.06^{b}	0.24^{a}	1.55^{b}	0.35^{b}
		Summer	2.34^{a}	0.26^{a}	1.36^{ab}	0.76	0.09^{a}	0.22^{a}	1.77^{a}	0.42^{a}
		Autumn	2.27^{b}	0.24^{a}	1.37^{a}	0.85	0.10^{a}	0.15^{b}	1.74^{ab}	0.37^{a}
Average data										
	Average		2.2	0.22	1.33	0.78	0.07	0.20	1.74	0.38
	SEM		0.06	0.01	0.04	0.03	0	0.01	0.05	0.01
Statistics Probabilities (p-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			0.0159	0.0341	0.0245	NS	<.0001	0.0114	0.026	0.0234
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	0.0346
Grazing x Season			NS	NS	NS	0.0271	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

^{a,b,c} Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; DM. DM: dry matter; CP: crude protein; NDF: neutral detergent fiber, ADF: acid detergent fiber; EE: ether extract; NFC: non-fibrous carbohydrate; OM: organic matter; GE: gross energy, NS: not significant.

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Interaction effect of grazing and nitrogen source was also detected for the excretion of GE (kg) as shown in the Table 7. However, when it was unfolded, no significative statistical effect was observed. The variables DM, CP, NDF, EE, NFC, TDN, OM and GE (kg), all had significative effect for season, with higher excretion values detected over the Summer, for all the variables.

Rumen Kinetics

Ruminal disappearance rate

As observed in the decomposition of the interaction (season x grazing) for the variable ruminal liquid mass per kg (Table 9), it was identified that animals supplemented with urea within Winter and Spring had greater liquid mass (15.9 and 18.7%, respectively) as compared to animals supplemented with nitrate (Figure 12). Similar trend was detected for the variable luminal liquid mass per LBW, and for the variable total rumen mass (kg) as seen depicted in the Figures 14 and 19.

Differently from what was observed for the liquid fraction, ruminal solid mass had effect of grazing within Summer season. As it is seen in the decomposition depicted in the Figure 16, animals kept in rotated grazing had greater content of solid luminal mass (+26.3%) as opposed to animals in deferred grazing method. Similar trend was observed for ruminal solid mass per LBW. Besides the previous interaction there was also an interaction of nitrogen source and season, which when unfolded, it was possible to see that animals supplemented with urea had greater rumen solid content within Spring (Figure 17).

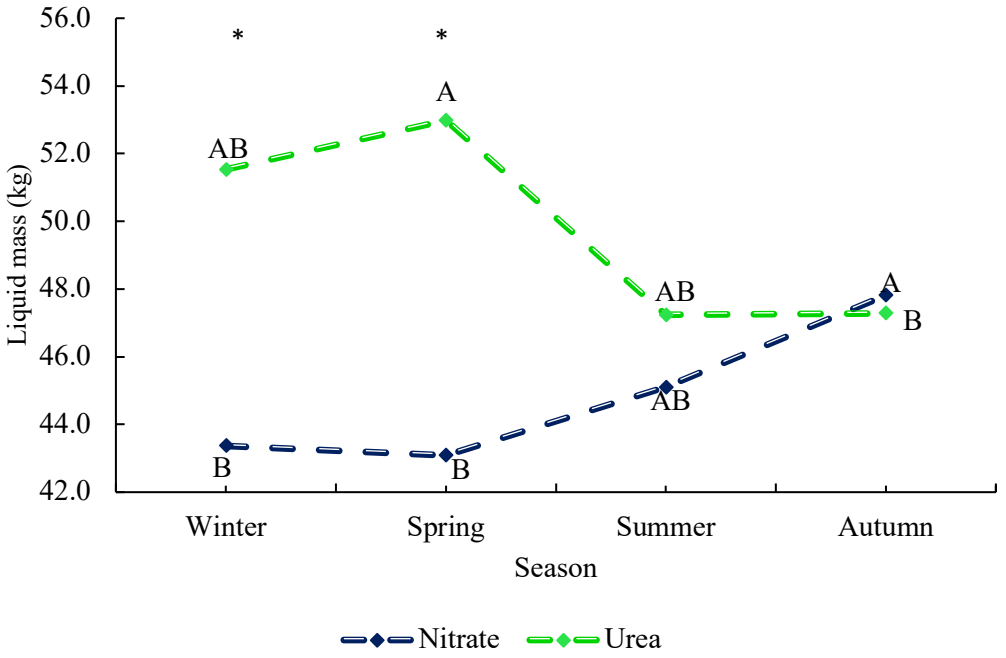
Disappearance rate by kg/h did indicate interaction effect for nitrogen and season of the year; however, when the interaction was decomposed no effect of treatment was observed. Despite of that, as seen on the Table 9, rumen disappearance rate by %/h and kg/h showed statistical effect of season, in which higher disappearance rate were observed during Summer and Autumn.

Table 9 - Rumen dynamics of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Fixed effects			Variables							
Grazing	N Source	Season	Liquid Mass		Solid Mass		Total Mass		Disappearance	
			(kg)	(% BW^{-1})	(kg)	(% BW)	(kg)	(% BW)	(%/h)	(kg/h)
Deferred			46.94	7.71	6.60	1.12	53.68	8.81	5.44	0.34
Rotated			47.65	8.23	7.12	1.27	55.11	9.44	5.12	0.33
	Nitrate		44.84	7.49	6.54	1.14	51.35	8.61	5.21	0.33
	Urea		49.76	8.44	7.18	1.24	57.44	9.64	5.36	0.34
		Winter	47.44	8.36	6.88	1.25	54.56	9.61	4.59^{b}	0.30^{b}
		Spring	48.03	8.35	7.14	1.27	55.25	9.50	4.04^{b}	0.27^{b}
		Summer	46.16	7.59	6.63	1.12	53.32	8.71	6.17^{a}	0.40^{a}
		Autumn	47.55	7.57	6.79	1.13	54.44	8.68	6.33^{a}	0.38^{a}
Average data										
	Average		47.32	7.91	7.01	1.17	54.34	9.08	5.30	0.26
	SEM		1.010	0.159	0.214	0.035	1.197	0.190	0.341	0.012
Statistic Probabilities (p-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			NS	0.0029	NS	0.0245	NS	0.0033	<.0001	<.0001
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			0.0114	0.0134	0.0002	0.0007	0.0005	0.0066	NS	NS
N source x Season			0.0027	0.0003	0.0031	0.0239	0.0045	0.0003	NS	0.0332
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

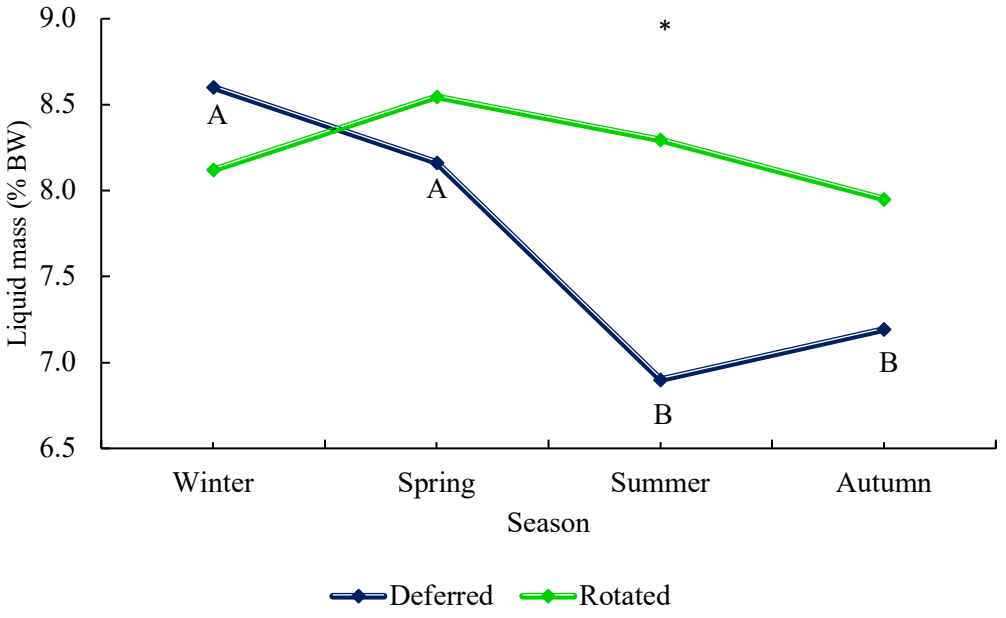
[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; BW: body weight, %/h: percentage per hour; kg/h: kilogram per hour; NS: not significant.

Figure 12 - Interaction effect of season and nitrogen source on ruminal liquid mass (kg) from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



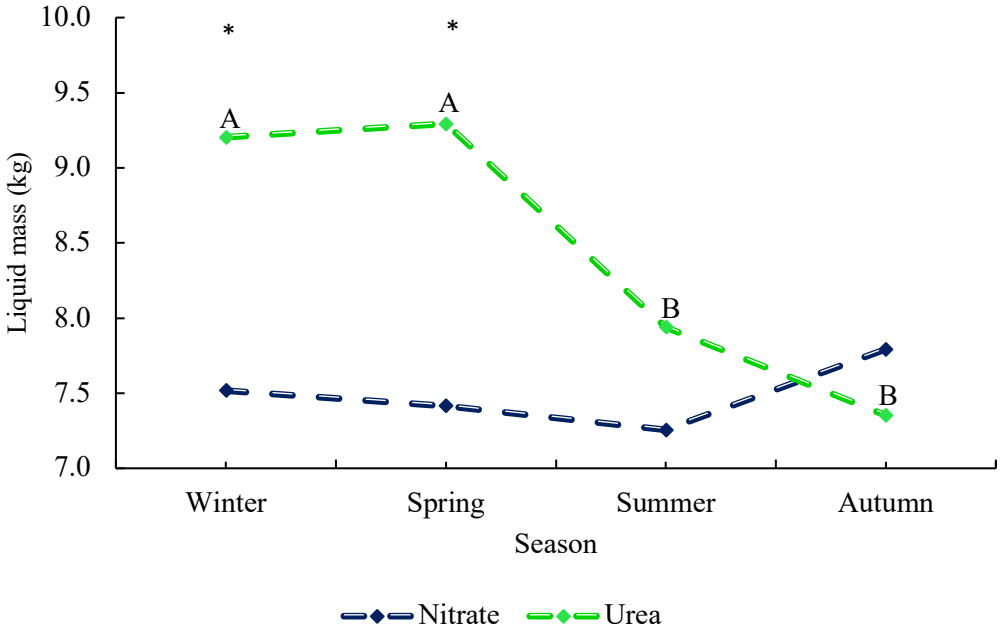
Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 13 - Interaction effect of season and grazing method on ruminal liquid mass per live body weight (kg) from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



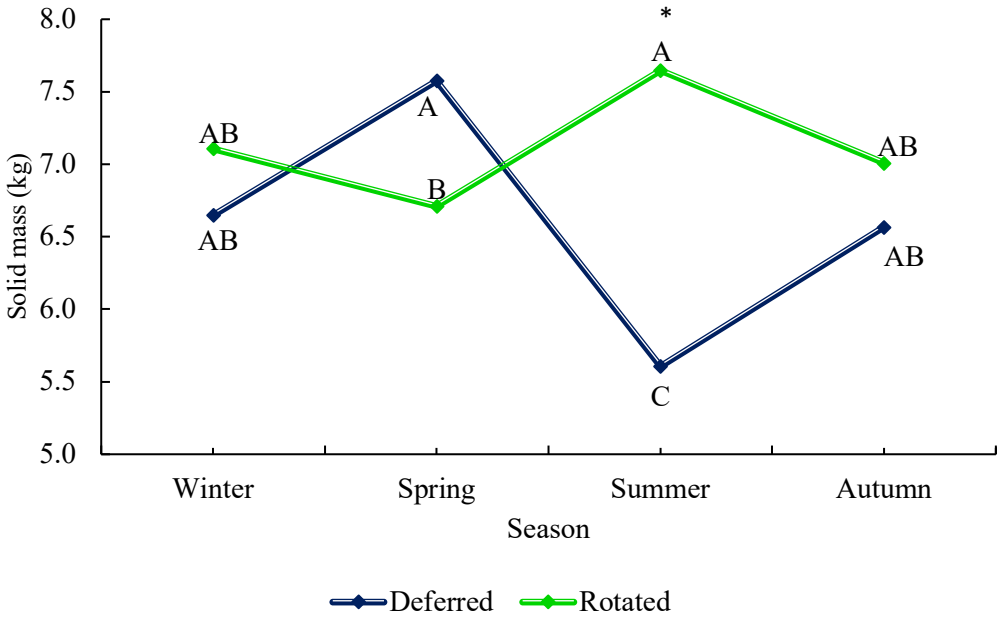
Capital letters within grazing method differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 14 - Interaction effect of season and nitrogen source on ruminal liquid mass (kg) from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



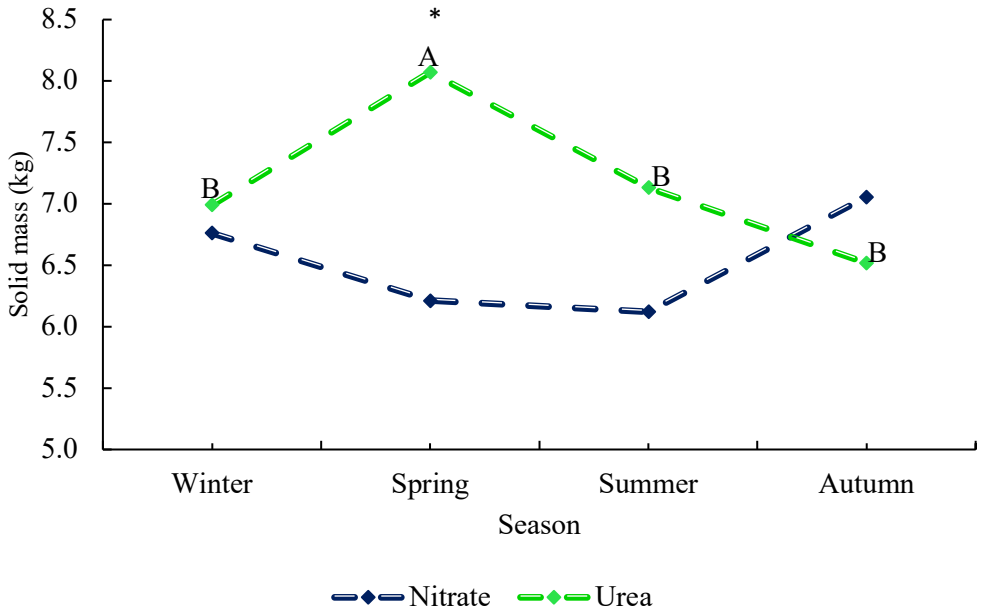
Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 15 - Interaction effect of season and grazing method on ruminal solid mass (kg) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



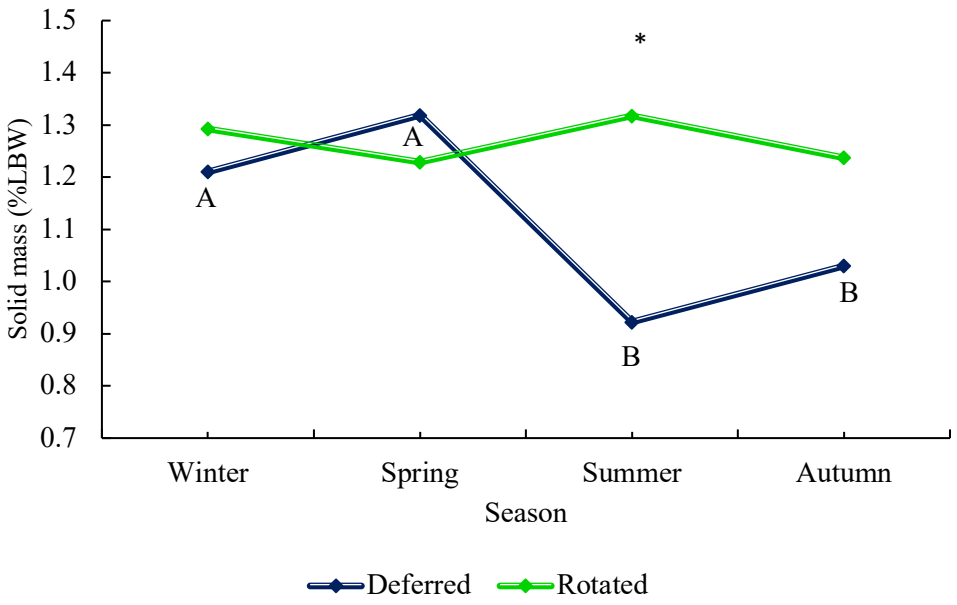
Capital letters within grazing method systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 16 - Interaction effect of season and nitrogen source on ruminal solid mass (kg) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



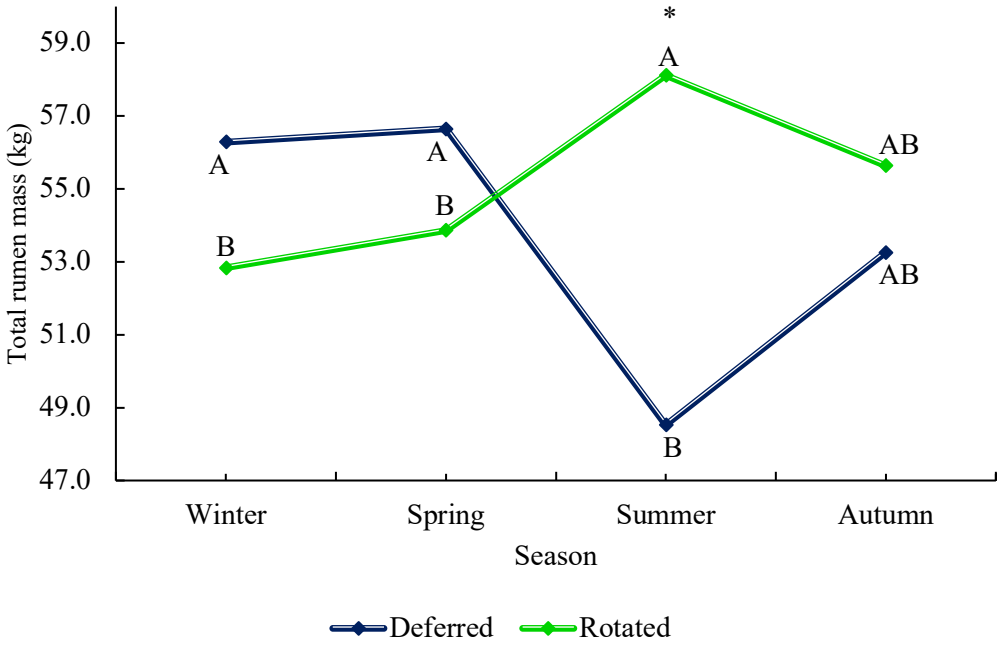
Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 17 - Interaction effect of season and grazing method on ruminal solid mass per % of live body weight from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



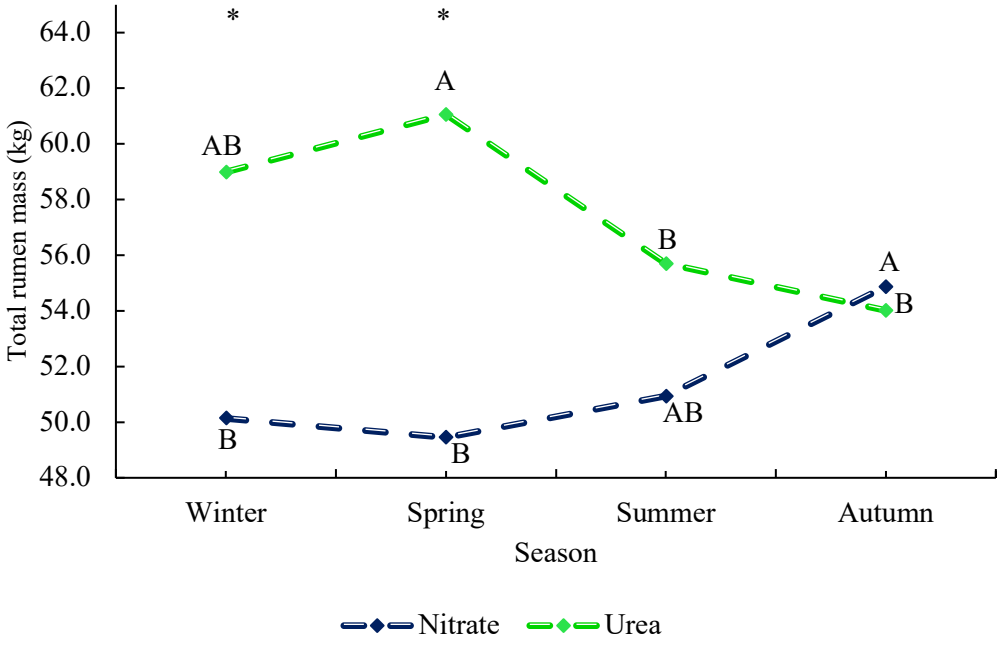
Capital letters within grazing method differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 18 - Interaction effect of season and grazing method on total ruminal mass (kg from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 19 - Interaction effect of season and nitrogen source on total ruminal mass (kg) from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within nitrogen source differs at P<0.05; * indicates statistical difference within season at P<0.05

Degradability parameters (DM, CP and NDF)

As seen on table 10, dry matter degradability parameters were mainly affected by season effect, with interaction effect for only two variables (PD and UnD %).

While the fraction a (%) displayed lower values on Summer, the fraction b had higher mean values during Summer and Autumn, and lower during Winter and Spring (Table 10). The rate in which the feed was effectively degradable per hour was not affected by treatment or interaction; However, it showed statistical effect for season with lower values during Summer. But despite of that, the average rate in which fraction b from all seasons were completely degradable in the rumen was at 6.0% per h^{-1}.

The potential degradability had interaction effect for nitrogen source and season and its decomposition is shown in the Figure 20. Animals supplemented with nitrate within Spring season displayed a PD (%) average value 6% higher than that observed for animals fed urea as the main source of non-protein nitrogen. Following a similar trend, the De2% of CP had also higher effective degradability when animals were fed nitrate within Spring (Figure 22), with no major change in the other seasons.

When it comes to the effect of season for degradability parameters of CP it was possible to see that fraction a and b showed lower values in Summer and Spring, and the parameters c (h^{-1}) had lower values in during Summer.

When evaluated the effective degradability at 2, 5 or 8% of passage rate, it was found statistical difference only at 2% from season effect, in which higher mean values in Summer and Autumn as opposed to Winter and Spring. The undegradable fraction was mainly affected by season with higher undegradable values detected on Winter, Spring, and Summer, and lower undegradable fraction in Autumn. Animals kept in rotated grazing had lower DM undegradable fraction within Autumn when compared to those in deferred grazing fraction (%).

For the nutrient crude protein (CP), shown in Table 11, degradability parameters were also mainly affected by season effect. Fraction a (%) displayed lower values on Summer and Autumn while fraction b had higher mean values during Summer and Autumn, and lower during Winter and Spring (Table 11). The lower rate in which the crude protein was effectively degradable per hour was detected from animals during Summer. The overall average rate in which fraction b was completely degradable in the rumen was of 3.5% per h^{-1}.

Crude protein (CP) rumen degradability rate had effect of season at 6 and 8% and as see on the table 11, lower values were observed over Summer while the highest De at 6 and 8% were observed on Winter and Spring.

NDF degradability had interaction effect of grazing and season for degradation rate of the potentially degradable fraction (h^{-1}) which is unfolded and depicted in the Figure 23. It was possible to understand that under rotated grazing, withing Spring season, the degradability rate (5.6%.h^{-1}) was 30.35% higher than that observed for animals under deferred grazing system of 3.9%.h^{-1}. This result is also a reflect of the high DM undigestible fraction present on deferred pastures within Spring as observed in the figure 21.

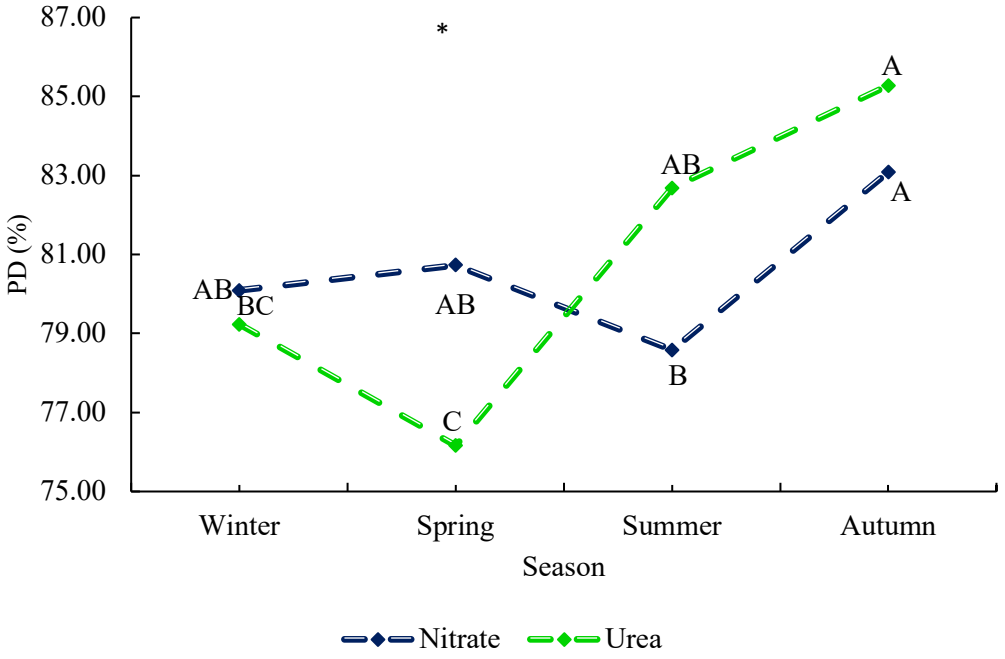
The NDF (%) content of deferred pastures showed higher potential degradability over Spring while within Autumn rotated grazing method displayed higher PD (%) as seen in the decomposition depicted in the Figure 24.

Table 10 - *In situ* degradability of DM of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Fixed effects			Variables							
Grazing	**N Source[1]**	**Season**	**a (%)**	**b (%)**	**c (h^{-1})**	**PD (%)**	**De2 (%)**	**De5 (%)**	**De8 (%)**	**Und (%)**
Deferred			26.36	52.65	0.07	80.62	66.50	55.62	49.74	19.42
Rotated			28.03	53.43	0.05	81.02	65.41	54.10	48.88	18.98
	Nitrate		27.45	52.82	0.07	80.76	66.53	55.70	49.77	19.24
	Urea		26.94	53.24	0.07	80.76	65.38	54.02	49.77	19.24
		Winter	29.81^{a}	50.14^{c}	0.04^{b}	79.66^{b}	63.74^{b}	52.76	47.50	20.34^{a}
		Spring	28.68^{ab}	49.16^{c}	0.06^{ab}	78.39^{b}	65.15^{b}	54.75	49.28	21.61^{a}
		Summer	23.88^{c}	55.10^{a}	0.09^{a}	80.68^{b}	66.04^{ab}	55.76	50.96	19.40^{a}
		Autumn	26.32^{b}	57.73^{b}	0.05^{b}	84.55^{a}	68.90^{a}	56.18	49.49	15.45^{b}
Average data										
	Average		27.02	53.56	0.066	80.95	66.19	55.25	49.45	19.04
	SEM		0.492	0.727	0.005	0.675	0.556	0.599	0.635	0.675
Statistics Probabilities (*p*-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			<.0001	<.0001	0.0188	0.0020	0.0020	NS	NS	0.0020
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	0.0108
N source x Season			NS	NS	NS	0.0380	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

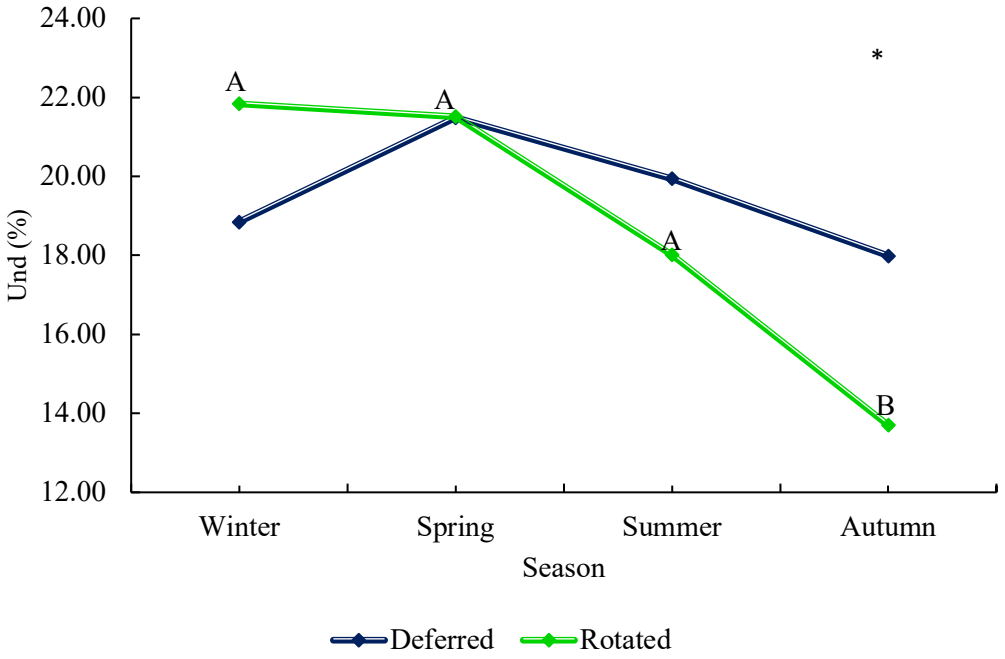
[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; **a:** Interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly; **b:** Potentially degradable fraction; **c:** Rate of degradation of the potentially degradable fraction; PD: Potential degradability (a + b); Und: Undigested fraction (100-PD). De2, De5 and De8% - rumen degradability rate. NS: not significant

Figure 20 - Interaction effect of season and nitrogen source on PD (%) of diet DM of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.



Capital letters within nitrogen source differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 21 - Interaction effect of season and grazing method on undegradable fraction of diet DM from of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.



Capital letters within grazing method differs at P<0.05; * indicates statistical difference within season at P<0.05

Table 11 - *In situ* degradability of CP of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Fixed effects			Variables							
Grazing	**N Source**	**Season**	**a (%)**	**b (%)**	**c (h^{-1})**	**PD (%)**	**De2 (%)**	**De5 (%)**	**De8 (%)**	**Und (%)**
Deferred			49.98	41.77	0.053	91.51	78.90	70.37	65.83	8.48
Rotated			51.16	43.80	0.048	90.81	79.72	70.88	66.46	9.71
	Nitrate		49.93	44.41	0.051	91.25	79.56	70.48	65.73	8.73
	Urea		51.21	41.17	0.050	91.08	79.05	70.76	66.56	9.46
		Winter	53.66^{a}	37.92^{b}	0.055^{a}	89.30^{b}	79.79	72.56^{a}	68.98^{a}	10.72^{a}
		Spring	55.29^{a}	38.61^{b}	0.058^{a}	90.47^{ab}	81.89	74.63^{a}	70.54^{a}	9.40^{ab}
		Summer	47.53^{b}	47.75^{a}	0.035^{b}	92.89^{a}	75.86	65.61^{c}	60.91^{c}	8.24^{b}
		Autumn	45.81^{b}	46.87^{a}	0.054^{a}	91.98^{a}	79.70	69.70^{b}	65.27^{b}	8.02^{b}
Average Data										
	Average		48.93	43.36	0.05	91.31	79.12	69.95	65.35	8.92
	SEM		1.36	1.30	0.003	0.59	0.52	0.77	0.89	0.58
Statistics Probabilities (*p*-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			0.0022	0.0104	<.0001	0.0075	<.0001	<.0001	<.0001	0.0286
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	0.0251	NS	NS	0.0359
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

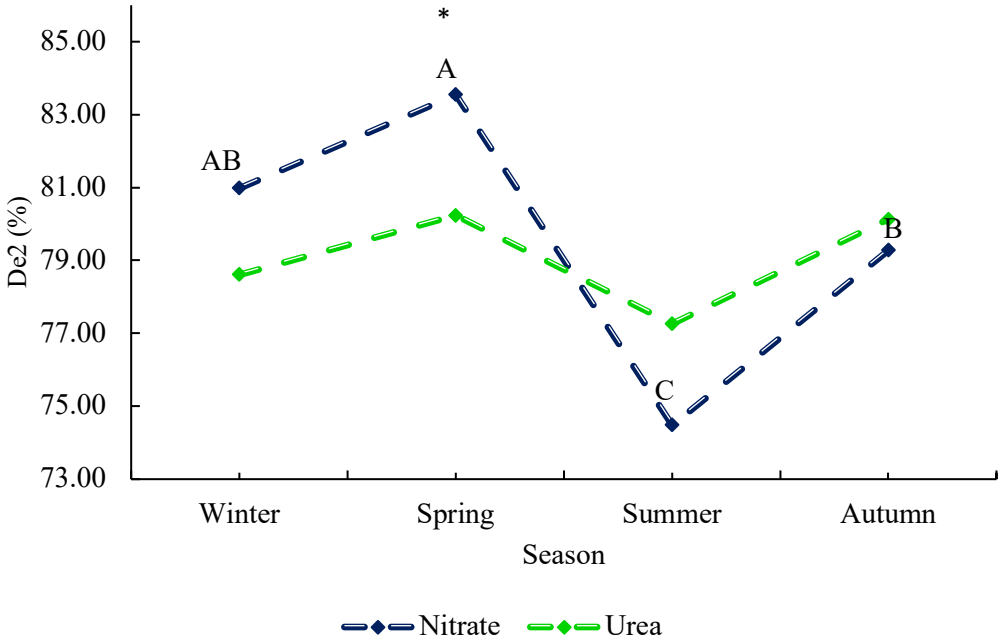
^{a,b,c} Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; **a:** Interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly; **b:** Potentially degradable fraction; **c:** Rate of degradation of the potentially degradable fraction; PD: Potential degradability (a + b); Und: Undigested fraction (100-PD). De2, De5 and De8% - rumen degradability rate, NS: not significant

Table 12 - *In situ* degradability of NDF of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed effects			Variables							
Grazing	**N Source**	**Season**	**a (%)**	**b (%)**	**c (h^{-1})**	**PD (%)**	**De2 (%)**	**De5 (%)**	**De8 (%)**	**Und (%)**
Deferred			11.99	68.37	0.046	80.60	59.54	45.27	37.31	18,96
Rotated			15.20	67.29	0.048	80.23	60.72	45.12	37.72	21.07
	Nitrate		12.84	67.34	0.048	79.73	59.99	45.48	37.53	20.33
	Urea		13.75	68.32	0.047	81.10	60.28	44.91	37.52	19.69
		Winter	20.25[a]	58.49[c]	0.044	78.90[b]	59.98	46.53	40.58	21.63
		Spring	16.43[b]	61.75[b]	0.047	75.94[b]	57.92	44.34	36.90	24.25
		Summer	10.38[c]	73.61[b]	0.045	84.31[a]	61.64	45.71	37.51	16.43
		Autumn	6.13[d]	77.47[a]	0.053	82.52[a]	61.00	44.19	35.10	17.74
Average Data										
	Average		12.82	68.30	0.046	80.89	59.98	45.19	37.74	19.42
	SEM		0.839	1.121	0.001	0.561	0.494	0.533	0.584	0.527
Statistic Probabilities (*p*-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			<.0001	<.0001	0.0003	<.0001	0.0167	NS	0.0008	<.0001
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	0.0034	0.0176	0.0385	0.0035	0.0221	0.0394
N source x Season			NS	NS	NS	NS	NS	NS	0.0226	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

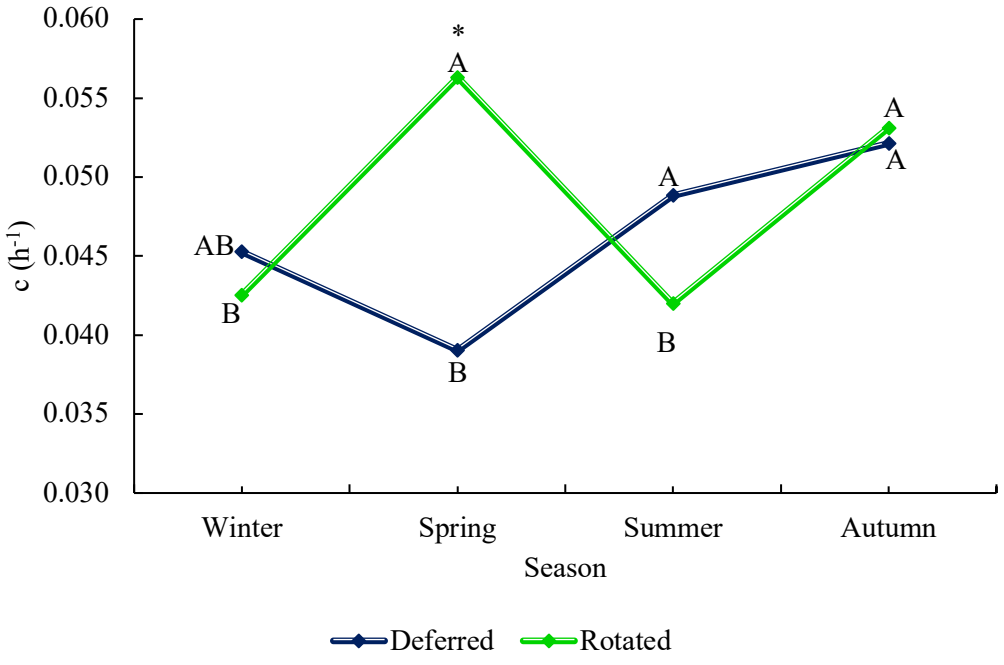
[a,b,c] Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; **a:** Interception of the curve at time zero, water-soluble and completely degradable fraction of analyzed nutritive component leaving the nylon bag rapidly; **b:** Potentially degradable fraction; **c:** Rate of degradation of the potentially degradable fraction; PD: Potential degradability (a + b); Und: Undigested fraction (100-PD). De2, De5 and De8% - rumen degradability rate, NS: not significant

Figure 22 - Interaction effect of season and nitrogen source on rate of CP rumen passage at De2% of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



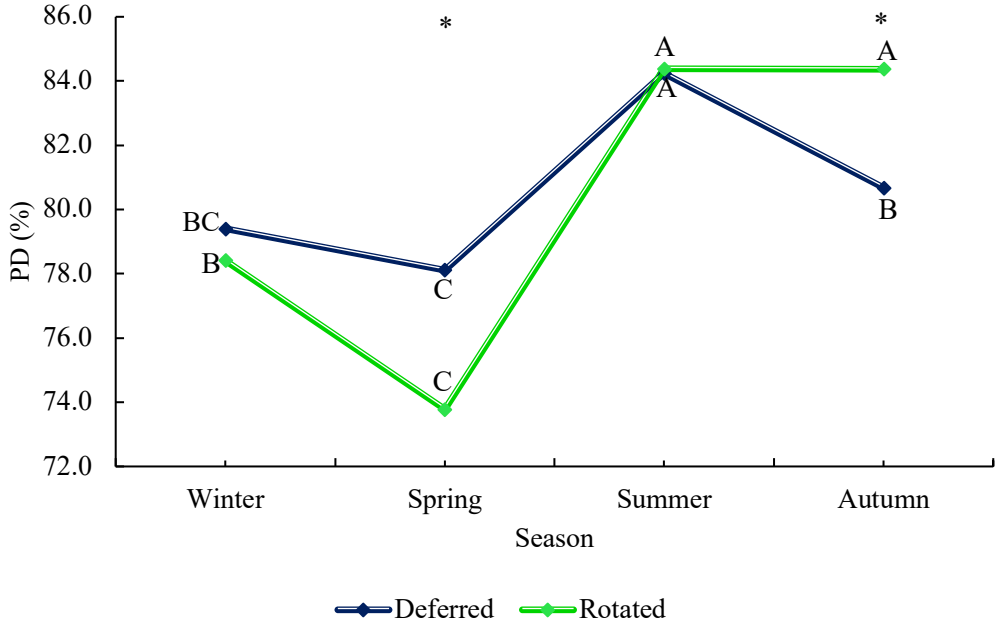
Capital letters within nitrogen source differs at P<0.05; * indicates statistical difference within season at P<0.05.

Figure 23 - Interaction effect of season and grazing method on rate of degradation of the NDF potentially degradable fraction (h^{-1}) of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing method differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 24 - Interaction effect of season and grazing method on PD (%) of diet NDF on rumen content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.



Capital letters within grazing method differs at P<0.05; * indicates statistical difference within season at P<0.05.

Ruminal microbial protein

When interaction effect of grazing and season for urine (L/day) was unfolded it was found difference of grazing system within season (Table 13). Season effect was noticed for UA (mmol/d), PuD (mmol/d), Al PuD (%) and for and EMNS (g/kg.OM). Lower UA (mmol/d) and PuD (mmol/d^{-1}) occurred over Winter and Spring while the highest excretion occurred in Summer and Autumn. The total PuD (mmol/d) represents the sum of allantoin and uric acid, allantoin by itself represents on around 97% of the total purine derivatives. Synthesis of microbial N (g/day) was 19.72% % higher on animals under deferred grazing method when compared to those kept under rotated grazing. EMNS also had grazing effect and following the pattern of synthesis of microbial N, the efficiency was also higher in animals under deferred grazing method when compared to rotated grazing.

EMNS also had season effect, and the highest efficiency of microbial protein synthesis by kg of digested organic matter were detect during Autumn (165.37 g/kg.OM) and Summer (160.62 g/kg.OM), which followed the same trend effect for synthesis of microbial N (g/day).

Table 13 - Urinary volume, excretion of its compounds and microbial nitrogen synthesis, and efficiency of microbial protein synthesis of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed Effects			Urine	Urinary compounds					Mic N	EMNS
Grazing	N Source	Season	(L/day)	Urea (g/day)	Al (mmol/d)	UA (mmol/d)	PuD (mmol/d)	Al PuD (%)	(g/day)	(g/kg OM)
Deferred			14.81	514.67	646.03	16.07	661.92	97.51	529.41	170.30
Rotated			13.52	402.73	525.27	14.94	540.53	97.66	424.93	136.70
	Nitrate		14.25	404.37	585.31	14.60	599.88	97.66	478.25	153.85
	Urea		14.09	513.03	585.99	16.42	602.57	97.51	476.06	153.15
		Winter	13.75	399.84	564.59	12.69^{b}	577.36^{bc}	97.94^{a}	461.51 ^{bc}	148.46^{bc}
		Spring	13.83	387.50	540.98	13.81^{b}	554.87^{c}	97.79^{ab}	433.80^{c}	139.55^{c}
		Summer	14.38	455.06	615.18	17.93^{a}	633.13^{ab}	97.20^{c}	499.30^{ab}	160.62^{ab}
		Autumn	14.70	592.40	621.86	17.60^{a}	639.54^{a}	97.40^{bc}	514.06^{a}	165.37^{a}
Average Data										
	Average		14.36	458.7	598.59	15.36	613.95	97.58	495.59	159.42
	SEM		0.162	59.35	12.09	0.964	12.81	0.120	10.60	3.410
Statistics Probabilities (p-value)										
Grazing			NS	NS	NS	NS	NS	NS	0.0362	0.0363
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			<.0001	NS	NS	0.0058	0.0448	0.0080	0.0210	0.0212
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			0.0322	NS	NS	NS	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N Source x Season			NS	NS	NS	NS	NS	NS	NS	NS

^{a,b,c} Different lowercase letters in the same column represent treatments that differ from each other ($p < 0.05$) by Fisher's test, Urine: Urinary volume; Al.: Allantoin; UA: uric acid; PuD: purine derivatives; Al PuD: allantoin percentage in total purine derivatives; Mic N: microbial nitrogen compounds synthesis; EMNS: efficiency of microbial protein synthesis; OM: organic matter. SEM: standard error of mean. NS: Not significant.

Discussion

Dry matter intake and apparent digestibility

Our findings shows that nitrate did not negatively impact in forage, supplement, or total dry matter feed intake (kg/day). Trettel (2021), who also worked with beef heifers under grazing systems having urea or nitrate as non-protein nitrogen supplementation, did not find negative effect of nitrogen source over feed intake. The author found an average DMI_F of 7.68 (kg/day) (Table 5), which was slightly superior when compared to findings by Fernandes (2019), who had beef cattle submitted to grazing (*Urochloa brizantha*) systems during dry and rainy season, with overall DMI average of 6.15 (kg/day). Martello et al. (2020) found a much lower feed intake for forage DMI of 4.56 (kg/day) for cannulated Nellore cattle allocated in grazing system in Urochloa brizantha pastures.

Despite of that, NPN intake (kg/day) were 32.5% superior when animals were grazing in rotated pastures as opposed deferred. Possibly the synergistic effect of supplementation and the greater quality of rotated pastures influenced in the higher digestibility of NDF (%), as observed in the Table 6. When compared to deferred, rotated grazing systems tend to have higher overall digestibility and higher concentration of nutrients in the leaves, which is often associated with how the grazing method is managed, prioritizing the forage use according to the ideal plant height and maturity (Eagle & Olander, 2012). Our findings show that animals under rotated grazing system had higher NDF (%) digestibility as opposed to deferred. Since the rotated grazing implicates in a deferment period of the pasture to allow it produces newly leaves, it is understandable that diet from rotated grazing system had higher digestibility for NDF (%).

A season effect was also observed for that same variable with lower (67.6%) values observed in Winter, while Summer and Autumn had the highest NDF digestibility of 77.3 and 74.3%, respectively. Season is an important factor that can alter nutrients digestibility over the year and, as it was observed in our experiment, season had effect over most of all variables.

ADF (%) digestibility was improved when animals were under rotated grazing and/or being supplemented with urea as the main source of non-protein nitrogen, as seen on the Figure 9. Assigned with lower digestibility of ADF for deferred grazing, we also encountered a higher ADF (kg) excretion from animals under deferred grazing within Spring season (Figure 11). The effect might be related to the fact that in pastures of rotated grazing, animals can be selective and thus the defoliation will prevail on high palatable leaves, the newly emerged

ones, which has lower concentration lignified structural carbohydrates (Hemphill 2020) and therefore, that can increase fermentation activity and thus improve the digestibility of the ADF (%). That statement was only true during Spring and Autumn, while no major effect was detected within the other seasons.

Rotated grazing also influences in higher digestibility of EE (%) not only when assessed the sole effect of grazing, but also when it interacts with season (Figure 13). According to our findings, rotated grazing influence in higher EE (%) digestibility when compared to deferred grazing within Spring and Autumn. That trend might be explained not only by the slightly higher concentration of that nutrient in the diet in rotated pastures when compared to the deferred, but also by the fact that in rotated grazing animals naturally harvest the most digestible portion from the pasture by selecting the leave's tips. As vastly reported on the literature and already mentioned before, rotated pastures when properly managed, provides a forage with higher nutrient concentration, higher digestible energy, and water-soluble carbohydrates (Eagle & Olander, 2012) once defoliation is supposed to happen at the optimal stage of height and maturity. In our 2 years trial, we also found that rotated grazing leads to higher crude energy digestibility when compared to deferred.

Despite of non-significant effect of nitrogen sources over feed intake and digestibility of the diet, it is valid to point out that supplementation of beef cattle has as one of the main goals the improvement in feed intake and the digestibility of the diet, since it accelerates the rumen passage and helps the breakdown of forage cell-wall, allowing the access on it by bacteria and protozoa. As observed by Martinez-Fernandez et al. (2020), who worked with cattle grazing in tropical pastures having supplementation or not, the Alpha diversity and Chao richness of the microbial community in the rumen increases when supplementation was adopted, and that plays a key role in the forage-cell's components degradation, and overall rumen kinetics.

Rumen degradability

The use of nitrate, as the main non-protein nitrogen source, led animals to have lower total solid rumen mass (kg) during season when compared to animals supplemented with urea. That same trend was detected to the variable total solid rumen mass per % of LBW.

During Summer season, abiotic (sun, nutrition, and humidity) and management variables can lead to successful foliar growth in a shorter length of time, especially in pasture

managed in rotational method. Thus, it is reasonable to assume that greater solid ruminal mass was observed in animals under rotated grazing within Summer when defoliation happens more intensively. And as seen in the Table 5, forage and DM intake were higher over the Summer season. Certainly, the recovering period of rotational grazing also play a key role in the ability to the leaf regrowth when compared to deferred grazing. Greater defoliation activity leads to reduced foliar surface to intercept sun light, and this associated with no proper recovery time can certainly affect the rate of forage growth.

Higher disappearance rate by %/h and by kg/h over Summer and Autumn might be justified by the fact that on both season pastures displayed overall higher forage availability and nutrients digestibility as well (Table 6). Greater content of dry matter in the rumen also leads to a constant interaction and pressure into the rumen wall and pillars, which have chemoreceptors that understands the ruminal volume size and its chemical composition. This effect cause changes in the rumen motility which ultimately leads the more dense and fine rumen content made up of concentrate and smaller degraded pieces of forage to leave the rumen, and thus possibly increasing the disappearance rate by %/h and by kg/h.

The greater intake and digestibility of diet over Summer and Autumn may have increased the disappearance rate as it can be one of the factors that foments the increase of microbial protein, which plays important role on the overall rumen digestibility and degradability as well.

Degradability parameters for forage-based diets will naturally change over season as it is directly related to the chemical quality of the diet. In our study, it was noticed that nitrogen source interacted with season for the variable potential degradability (PD %) of the DM, which was higher when animals were fed nitrate within Spring (dry season) as opposed to the use of urea. Higher PD (%) for animals when fed nitrate within Spring (Figure 20), might be explained by a possible improvement of the overall digestibility and degradability of the nutrients since in Spring season animals had higher supplement feed intake as compared to the Summer, for instance, (Table 5). The intake of nitrate by means supplementation leads to the reduction of it to ammonia, which directly contributes to the microbial protein synthesis; however, besides that, the metabolic path in which nitrate is reduced to ammonia leads to a higher flow of negative Gibbs energy that is incorporated into the rumen and furnish energy for microorganism's growth, transport of substrate and mobility (Ungerfeld, 2020).

Following a similar trend, higher CP degradability rate at 2% (Figure 21) showed to be superior in the rumen of animals feed nitrate as opposed to the use of urea within the Spring

season, and as previously discussed the supplementation plays a major role on the obtained result. Despite of that results, the majority of degradability parameters for CP displayed significant effect of season.

It was notably the difference for the variable b, potential degradable fraction, which had lower values in Winter and Spring and higher during Summer and Autumn. Surprisingly, Summer had the lowest rate of degradation (3.5%/h) of the fraction b (potential degradable fraction), while no major effect was noticed among the other seasons. The decrease in 40.67% in the supplement dry mater intake over the Summer and increase of 38.55% for forage DMI (Table 5) could be a reason for the lowest rate of degradation (3.5 %/h) of the fraction b, since greater proportion of the CP during that season came from forage.

In rotated grazing systems the ruminal degradation rate of fraction b for NDF was 30.35% higher than that of deferred systems within Spring season, which might have a direct effect of the greater intake of NPN (kg/day) from animals kept in rotated method, and the higher digestibility of the NDF (%) content from those experimental units as well. The intake behavior of animals in rotated grazing might play a role on this too. When animals are rotated to a "deferred" grazing area, there is great availability of new leaves and that is the main target of the cattle, as they harvest of foliage's tips, which is more digestible and nutritious. In the same experiment that our study was carried out, Lelis (2021) evaluated forage production and quality on deferred and rotated grazing methods and during Spring season the authors observed that rotated grazing pastures had 40% more leaves and 27.7% more CP when compared to deferred. Possibly, the higher apparent digestibility of the NDF for rotated grazing method (Table 6) and higher availability of CP (27.3%) could have led to higher fermentation activity by the rumen bacterial community, which consequently can affect the rate of degradation of the potential degradable fraction of NDF, culminating in the result of our findings. That trend does not happen in the deferred grazing method since it is a continuous grazing, where most of the forage leaves were already under harvesting.

NDF potential degradability lower PD (%) in deferred pastures Autumn season. This result is mainly justified by the characteristics of the grazing method and its correlation with the season. Deferred pastures certainly will provide better forage availability over Winter, and Spring (as it was deferred for that porpoise), and Summer but it tends to decrease it in Autumn. Since grazing is continuous and defoliation happens with no pause, even with the influence with the last rain of Summer, forage quality tends to pike down in Autumn, especially because

in Autumn, as observed by Lelis (2021) there is greater proportion of steams in deferred pastures.

Ruminal microbial protein synthesis and efficiency

Interaction effect of grazing and season for the variable Urine (L/day) did not reveal statistical effect within season and, thus, no treatment effect was detected for that variable. Season effect was noticed for UA (mmol/d), PuD (mmol/d), Al PuD (%) and for EMNS (g/kg.OM). Lower UA (mmol/d) and PuD (mmol/d) occurred over Winter and Spring, while the highest excretion occurred in Summer and Autumn. The total PuD (mmol/d) represents the sum of allantoin and uric acid; allantoin by itself represents on around 97% of the total purine derivatives.

Nitrate can work as an important source of non-protein nitrogen that will play important role in the incorporation of NH_3 in the rumen environment and in the reduction of methane emission (Zurak et al., 2023). The reduction of nitrate to ammonia represents a metabolic path that is thermodynamically favourable and incorporates more energy into the rumen when compared to the reduction of CO_2 to CH_4, for instance, and that can increase the overall flow of microbial protein in the rumen (Ungerfeld, 2020). Despite of that, no effect of nitrogen source was detected for urine, excretion of urinary compounds, microbial nitrogen synthesis and efficiency of microbial protein synthesis. Despite of nitrate had lower NPN intake (kg/day) yet no effect on microbial protein synthesis nor on efficiency of microbial N synthesis were detected. Based on this finding, we understand that ammonium nitrate is a potential and suitable NPN source for beef cattle in grazing system.

On the other hand, grazing method had influence over microbial N synthesis (MicN) and efficiency of microbial N synthesis (EMNS) with 19.72% lower MicN and EMNS detected for animals under rotated grazing when compared to deferred grazing.

A possible reason for the lower EMNS (g/kg.OM) in rotated grazing is that animals under rotated grazing, as mentioned before, have access to a pasture with a profile of forage with higher content of nutrients, and higher concentration ratio of cell soluble compound to structural compound, which makes the forage highly concentrated in nutrients and, thus, more digestible, as observed in our findings (Table 6; Figures 9 and 10).

In this context animals would rather prefer the new green leaf's harvesting, a great source of PDR than approaching the supplementation. Possibly, this led to lower rate of

conversion of protein to ammonia, and thus resulted in a lower MicN when animals were in rotated grazing. Despite of that assumption, no significative effect was detected on supplementation intake. The most likely and a probable second reason that adds up to justify the lower microbial N synthesis (MicN) and efficiency of microbial N synthesis (EMNS) was the greater rumen solids content from animals in rotated pasture, especially during Summer season (Figure 19). As previously mentioned, the pressure of fiber content on a full rumen into the rumen wall influences ruminal motility, which increase the disappearance rate by %.h and by kg/h. Theoretically, greater amount of ruminal content leaven the rumen means lower retention of microbial protein, degradability and consequently lower EMNS as well.

Cattles behaviour in continuous grazing is different and since they have full time availability of forage for grazing, the more digestible and palatable leaves are constantly taken. This, associated with extrinsic factors that do not contributes to forage growth along the seasons can lead to the pasture have lower content of new and nutritious leaves, higher content of more lignified and recalcitrant forage compounds that tends to take greater time within the rumen.

Conclusion

Ammonium nitrate can be used as a non-protein nitrogen source in intensified grazing systems. It did not cause negative impact on intake of forage and supplement as well as for parameters of digestibility, nutrients intake and excretion. Both supplementations seem to do not greatly affect kinetics parameters; however, based on our findings, when urea and nitrate were used as the main nitrogen source an equal contribution to microbial protein synthesis and its efficiency were observed, which is a result that stands-out placing ammonium nitrate not only as an important supplementation source that does not cause negative effect on feed consumption, of Nellore beef cattle in grazing systems, and still have its contribution at metabolic level in the rumen, furnishing the synthesis of microbial protein.

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CHAPTER 3 – Grazing systems with adoption of ammonium nitrate positively affect Nellore beef cattle rumen fermentation and reduce enteric methane emissions.

Abstract

The main goal of this experiment was to investigate the effects of intensified grazing systems (deferred or rotational grazing methods) with non-protein nitrogen supplementation (ammonium nitrate or urea) for beef cattle during different seasons on fermentation parameters, as pH, and metabolites, such as methane emission, nitrogen ammonia and short chain fatty acids concentration and production. To conduct the experiment, it was used eight Nellore female cows were used as experimental animals, and randomly allotted to 8 paddocks. Each treatment was allotted to a paddock in a randomized block design, composed by two different grazing methods with supplementation of ammonium nitrate or urea making up a factorial design, in which seasons of the year was also included. The experimental treatments were as follows: Rotational grazing with urea supplementation, rotational grazing with nitrate supplementation, deferred grazing with urea supplementation and deferred grazing with ammonium nitrate supplementation. All variables were collected during four seasons over two years. Data was analyzed using the mixed procedure. Nitrate supplementation did not significantly affect acetic acid production, but it led to a significant reduction of 23.13% in the release of energy as acetic acid within the Summer season. Nitrate supplementation resulted in a 28.8% reduction in propionic acid concentration compared to urea during the Autumn. The concentrations of butyric acid were higher in animals under rotated grazing systems during the Spring and Autumn seasons. Additionally, when animals were supplemented with urea during the Summer, there was a 33% increase in butyric acid production, leading to greater gross energy release in the rumen. The study also observed a 13.1% reduction in methane production and a subsequent 15.7% decrease in energy release in the rumen of animals supplemented with nitrate when compared to urea. Rotated grazing systems contributed to a 21.3% reduction in methane yield during the Summer season. However, when nitrate was used with animals in deferred grazing systems during the Spring, it was observed a reduction of 26.11% relative energy loss (REL %). Our study suggests that rotated grazing did not compromise the concentration of ammonia nitrogen in the rumen, and it improved the overall short chain fatty acids concentration and production which can then furnishing the ruminants' energetic needs with greater amount of energy release in the rumen. Mitigation of enteric methane yield was also observed when

animals were subjected to rotated grazing over the Summer season. Coupled with rotated grazing, ammonium nitrate showed as an appealing technology to be adopted when it comes to reduction of methane yield in beef cattle production. Thus, the supplementation of ammonium nitrate for Nellore beef cattle positively contributed to the reduction of ruminal methane production and partially contribute to mitigate the negative impact from beef cattle production in tropical regions.

Keywords: Electron-sink, grazing, methane mitigation,

Introduction

Agricultural policies have been studied by the scientific community, and its implementation are considered, by the scientific community, as feasible by means strategics approaches which could then mitigate greenhouse gases (GHG) emission from agricultural sector. It is understood that these approaches can fall into two major area, the supply side and demand side. The first is reducing GHG emissions by means increasing productivity coupled with the efficiency in input use by means adoption of proper technologies and management of them. The second is through reducing indirect emissions from inappropriate expansion of land and its use. Proper use of lands lead to increase on carbons stocks in agricultural soils, which can be attained by great soil management and restoration of degraded lands allowing increase in soil carbon sequestration on cropland and grassland (OECD, 2022).

Adoption of technologies in Livestock production has been studied and taken for increasing productivity coupled with less impact in the environment. Withing a beef cattle factory, the main way to do that can be by means reduction of the enteric methane emission.

Enteric fermentation, as it is most refereed in reports regarding anthropogenic GHG emissions, is a physiological activity that occurs in the rumen of a ruminant animal. Ruminants can even be considered as privilege animals since through enteric fermentation, supported by a synergistic action of anaerobic microorganisms in the rumen, they are able to convert complex polysaccharides into valuable products such as acetic, propionic and butyric acids, as source of energy to the ruminant metabolism. However, besides these metabolites, there is also generation of hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4). CH_4 is often considered as a necessary energetic loss of ruminant's metabolism that account for about 2 to 12% of gross energy (Beauchemin et al., 2020).

Despite of the importance of methane production to ruminant's fermentative metabolism, it represents a source of GHG that negatively contributes to global warming and thus to the scrutinization over the beef cattle production chain.

The most recent estimative of greenhouse gases emissions (GHG), released in a report from IPCC (2021), indicated that from all total world's anthropogenic activities GHG emissions to the atmosphere, expressed as $GtCO_2eq$, agriculture was responsible for 23% whit enteric fermentation accounting for 5% of that emission. In the last update from the Ministry of Science, Technology, and Innovations (MCTI) indicated that Brazilian agriculture was

responsible for about 35.8% of the total GHG emissions (Gt CO2eq) to the atmosphere, and that withing the agricultural sector enteric fermentation was responsible for 58%.

Knowing how the Brazilian livestock production is performed, we understand that there is possibility to reduce the impact it brings to the environment. One of the main way to achieve that is by modulating the ruminal fermentation and incorporating the available molecular hydrogen, freely released in the rumen by phosphorylation process (Ungerfeld, 2020), into more valuable metabolites.

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 from acetate and butyrate production, but also the available hydrogen from re-oxidation of reduced cofactors (NADH, NADPH and FADH) and the reduction of pyruvate to acetyl-CoA that will furnish methane synthesis (Eugene et al., 2021). According to Morgavi et al. (2023) it is estimate that 50 to 80% of the dissolved hydrogen in the rumen is converted to methane, while 20 to 30% can be used in propionate synthesis.

Literature has extensively showed the effect of supplementing beef cattle with calcium nitrate, especially in feedlot systems (Hulsholf et al., 2012; Newbold et al., 2014; Lee et al., 2015; Duthie et al., 2018; Tomkins et al., 2018; Troy et al., 2015; Capelari 2018 and Alemu et al., 2019). However, the supplementation of Nellore beef cattle wit ammonium nitrate under intensified grazing systems and its effect of rumen metabolites is not well described.

The incorporation of molecular hydrogen into fermentative metabolites by reducing nitrate to ammonia can be nutritionally advantageous as it leads to less digestible energy losses as CH_4 production (Lan & Yang, 2019). Ammonium nitrate, despite of its lower concentration of nitrogen (34%) in the composition as compared to urea (46%), its market trading price is cheaper than urea (Trading Economics, 2023). Therefore, it is important to assess whether utilization of ammonium nitrate supplementation for beef cattle can mitigate the methane generated from beef cattle in tropical grazing systems. Positive results in terms of improving ruminal performance, favorable change in ruminal fermentation and lower enteric methane production might give indicative of a profitable system production for tropical areas that would not only be economically appealing but also environmentally.

We understand that if coupled with decreased methane production and no major change on short chain fatty acids production is detected, nitrate brings positive results regarding its main purpose. Thus, the hypothesis of this study is that adoption of intensified grazing methods associated with nitrate supplementation as the main non-protein nitrogen source during different

seasons have positive effect on Nellore beef cattle ruminal metabolism, and thus, mitigation of enteric methane emission can be achieved with no major changes on fermented products when compared to the use of urea as the main source of supplementation.

Objective

The objective of this experiment was to investigate the effects of intensified grazing systems, deferred or rotational grazing methods associated with nitrate supplementation, for beef cattle during different seasons on fermentation parameters as pH, and metabolites, such as methane emission, nitrogen ammonia and short chain fatty acids yield and concentration, when compared to the use of urea.

Material and Methods

Location

The experiment was carried out at College of Veterinary Medicine and Animal Science (FMVZ/USP), Pirassununga, Sao Paulo State, Brazil, for two years, in between June of 2019 and April of 2021. The experimental animals were handled and managed according to the Ethic Committee on Animal Use on Research (FMVZ/USP). A total of 8 Nellore female cows, of approximately 551 ± 7.01 kg of BW were used as experimental animals for rumen fermentation data (cannulated animals).

Experimental design, pasture system and treatments

The experimental animals were randomly allotted to 8 paddocks. Each treatment was allotted to a paddock in a randomized block design (blocks were formed as a function of terrain location) for two years (total of four replicates). Treatments is composed by combination of two different grazing systems with supplementation of ammonium nitrate (NH_4NO_3) or urea within each season, they are as follows: 1) Deferred grazing system with NH_4NO_3 supplementation (DGN), 2) Deferred grazing system with urea supplementation (DGU), 3) Rotational grazing system with NH_4NO_3 supplementation (RGN), and 4) Rotational grazing system with urea supplementation (RGU).

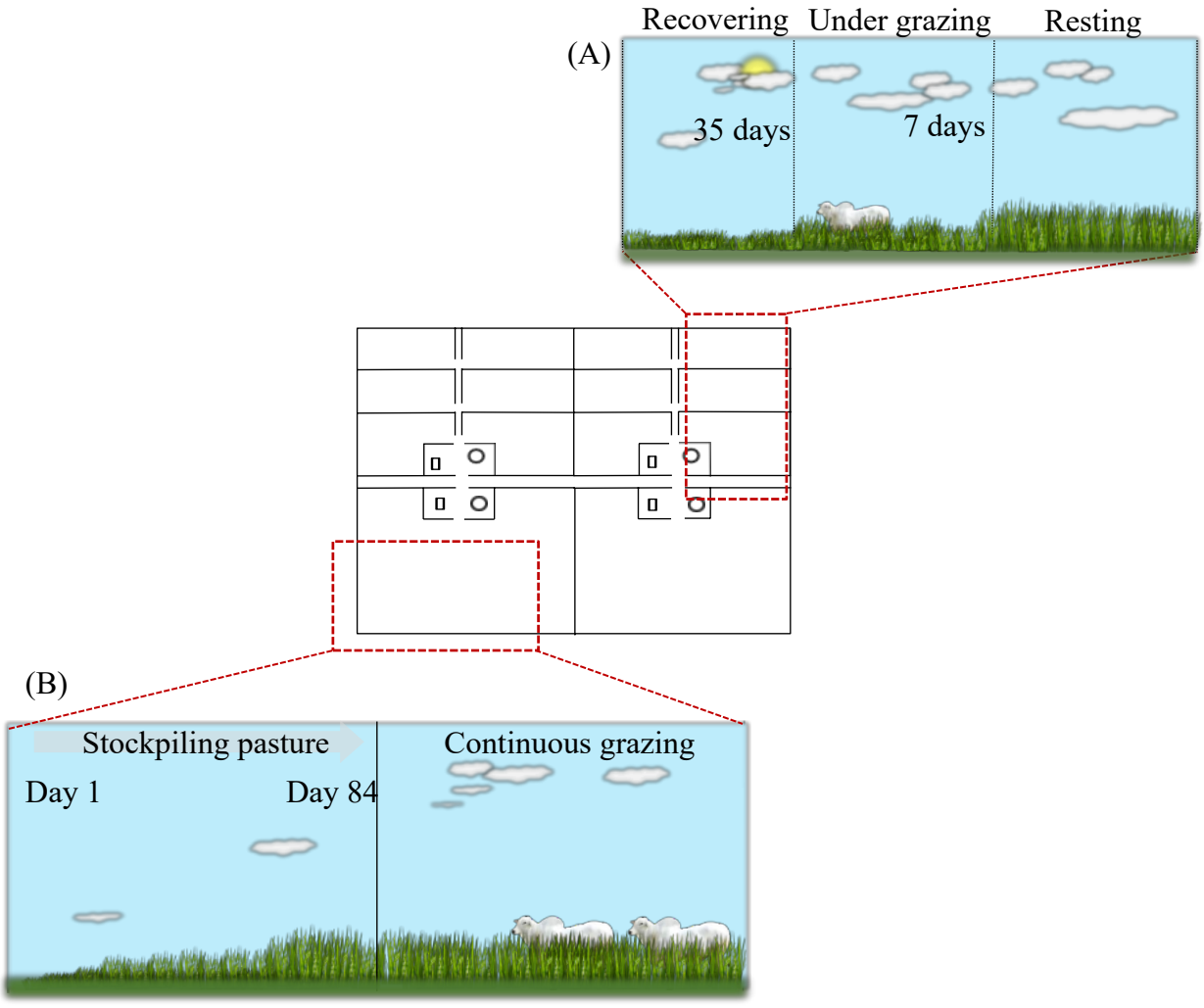
The experimental area as seen on Figure 25, has 26.5 ha^{-1} divided by management corridors. The area was established in 1999 with Urochloa (syn. Urochloa brizantha) brizantha. Additionally, 13.1 ha^{-1} was used for allocation of extra animals used to adjust stocking rate. Fertilization procedures were adopted along the experimental period following soil's recommendation.

In January of 2019, previously to the beginning, experimental area was fertilized with 50 kg ha^{-1} of nitrogen and 50 kg ha^{-1} of potassium. In November of 2019, the pastures were fertilized with 53 kg ha^{-1} of nitrogen and 57.5 kg ha^{-1} of sulfur, using ammonium sulfate. In January and March of 2020, ammonium nitrate fertilizer was applied in the amount of 56.7 kg ha^{-1} of nitrogen in each post-grazing rotated paddocks, and in the deferred pastures it was carried at once, on the same day when the last paddock of the rotated pastures was fertilized.

The deferred pastures, where stockpiling was adopted, were locked for 84 days at the end of rainy season in the first and second year (at the end of March). The management of grazing in the deferred systems was simple and animals were introduced into the systems when

stockpiling was ceased, then animals were left to continuously grazing. Each rotational system was comprised by 6 even paddocks, where animals were left under grazing in each paddock for seven defoliation days with a resting period of 35 days.

Figure 25 - Schematic representation of the rotational and deferred grazing systems with indication of recovering period, under grazing, resting period and deferred period.



Independently of the systems, animals had easy access to fresh water and to the formulated supplement, which has its composition described on table 14.

Table 14 - Composition and proportion of each ingredient used to prepare supplement for the adaptation, rainy and dry season using urea or nitrate as nitrogen source.

Ingredients		Adaptation		(Dry season)		Rainy (Season)	
		Urea	Nitrate	Urea	Nitrate	Urea	Nitrate
		(%)	(%)	(%)	(%)	(%)	(%)
Ground corn		55	55	48	45	72	69
Urea		10		22		13	
Salt		20	15	15	10	7	5
Mineral mixture[1]		15	15	15	15	8	8
Ammonium nitrate			15		30		18
Nutritional composition							
CP	(%)	33.14	33.49	66.34	61.13	43.01	43.34
TDN	(%)	48.22	48.22	42.02	39.46	63.13	60.5
EE	(%)	1.60	1.60	1.39	1.31	2.09	2.00
NDF	(%)	4.35	4.35	3.79	3.56	5.69	5.45
ADF	(%)	1.43	1.43	1.25	1.17	1.87	1.79
Ca	(%)	2.70	2.70	2.69	2.69	1.45	1.45
P	(%)	2.54	2.54	2.52	2.52	1.47	1.46
Na	(%)	7.81	5.86	5.86	3.91	2.74	1.96

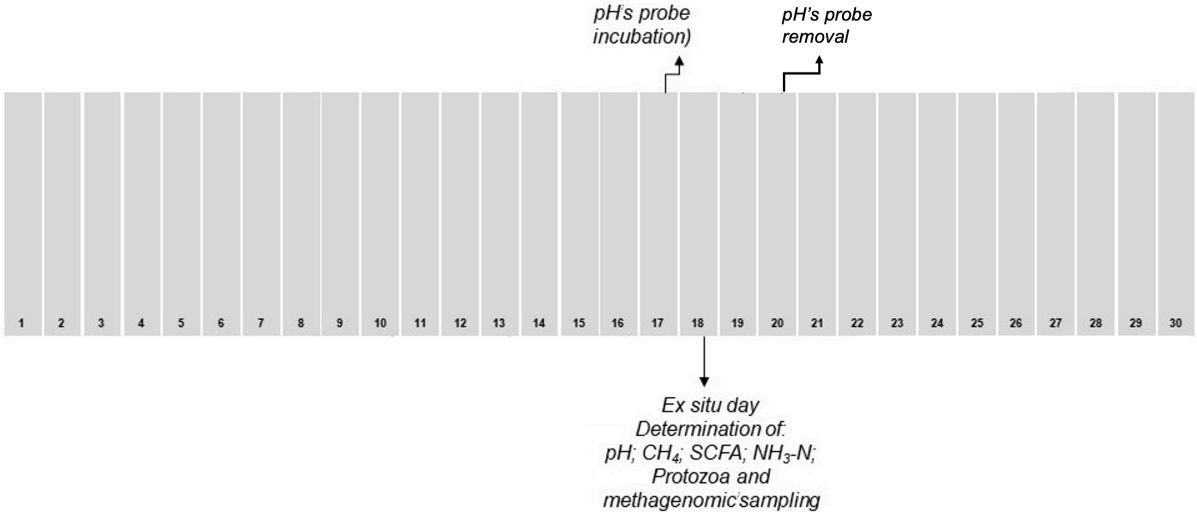
[1]Minerthal®**Estimated Macro and micromineral composition for the urea and nitrate supplement adopted in adaptation period and dry season:** 1.93 g/kg of potassium, 0.77 g/kg of magnesium, 3.29 g/kg of sulfur, 12.30 mg/kg of cobalt, 342.45 mg/kg of copper, 16.79 mg/kg of iodine, 402.90 mg/kg of Iron, 291.00 mg/kg of molybdenum, 3.36 mg/kg of selenium, 812.70 mg/kg of zinc. **Estimated Macro and micromineral composition for the urea supplement adopted in rainy season:** 2.52 g/kg of potassium, 1.01 g/kg of magnesium, 2.22 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. **Estimated Macro and micromineral composition for the ammonium nitrate supplement adopted in rainy season**: 2.42 g/kg of potassium, 0.97 g/kg of magnesium, 2.19 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. CP: crude protein; TDN: total digestible energy, EE: ether extract, NDF: neutral detergent fiber; ADF: acid detergent fiber; Lig: lignin; EE: ether extract; Ca: Calcium, P: phosphorous, Na: sodium.

Animals were adapted to the supplementation of ammonium nitrate and previously to this experiment other studies were carried out evaluating the inclusion of different dosages of nitrate into the diet and no intoxication was detected. Despite of that, Methylene blue antidote was readily available in any case of intoxication sign.

Experimental period

All variables were collected during four periods of two years (Winter, Spring, Summer and Autumn). In the following schematic representation, it is shown all the activities scheduled in each month (the second month of the season) of each season.

Figure 26 - Schematic representation of the activities scheduled in each sampling month of each season.



In the first month of the season all animals were under adaptation on its respective experimental units.

Ruminal pH evaluation

The pH was continuously measured on each experimental period by using a data logger (model T7-1 LRCpH, Dascor, CA), which consisted of a pH probe housed in a water-resistant capsule and an electrode protected by a structure that allowed the passage of particles and liquid while protecting the electrode from encountering the rumen epithelium. Two 900 g weights are coupled to each probe to ensure that it remains in the ventral sac of the rumen. Each data logger was programmed to measure the pH every 10 minutes for 24 hours. This allowed the calculation of the variables: minimum, medium, and maximum daily pH, time at which pH remained below 6.4; 6.6; 6.8 and 7.0 and pH area below 6.4; 6.6; 6.8 and 7.0 according to Moya et al. (2011).

Before and after introducing the probes in the rumen, they are calibrated in solutions of pH 7.0 and 4.0. The calibration allowed the calculation of a slope and an intercept before and after the test to adjust the measured data. The area under the curve was calculated by multiplying the absolute value of the deviations in pH by the time (min) spent below the threshold established for each measurement, and divided by 60, being expressed as pH unit per hour, according to Moya et al. (2011).

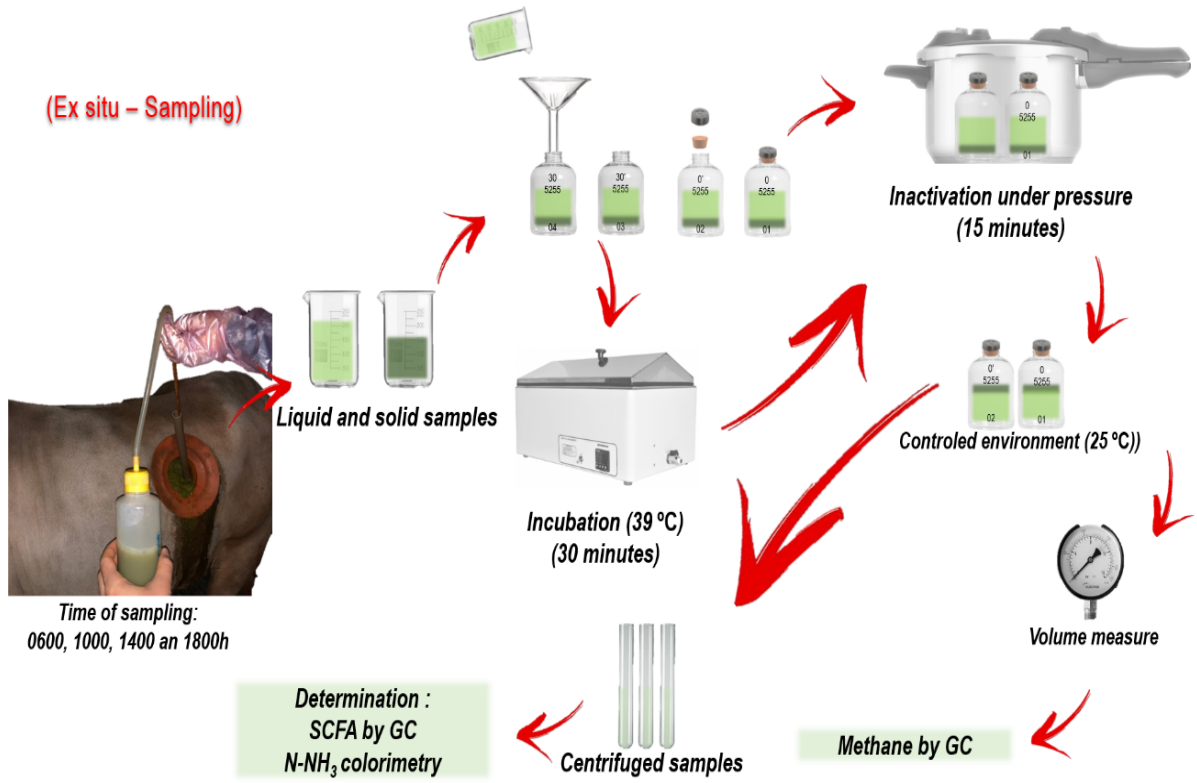
Ex situ assay to evaluation of ruminal fermentation products

The evaluation of rumen fermentation products was performed using the *ex-situ* technique described by Rodrigues et al. (2012) and Perna Junior et al. (2017). This novel technique consists in adding samples of rumen content into flasks (micro-rumen) which are incubated in a water bath (39 ºC), simulating the prevailing conditions of the rumen (presence of microorganisms, anaerobic environment, temperature of 39 °C, natural saliva, physiological rumen pH) for 30 minutes.

Sampling of rumen content

The rumen content for the measuring of CH_4, SCFA, NH_3-N as well as total protozoa counting was collected on day 18 of each experimental period, at 0600, 1000, 1400 and 1800 h from that day (Figure 27). To procced the sampling fistulated animals were tied in the paddocks for sampling. At this point, samples were taken as liquid and solid phase from three distinct points of the rumen by a semi-automatic vacuum pump and manually collected, respectively.

Figure 27 - Schematic representation of the procedure used in the ex-situ technique developed by Rodrigues et al., (2012).



Both fractions were added into the flasks (about 10 g of the solid fraction and 20 mL of the liquid fraction) with the aid of a funnel and a plastic stick as seen on the Figure 27. The flasks were then capped with rubber stoppers and sealed with aluminum sealing wax through specific pliers. Afterwards, they were "washed" with CO_2 by means of two needles for gas inlet and outlet to ensure anaerobiosis.

Four flasks per cow were prepared for each sampling time, two of which were immediately inserted into autoclave to inactivate the fermentative process (under temperature and pressure) for 15 minutes. The other two flasks were immediately incubated for 30 minutes in a thermostatic bath at 39°C. At the end of the incubation time the fermentative process was inactivated under temperature and pressure for 15 minutes.

Methane assay

To assess the methane concentration from ruminal content as well as its production four penicillin bottle of 50 mL capacity were used, two of them as blank and two denominated as incubated. The bottles were filled by 30 mL of ruminal content and capped with rubber corks, sealed, and washed with CO_2 by means of needles for input and output to ensure an anaerobic environment. The material then is submitted to incubation and after 30 minutes, fermentation is going to be block by autoclaving (under pressure and elevated temperature for 15 minutes).

To measure the total gas volume generated in incubated and blank bottles a pressure transducer (Data logger Universal® - logger model AG5000) connected to a syringe with a needle is used. The total gas volume is a result of sum of volume obtained at the transducer plus the head space. The determination of methane concentration is done through gas chromatography (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy), injecting 0.5 mL of gas from each bottle, according to Kaminski et al. (2003) in a controlled temperature environment (25°C).

To quantify the methane production, the total gas volume (mL) was multiplied by its respective concentration in the gas phase (mmol/mL) attained from the incubated bottles and subtracted from the non-incubated bottles (blank) using the following equation:

$$\text{Prod. } CH_4 = (\text{Con. } CH_4 \times \text{Total Gas Vol.})\ T_{30} - (\text{Con. } CH_4 \text{ x Total Gas Vol.})\ T_0$$

Where:

Prod. CH_4: methane production at the time between 30 minutes and zero (0) minute of incubation.

Con. CH_4: concentration of methane (mmol/mL).

Total Gas Vol.: total gas volume was obtained by the sum between the volume obtained at the transducer plus the head space (mL).

T_{30}: incubation time of 30 min.

T_0: blank: incubation time of 0 min.

All attained values for CH_4 were expressed based on the solid content of the bottle.

Short chain fatty acids evaluation (SCFA)

To assess the concentration of SCFA a fraction from ruminal fluid from each bottle previously autoclaved is submitted to centrifuge for 15 minutes at 5000 rpm, after which, 2.0 mL of the supernatant is sampled and added into it 0.4 mL of formic acid (P.A), the material is preserved at 4˚C up to the moment of being assessed for concentrations of acetic, butyric, and propionic acid as suggested by Erwin et al. (1961). The concentration of each fatty acid is detected by gas chromatography (Focus GC, Thermo Scientific®, Rodano, Milan, Italy) using a glass column with 1.22 m in length and 0.63 cm in diameter packed with 80/120 Carbopack B-DA/4% (Supelco, Sigma-Aldrich®, St. Louis, MO).

The quantification of SCFA is attained by multiplying the liquid volume (mL) and the concentration of each SCFA (mmol/mL) obtained in the incubated bottle, subtracted from that obtained on the blank bottle using the following equation:

$$\text{Prod. SFCA} = (\text{Con. SCFA} \times \text{Total Li. Vol})\, T_{30} - (\text{Con. SCFA} \times \text{Total Li. Vol})\, T_0$$

Where:

Prod. SFCA: SCFA production now between ruminal content injection in bottle and inactivation.

Con. SCFA: SCFA concentration (mmol/mL).

Total Li. Vol.; Total volume of liquid in the flask.

Vol.: total liquid volume at penicillin bottle obtained by weight difference between before and after the oven (mL).

T_{30}: incubation time of 30 min.

T_0: blank: incubation time of 0 min.

All attained values for SCFA are expressed as solid basis of the bottle (grams and kilograms).

Relative energy loss (REL)

Energy release estimative is determined as described by Rodrigues et al. (2012), which says that the concentration of each SCFA assessed is multiplied by its heat combustion as a mean to express methane production as a function of acetic, butyric, and propionic acid. Therefore, the relative energy loss is the ratio between the energy in the methane produced and the energy sum in all the quantified fermentation products, which are expressed as a percentage. To accomplish that, it is assumed the following heat combustion factors: 3.49, 4.98, 5.96, 13.16 and 0.0 kcal/g for acetic, propionic, butyric, CH_4 and CO_2, respectively. Then, the relative energy loss was calculated using the following equation:

$$REL\ (\%) = 100 \times [\ \text{Ɛ}CH_4 /(\text{Ɛ}CH_4+\text{Ɛ}C_2+\text{Ɛ}C_3+\text{Ɛ}C_4\ )]$$

Where:

REL.: relative energy loss (%).

$\text{Ɛ}CH_4$: methane energy (kcal/g or kcal/mol).

$\text{Ɛ}C_2$: acetic acid energy (kcal/g or kcal/mol).

$\text{Ɛ}C_3$: propionic acid energy (kcal/g or kcal/mol).

$\text{Ɛ}C_4$: butyric acid energy (kcal/g or kcal/mol)

Energy release estimative

Gross energy intake (GEI) was obtained by multiplying DMI (kg) and diet GE (Mcal/kg). The amount of energy release into the rumen in form of acetate, propionate, butyrate and CH_4 (Mcal/ani.day) was calculated by multiplying the crude amount (g/kg.day) of these metabolites (g/kg.day) with their respective combustion heat (Mcal/g) and then multiplied by rumen solid mass (kg).

To express the energy release in the rumen as a percentage of GEI or digestive energy (DE), fermented metabolites released in the rumen (Mcal/ani.day) were divided by GEI (Mcal/ani.day) or DE (Mcal/ani.day) and then multiplied by 100. Methane release in the cecum and colon (C&C) was considered as 5% of the total CH_4 release as Enteric methane is mainly

generated in in the rumen (95%) and, to a smaller extent (5%), in the low gut. The fermentation heat (FH) and microbial ATP were estimated from the ration among of SCFA produced according to Owens and Basalan (2016).

To estimate the energy release in the intestine (Mcal/ani.day), it was used GEI (Mcal/ani.day) to subtract the energy that comes from SCFA and CH_4 from rumen (Mcal/ani.day) plus feces' GE (Mcal/ani.day), CH_4 release in the cecum and colon (Mcal/ani.day), and FH following the equation:

$$ERI = GEI - (ЄC_2 + ЄC_3 + ЄC_4 + feces'GE + C\&C\ CH4 + FH + mATP)$$

Where: ERI: energy release in the intestine (Mcal/ani.day); GEI: gross energy intake (Mcal/ani.day); $ЄC_2$: acetate energy (Mcal/ani.d); $ЄC_3$: propionate energy (Mcal/ani.day); $ЄC_4$: butyrate energy (Mcal/ani.day); Feces GE: energy release in the feces (Mcal/ani.day); C&C CH_4: CH_4 release in cecum and colon (Mcal/ani.day); FH: fermentation heat; mATP: microbial ATP.

To obtain the energy release in the intestine as percentage of GE or DE, the amount of energy release in the intestine expresses as Mcal/ani.day were divided by GEI (Mcal/ani.day) or DE (Mcal/ani.d) and then multiplied by 100. The energy released in feces, expressed in terms of percentage of GEI, was obtained dividing feces' energy content (Mcal/ani.day) by GEI (Mcal/ani.day) and then multiplying by 100.

Ammonia nitrogen concentration (NH_3-N)

To determine total ammonia concentration, 1.0 mL of sulfuric acid at 1 mol. L^{-1} is added into a tube with 2.0 mL of centrifuged sample. Up to the colorimetric analyses the tubes are kept frozen as described by Kulasek (1972) and adapted by Foldager (1977). The balance was determined by subtracting NH_3-N concentration after 30 minutes of incubation from the baseline (blank). With this procedure it is possible to evaluate whether the balance of ammonia production in the rumen is positive or negative. Ammonia nitrogen concentration' data are expressed as mg/dL per hour and its determination is obtained using the following equation:

$$NH_3\text{-N balance (mg/dL.h)} = [\text{Conc. 30 min (mg/dL)} - \text{Conc. 0 min (mg/dL)}] \times 2$$

Where: Conc. 30 min = NH_3-N concentration in incubated flasks; Conc. 0 min = NH_3-N concentration in non-incubated flasks.

Statistical analysis

Data was statistically analyzed using the online version of the software Statistical Analysis Systems – OnDemand for Academics SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Previously to the statistical analysis, the data was assessed for the presence of disparate information ("outliers") and the normality assumption of the residuals was assessed by means the Shapiro-Wilk test. When the normality assumption was not accepted, the logarithmic or the square root transformation was applied.

Data was analyzed according to the mixed procedure (PROC MIXED), in which season was considered as repeated variable (split-plot in time). A total of 15 different covariance structures were tested, and the chosen one was based on the lower value of Corrected Akaike Information Criterion (AICC) (Wang and Goonewardene, 2004).

The model includes the effect of grazing method, nitrogen source, period of the year (Winter, Spring, Summer and Autumn) and the interaction between grazing method, nitrogen source and season of the year. The effects of block were considered as random factor.

$$Y_{ijkl} = u + b_i + g_j + n_k + (gn)_{jk}\, e_{(1)ijk} + s_l + (sg)_{lj} + (sn)_{lk} + (sgn)_{ljk}\, e_{(2)ljk}$$

Where:

Y_{ijkl}: experimental answer
u: Constant
b_i: Effect of the block
g_j: Effect of grazing
n_k: Effect of nitrogen source
$(gn)_{jk}$: Interaction effect of grazing and nitrogen source
$e(1)_{ijk}$: Random error
s_l: Effect of season
$(sg)_{lj}$: Interaction effect of season and grazing
$(sn)_{lk}$: Interaction effect of season and nitrogen source

$(sgn)_{ljk}$: Interaction effect of season, grazing and nitrogen source.

$e(2)_{ljk}$: Random error

In the presence of interaction, effects of one factor inside the other were evaluated using the SLICE command of Mixed Procedure. All means were presented as least squares means and statistical differences by treatment effects were obtained by pairwise difference test (PDIFF) using the Fisher test considering a significance of $P \leq 0.05$.

Results

Parameters of ruminal pH

Analyzing all pH parameters, shown on table 15, it is seen that nitrogen source and season had effect over mean pH values as well as for minimum pH. Animals supplemented with nitrate had higher mean and minimum ruminal pH as compared to those fed urea as the main nitrogen supplementation source by 0.15 and 0.23 pH units, respectively. Grazing method also influence in ruminal pH and as seen on Table 15; animals kept in rotated grazing systems had lower ruminal pH as compared to deferred grazing.

The pH parameters such as time of pH (min/day) and area (h.pH/day) explain how intense is the effect of the pH over the rumen environment. A statistical effect nitrogen source for time of pH showed that nitrate influenced ruminal pH to stay below 6.6 by 431 (min/day) while the rumen content of animals fed urea had its pH bellow 6.6 for 788 minutes, which was approximately 45% more than that observed for animals fed nitrate.

The intensity in which pH remained below a certain range was measured as pH area (h.pH/day), and it was possible to see that the extent in which ruminal pH of animals under rotated grazing remained below 6.4 was 74% greater and thus more intense when compared to animals under deferred grazing systems. Same trend was detected when both nitrogen sources were contrasted as urea influenced in a greater pH area (h.pH/day), being 57% more intense (Table 15).

Rumen pH values of Nellore cattle under different grazing systems having different nitrogen sources along different seasons displayed effect for season with higher mean and minimum pH values during Spring and lower values in Summer and Autumn, as seen on Table 15. Overall, the time of pH (min/day) and area of pH (h.pH/day) had similar season effect with greater time and area happening during Summer and lower in Spring (Table 15).

Table 15 - Ruminal pH values of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.

Fixed factors			pH day			Time of pH, min/day				Area, h.pH/day			
Grazing	**N Source**	**Season**	**Mean**	**Min**	**Max**	**<6.4**	**<6.6**	**<6.8**	**<7.0**	**<6.4**	**<6.6**	**<6.8**	**<7.0**
Deferred			6.72	6.32	7.06	186	392	824	1185	0.54	5.79	3.74	7.3
Rotated			6.60	6.15	6.97	427	827	993	1231	2.08	6.62	6.63	10.2
	Nitrate		6.74	6.35	7.07	209	431	771	1136	0.78	3.67	3.78	7.08
	Urea		6.59	6.12	6.96	404	788	1046	1280	1.85	8.74	6.59	10.42
		Winter	6.67^{ab}	6.29^{ab}	6.99	303^{ab}	574^{ab}	937^{ab}	1244^{ab}	1.25^{b}	2.69^{b}	4.94^{ab}	8.57^{ab}
		Spring	6.82^{a}	6.42^{a}	7.12	87^{b}	314^{b}	626^{b}	1077^{b}	0.18^{b}	0.72^{b}	2.29^{b}	5.07^{b}
		Summer	6.57^{b}	6.11^{b}	6.96	501^{a}	782^{a}	968^{a}	1137^{ab}	2.61^{a}	18.46^{a}	7.63^{a}	11.06^{a}
		Autumn	6.58^{b}	6.11^{b}	6.99	333^{ab}	766^{a}	1101^{a}	1372^{a}	1.20^{b}	2.96^{b}	5.88^{a}	10.28^{a}
Average data													
	Average		6.66	6.23	7.01	306.40	572.65	900	1194.5	1.31	6.20	5.18	8.74
	SEM[1]		0.038	0.048	0.032	48.432	63.725	66.211	51.65	0.287	2.045	0.634	0.786
Statistics Probabilities (*p*-value)													
Grazing			NS	0.049	NS	NS	NS	NS	NS	0.004	NS	0.023	0.035
N source			0.025	0.009	NS	NS	0.008	NS	NS	0.041	NS	0.027	0.017
Season			0.004	0.008	NS	0.013	0.011	0.029	0.039	0.004	0.005	0.002	0.002
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant. < indicates: pH bellow at 6.4, 6.6, 6.8 or 7.0

Fermented products

Short Chain Fatty Acids (SCFA)

Table 16 shows the concentration of fermented products before and after incubation of 30 minutes and the respective production from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons in both years.

It was detected, for acetic acid before and after incubation, an effect for grazing method with higher values (+6.06%) in ruminal content of animals under rotated grazing when compared to deferred. Despite of that, when it comes to the acetic acid difference (mmol/L), it was possible to detect only effect of season, whit higher production of acid (mmol/L) over Summer and Autumn (Table 16).

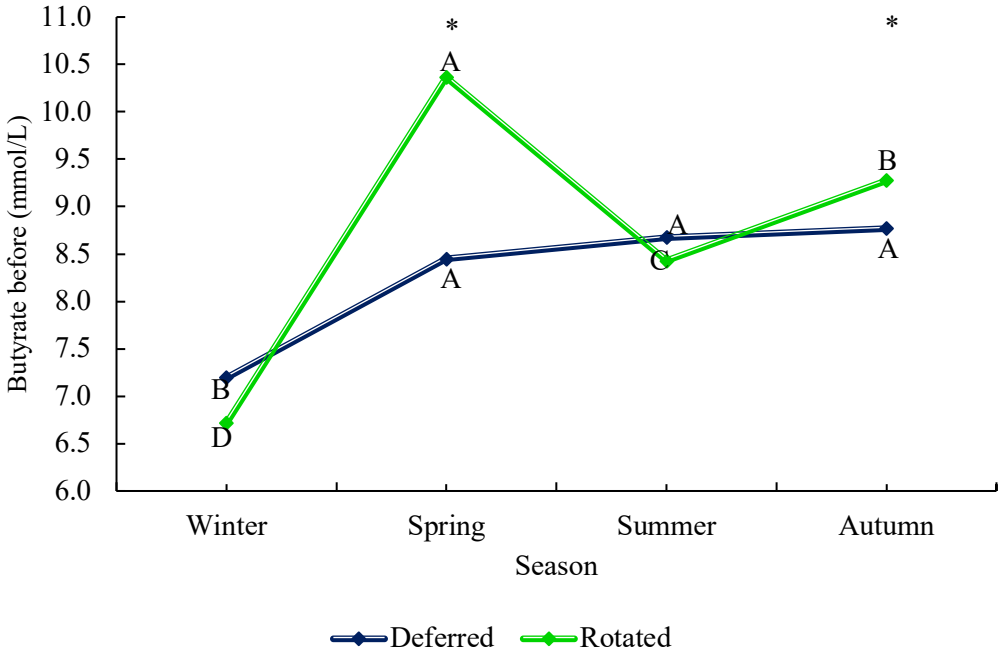
For the variable butyric acid concentration (mmol/L), it was observed effect of grazing method, in which the butyric acid concentration before and after incubation had higher values for ruminal material from animals under rotated grazing. However, it was detected interaction effect for grazing method and season, and its decomposition indicating the differences is displayed in the figures 28 and 29.

Table 16 - Short chain fatty acids (SCFA) production from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed factors			Variables											
			Acetic Acid			Propionic Acid			Butyric Acid			Total SCFA		
Grazing	N Source	Season	Before (mmol/L)	After (mmol/L)	Diff (mmol/L)	Before (mmol/L)	After (mmol/L)	Diff (mmol/L)	Before (mmol/L)	After (mmol/L)	Diff (mmol/L)	Before (mmol/L)	After (mmol/L)	Diff (mmol/L)
Deferred			71.19	75.09	4.29	16.76	18.05	1.14	8.24	9.11	0.70	95.90	103.55	6.12
Rotated			75.75	79.75	4.11	18.77	19.39	1.13	8.76	9.09	0.60	102.85	105.32	5.64
	Nitrate		74.04	77.724	4.09	17.72	18.73	1.08	8.46	9.15	0.59	99.69	105.06	5.70
	Urea		72.90	77.126	4.31	17.81	18.71	1.19	8.53	9.05	0.70	99.06	103.80	6.06
		Winter	73.32	76.899	3.62^{c}	16.38^{b}	17.17^{b}	0.87	6.98	7.30	0.43	96.59^{b}	100.56^{b}	4.80^{b}
		Spring	74.94	79.122	4.01^{bc}	18.37^{a}	19.52^{a}	1.19	9.43	10.19	0.77	102.53^{a}	107.88^{a}	5.89^{a}
		Summer	74.53	77.511	4.37^{ab}	18.31^{a}	18.92^{a}	1.26	8.56	9.00	0.67	100.77^{ab}	104.16^{ab}	6.17^{a}
		Autumn	71.08	76.168	4.78^{a}	17.99^{a}	19.27^{a}	1.22	9.02	9.91	0.72	97.61^{b}	105.12^{ab}	6.66^{a}
Average data														
	Average		73.47	77.43	4.18	17.69	18.79	1.16	8.51	9.14	0.66	99.37	105.15	5.96
	SEM[1]		0.701	0.786	0.223	0.250	0.266	0.051	0.163	0.191	0.038	0.963	1.113	0.206
Statistics Probabilities (p-value)														
Grazing			0.0232	0.0354	NS	0.0005	NS	NS	NS	NS	NS	0.0002	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Season			NS	NS	0.0033	0.0006	0.0005	0.0025	<.0001	<.0001	NS	0.071	0.008	0.003
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	<.0001	<.0001	NS	NS	NS	NS
N source x Season			NS	NS	NS	NS	NS	0.0389	NS	NS	NS	NS	NS	NS
Grazing x N source x Season			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant; Diff: difference.

Figure 28 - Interaction effect of season and grazing system on butyric acid concentration (mmol/L) from ruminal liquid before incubation of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

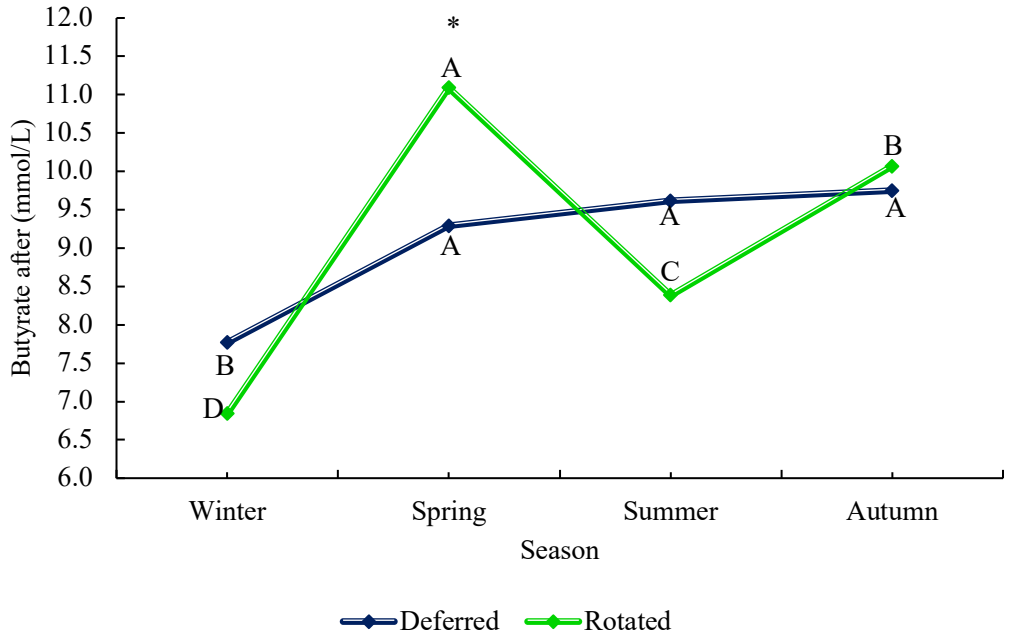


Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05.

Rotated grazing system influenced in higher butyric acid (mmol/L) concentrations before the incubation time within Spring and Autumn when contrasted with deferred grazing. The extent of difference between both grazing methods were 18.5 and 5.5%, respectively, as decomposed in the Figure 28. It is visual the fluctuation of butyric acid (mmol/L) concentrations (Figure 28 and 29) along the season from rumen content of animals under rotated grazing method. As seen in the figure 29, the concentration had an increase of +33.9% from Winter to Spring, a decrease of -17.09% from Spring to Summer and an increase of +11.2% when going to Autumn. That trend was not observed for animals under deferred grazing.

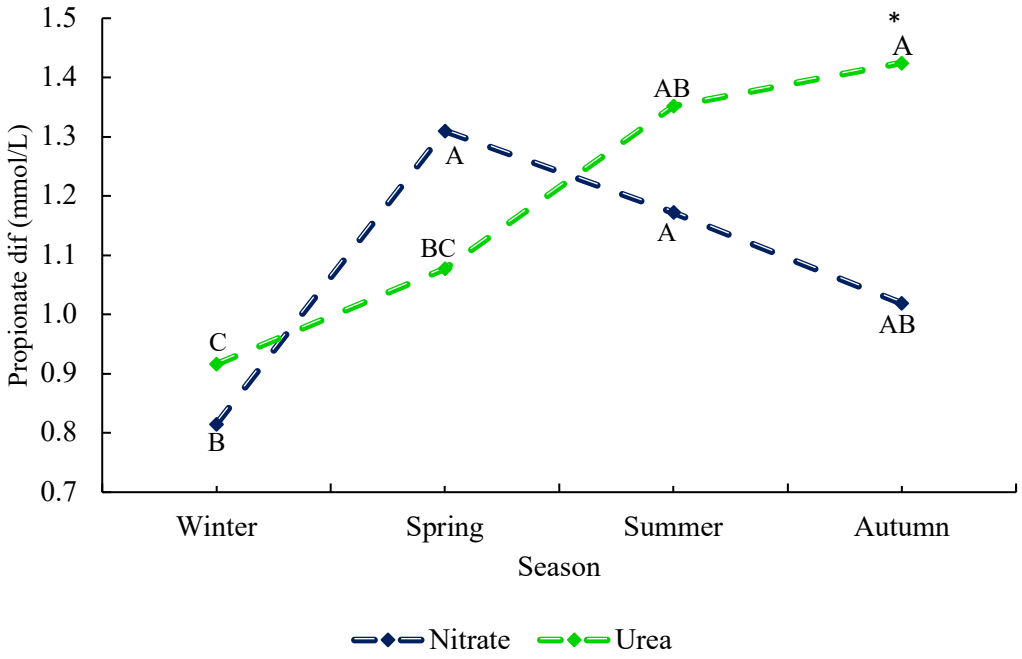
An equal trend was observed for the variable butyric acid (mmol/L) concentrations after incubation, except for a single detail of no effect of grazing within Autumn season, as seen in the decomposition in the Figure 29.

Figure 29 - Interaction effect of season and grazing method on butyric acid concentration (mmol/L) from ruminal liquid after incubation 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

Figure 30 - Interaction effect of season and grazing method on propionic acid concentration (mmol/L) from ruminal liquid difference of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Propionic acid concentration after, before and its difference was clearly affected by season. As seen on Table 16 lower values were detected on ruminal content of animals in Winter as compared to the other seasons. In terms of total short chain fat acids production, there was also a season effect, with lower values observed in Winter as compared to the other seasons. Despite of that, the concentration of propionic acid to the variable difference (mmol/L) had an interaction effect of nitrogen source and season (Figure 30), in which greater propionic concentration was observed within Autumn when animals were supplemented urea as the main NPN source.

On Table 17 it is displayed the obtained data for short chain fatty acids (SCFA) production from ruminal content of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons and year.

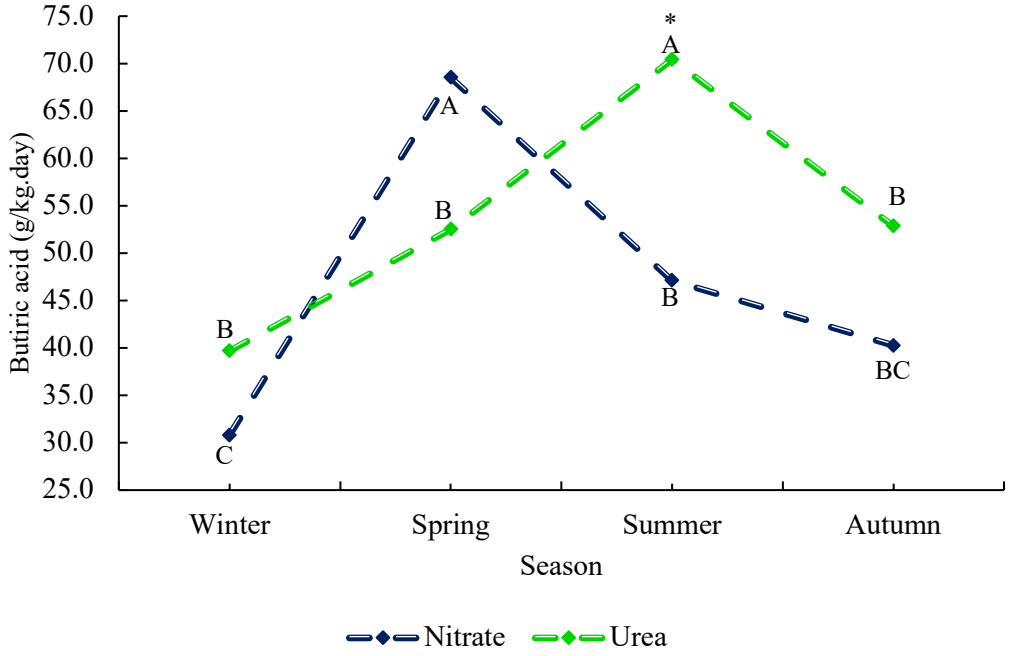
An interaction effect was detected for the variable butyric acid (g/kg.day), in which urea seemed to strongly increase butyric acid production within Summer, as seen in the decomposition depicted in the Figure 31. Overall, for short chain fatty acid production in g/kg.day, it was noticed strong season effect for acetic, propionic, butyric, and total short chain fatty acids production. Acetic acid had higher production in Summer and lower in Winter, remarkably different from the other products.

Table 17 - Short chain fatty acids (SCFA) production from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed factors			Variables							
			Acetic	Prop	Butyric	Total	Acetic	Prop	Butyric	Total
Grazing	N Source	Season	(mmol/g.day)	(mmol/g.day)	(mmol/g.day)	(mmol/g.day)	(g/kg.day)	(g/kg.day)	(g/kg.day)	(g/kg.day)
Deferred			3.73	0.98	0.61	5.22	224.23	69.97	51.42	338.28
Rotated			3.70	1.01	0.63	5.20	222.36	72.30	49.11	340.22
	Nitrate		3.61	0.95	0.55	4.99	217.37	68.37	46.66	325.80
	Urea		3.81	1.04	0.69	5.44	229.22	73.90	53.86	352.70
		Winter	3.01^{c}	0.73^{c}	0.42	4.08^{c}	182.91^{c}	53.64^{c}	35.23	264.63^{c}
		Spring	3.37^{bc}	1.01^{b}	0.71	4.99^{bc}	202.73^{bc}	74.09^{b}	60.52	329.76^{b}
		Summer	4.52^{a}	1.23^{a}	0.67	6.37^{a}	271.26^{a}	91.19^{a}	58.76	416.68^{a}
		Autumn	3.94^{ab}	1.01^{b}	0.69	5.42^{ab}	236.28^{ab}	65.61^{bc}	46.55	345.93^{b}
Average data										
	Average		3.73	0.99	0.60	5.23	224.31	71.26	49.77	340.84
	SEM[1]		0.150	0.045	0.035	0.213	8.981	3.245	2.643	13.747
Statistics Probabilities (p-value)										
Grazing			NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS
Season			0.0071	0.0001	0.0012	0.0007	0.0017	<.0001	0.0002	0.0006
Grazing x N source			NS	NS	NS	NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	NS	NS	NS	NS
N source x Season			NS	NS	0.0131	NS	NS	NS	0.0119	NS
Grazing x N source x Season			NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant.

Figure 31 - Interaction effect of season and nitrogen source on butyric acid production (g/kg.day) from ruminal liquid after incubation of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05

Fermented products' gross energy

In the Table 18 it is described the mean values for fermented products' gross energy from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons. An interaction effect was observed for the variable butyric acid (kcal/kg.day) and its decomposition is unfolded and depicted in the Figure 33. Higher gross energy as butyric acid (kcal/kg.day) within Summer (+28.5%) and Autumn (+23.8%) were generated when animals were subjected to rotated pastures as opposed to animals in deferred grazing systems. Methane expressed as gross energy (kcal/kg.day) also had an interaction effect from grazing method and season. In the decomposition is possible to identify that higher methane crude energy (+21.1%) is generated when animas are kept in deferred grazing systems within Summer.

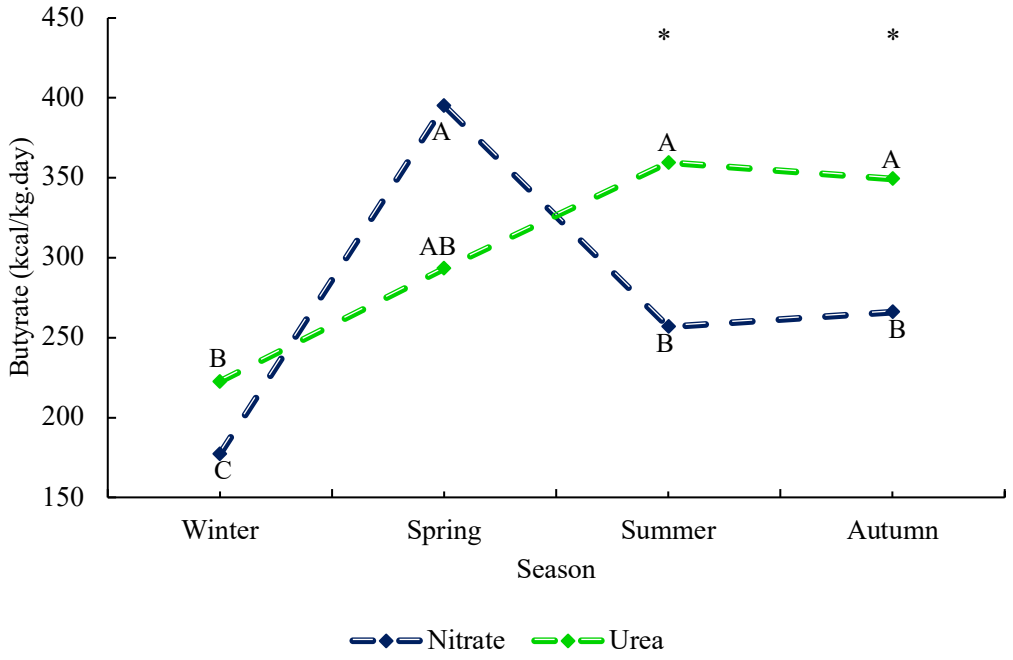
It is noteworthy the mention of the constant increase in methane crude energy from Winter to Summer within deferred grazing. It was observed an overall increase of 36.70%, from 220.34 to 348.10 kcal/kg.day.

Table 18 - Fermented products' crude energy from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed factors			Variables				
Grazing	N Source	Season	Acetic (kcal/kg.day)	Propionic (kcal/kg.day)	Butyric (kcal/kg.day)	Total (kcal/kg.day)	Methane (kcal/kg.day)
Deferred			791.37	356.53	306.37	1434.59	295.72
Rotated			742.36	340.81	294.02	1304.54	263.68
	Nitrate		769.57	334.49	277.01	1318.93	273.31
	Urea		764.16	362.86	323.39	1420.2	286.09
		Winter	665.66^{c}	277.30^{c}	204.74	1078.05^{c}	202.25
		Spring	727.14^{bc}	343.38^{bc}	354.28	1357.96^{b}	304.55
		Summer	873.27^{a}	395.39^{a}	318.32	1494.41^{ab}	314.77
		Autumn	801.39^{ab}	378.62^{ab}	323.45	1547.85^{a}	297.23
Average data							
	Average		772.36	353.78	297.49	1397.02	280.93
	SEM		24.767	13.998	15.275	48.019	8.299
Statistics Probabilities (p-value)							
Grazing			NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS
Season			0.003	0.0002	0.0001	<.0001	<.0001
Grazing x N source			NS	NS	NS	NS	NS
Grazing x Season			NS	NS	NS	NS	0.017
N source x Season			NS	NS	0.0079	NS	NS
Grazing x N source x Season			NS	NS	NS	NS	NS

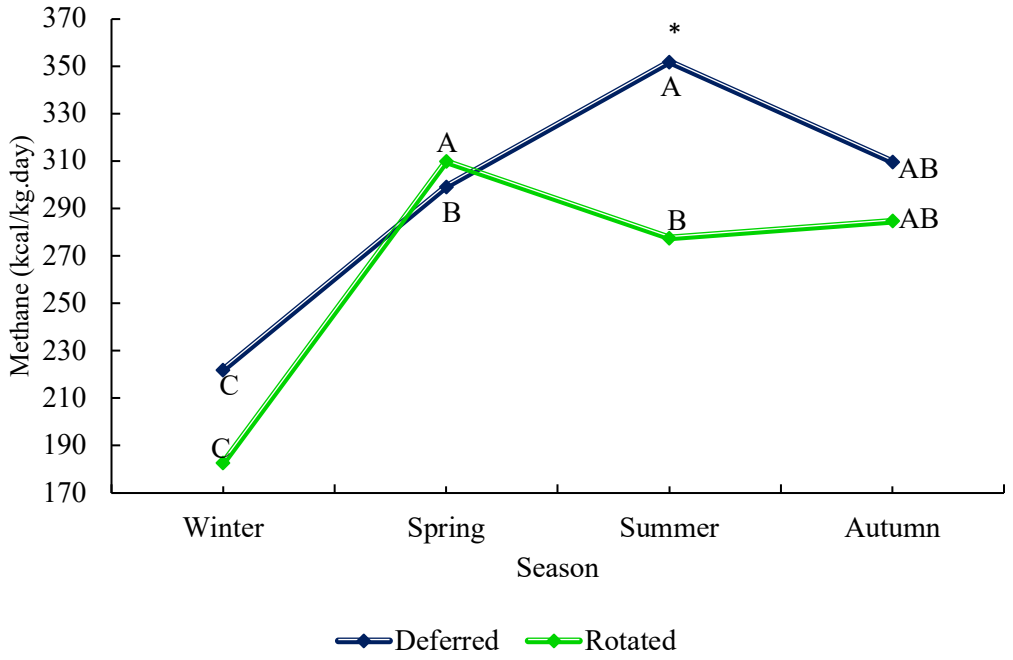
^{a,b,c} Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant.

Figure 32 - Interaction effect of season, and nitrogen source on butyric acid crude energy kcal/kg.day 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

Figure 33 - Interaction effect of season, and grazing method on methane crude energy kcal/kg.day 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

Methane yield

Significant effect of season, nitrogen source and interaction of grazing method and season were detected, as seen on the table 18. Methane yield per g/kg.day was affected by the nitrogen source in which was notably the nitrate effect on reduction of methane yield by 13.1% as seen on Table 19. 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 (g/kg.day).

Table 19 - Methane yield from ruminal liquid of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

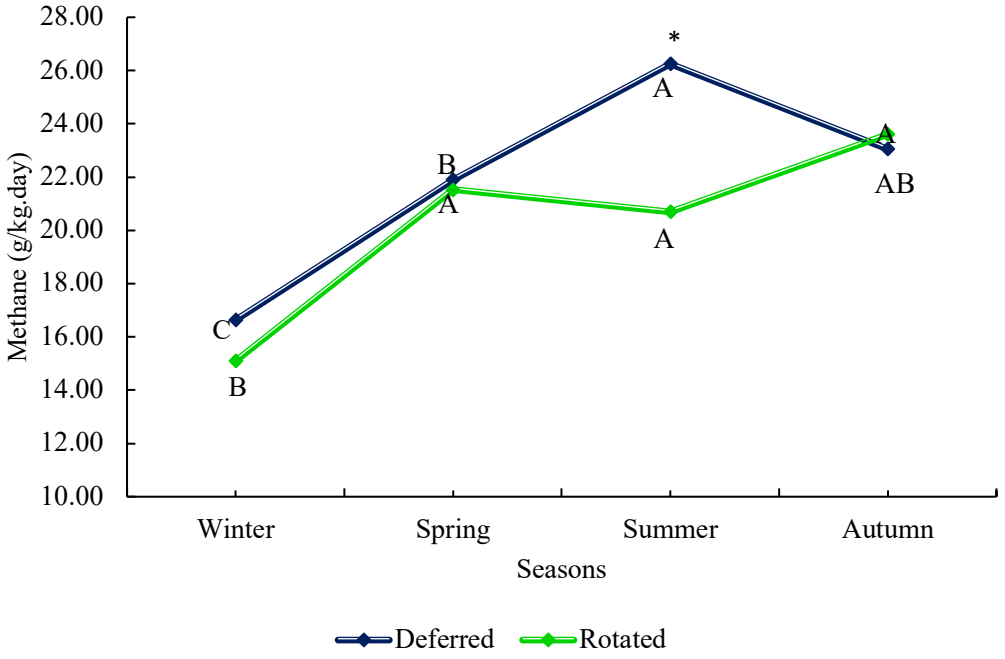
Fixed factors			Variables			
Grazing	**N Source**	**Season**	**CH_4 (mmol/g.h)**	**CH_4 (mmol/kg.day)**	**CH_4 (g/kg.day)**	**REL (%)**
Deferred			0.057	1.36	21.71	18.82
Rotated			0.052	1.27	20.24	20.32
	Nitrate		0.050	1.22	19.51	18.81
	Urea		0.059	1.40	22.44	20.33
		Winter	0.041	0.99	15.77	17.56
		Spring	0.056	1.33	21.32	19.56
		Summer	0.061	1.47	23.47	20.73
		Autumn	0.061	1.46	23.33	20.44
Average data						
Average			0.06	1.32	21.11	19.05
SEM			0.002	0.040	0.638	0.415
Statistics Probabilities (*p*-value)						
Grazing			NS	NS	NS	NS
N source			0.0054	0.011	0.0112	0.0231
Season			<.0001	<.0001	<.0001	0.0013
Grazing x N source			NS	NS	NS	NS
Grazing x Season			0.0399	0.0092	0.0090	<.0001
N source x Season			NS	NS	NS	0.0114
Grazing x N source x Season			NS	NS	NS	0.0018

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; REL: Relative Energy Loss (%); NS: not significant.

Methane (g/kg.day) had interaction effect of grazing method and season, which was decomposed and depicted in the figure 35. Animals produced 21.15% more methane under deferred grazing method when compared to animals kept in rotated grazing systems within Summer.

Despite of season being a secondary effect, it was very noticeable the increase in methane production over the season, as seen in the figure 35. Animals under rotated grazing method increase production in 30% from Winter to Spring, while animals under deferred grazing method had an increase of 24.1%, which kept increasing until Summer, moment which animals under rotated grazing had a big drop in methane production.

Figure 34 - Interaction effect of season and grazing method on methane g/kg.day production from ruminal content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

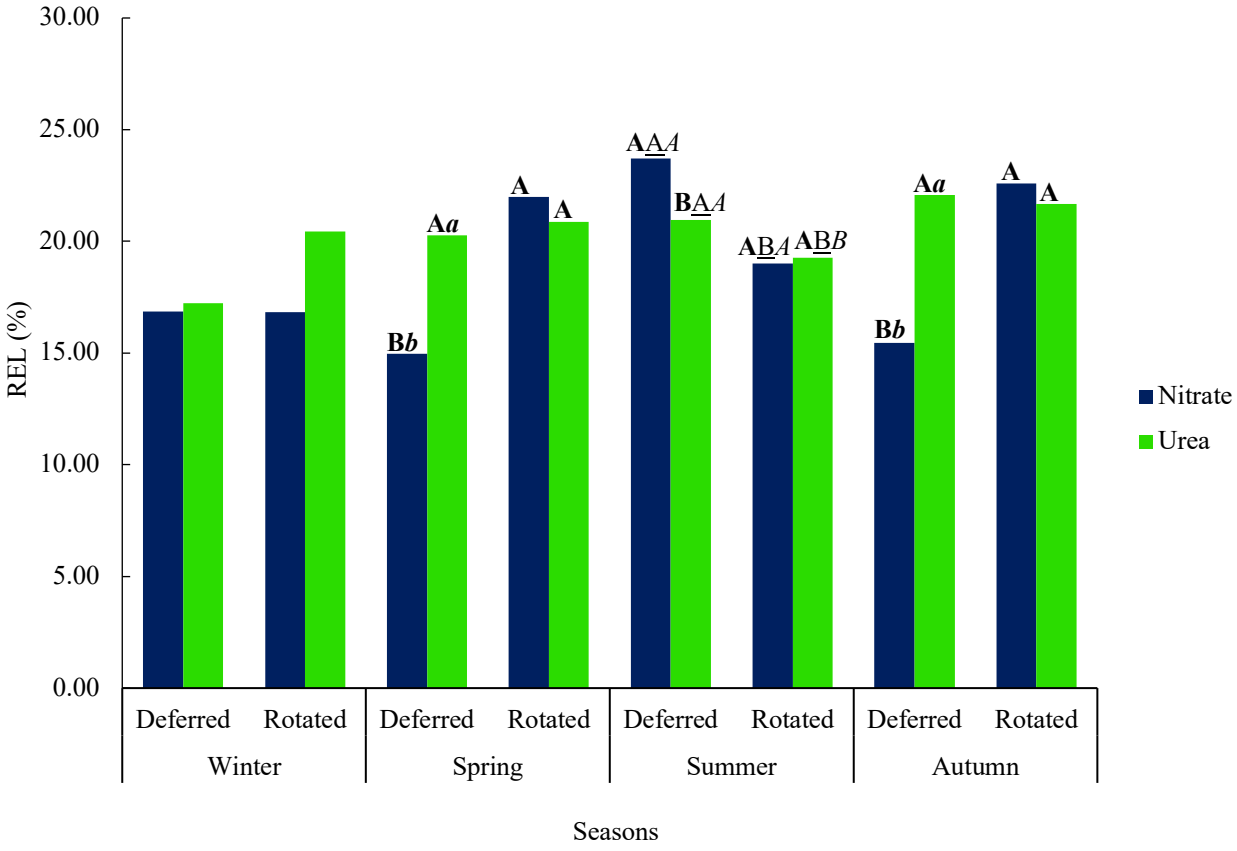


Capital letters within grazing systems differs at P<0.05; * indicates statistical difference within season at P<0.05

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.

The interaction for relative energy loss was decomposed (Figure 37) and it was noticed that when animals were fed nitrate, as the main source of non-protein nitrogen, under deferred grazing systems, lower amount of energy was lost as compared to animals kept fed urea, within Spring season (Figure 37). When contrasted the effect of grazing method within nitrogen source (urea or nitrate) no major effect was observed.

Figure 35 - 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.



Different bold capital letters within season indicates difference between nitrogen sources at P<0.05. Different capital underlined letters within season indicates difference between grazing system at P<0.05. Different italic capital letters within nitrate indicates difference between grazing at P<0.05 (within season) at P<0.05. Different capital letters in italics underlined within urea indicates difference between grazing (within season) at P<0.05

That same trend was not observed when the data was assessed within Summer, in that occasion, animals fed urea showed to be more efficient. Animals under rotated grazing systems also displayed lower loss relative energy (%) when compared to animals under deferred grazing fed nitrate and/or urea.

When animals were fed nitrate under rotated grazing systems, it was possible to observe a reduction of 32.8% in relative energy loss (%) when compared to animals under deferred grazing within Summer season. The same trend was detected when studied the urea slice comparing both grazing systems; however, in a lower intensity with 19.7% less relative energy loss for animals under rotated grazing within Summer. Nitrate seemed also to reduce the energy loss of animals kept in deferred grazing in the seasons Spring and Autumn. The extent of reduction was 20.03 and 24.23%, respectively, when compared to animals fed urea, as the main source of non-protein nitrogen (Figure 35).

Ammonia nitrogen concentration (NH_3-N)

Total ammonia nitrogen concentration from ruminal liquid of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons are found on Table 20. It can be seen a consistent, and already expected effect of season upon the variable concentration of NH_3-N in mg/dL at 0 min and 30 min of fermentation. No treatment or season effect was detected to the NH_3-N balance. Despite of that, interaction effect was observed for concentration of NH_3-N in mg/dL at 0 min and 30 min (Figures 36).

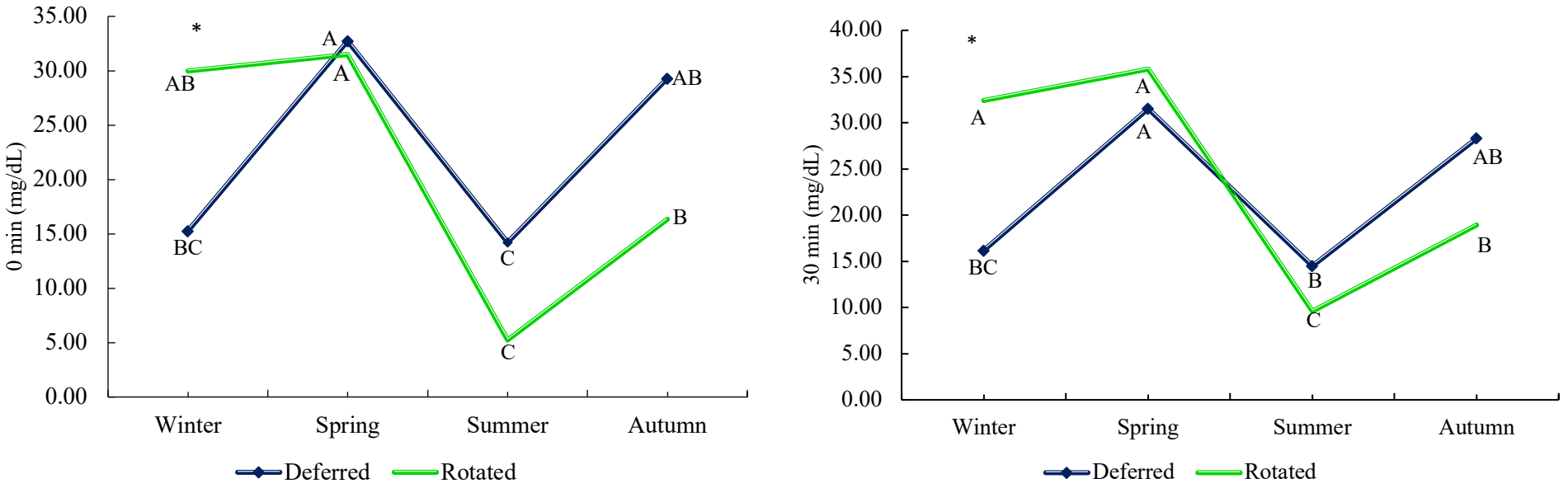
Table 20 - Concentration and balance of ammonia nitrogen (NH_3-N) from ruminal content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Fixed factors			Variables		
			Concentration		
Grazing	**N Source**	**Season**	**0 min (mg/dL)**	**30 min (mg/dL)**	**Balance (mg/dL)**
Deferred			23.16	22.61	1.62
Rotated			20.20	24.17	1.91
	Nitrate		20.60	21.57	0.84
	Urea		22.75	25.21	2.69
		Winter	22.05	24.27	3.28
		Spring	32.11	33.65	1.94
		Summer	9.72	12.04	0.41
		Autumn	22.82	23.59	1.44
Average data					
	Average		22.05	23.8	2.22
	SEM		2.026	2.101	0.762
Statistics Probabilities (p-value)					
Grazing			NS	NS	NS
N source			NS	NS	NS
Season			0.0003	0.0018	NS
Grazing x N source			NS	NS	NS
Grazing x Season			0.0070	0.0045	NS
N source x Season			NS	NS	NS
Grazing x N source x Season			NS	NS	NS

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test..
NH_3-N: ammonia nitrogen, N Source: nitrogen source; SEM: Standard error of mean; NS: not significant.

Animals under rotated grazing systems within Winter season had 59.15 and 50.09 more ammonia nitrogen concentration at the time 0 and 30min mg/dL when compared to ruminal content of animal under deferred grazing.

Figure 36 - Interaction effect of season and grazing method for ruminal ammonia nitrogen concentration mg/dL production 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

Energy partitioning

Grazing method interacted with season of the year over the variable energy release in the rumen in form of acetic acid (Mcal/cow) as seen in the decomposition depicted on the Figure 37.

Animals under rotated grazing within Summer and Autumn season displayed higher release of energy (+27.8 and 21.7%, respectively) into the rumen in form of acetic acid when compared to animals kept in deferred pastures. In the other seasons it was not found significative effect of treatment.

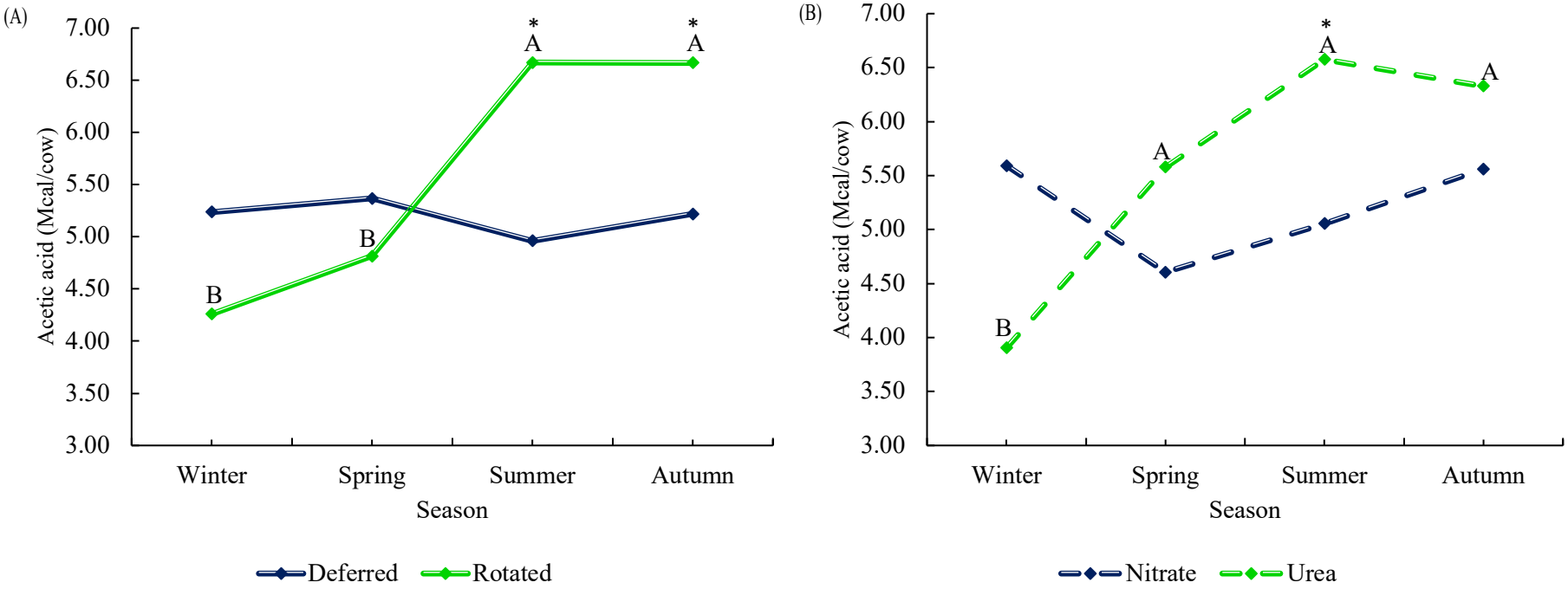
Nitrogen source also interacted with season (Table 21) and its decomposition presented in the Figure 37 showed that urea had effect over energy release in the rumen in the form of acetic acid within Summer with 23.13% more energy release into the rumen.

Overall, total short chain fatty acids (SCFA) also had interaction effect for grazing system and season of the year for energy release in the rumen expressed as Mcal/cow in which animals kept in rotated pasture displayed higher (20.26%) energy release in the rumen (Mcal/cow) within Summer, as opposed to animals under deferred grazing system.

Rotated grazing pastures influenced in higher release of energy into the rumen in the form of butyric acid as Mcal/cow, gross energy (GE%) and digestible energy (DE%) by 22.5, 26.9 and 28.55%.

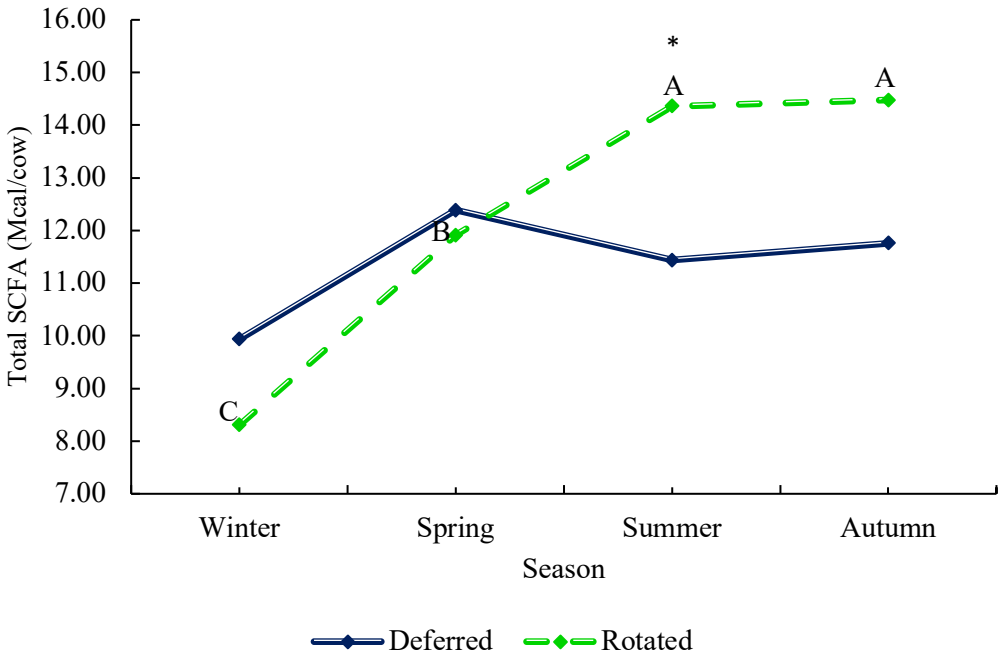
Nitrate promoted about 17.2% less digestible energy release into the rumen in for of methane as compared to urea (Table 21).

Figure 37 – (A) Interaction effect of season and grazing and (B) interaction effect of season and nitrogen source for ruminal energy release as acetic acid (Mcal/cow) 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 method (A) or nitrogen source (B) differs at P<0.05; * indicates statistical difference within season at P<0.05

Figure 38 – Interaction effect of season and grazing and effect of season and nitrogen source for ruminal energy release as total SCFA (Mcal/cow) 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

Table 21. Estimative of energy release from fermentative products of ruminal content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons.

Variables, day	Grazing		N Source		Season				Statistics Probabilities (*p*-values)						
	Deferred	Rotated	Nitrate	Urea	Winter	Spring	Summer	Autumn	G	N	S	G*N	G*S	N*S	G*N*S
Rumen mass, kg	6.63	7.04	6.63	7.04	6.90	7.02	6.61	6.80	NS	NS	NS	NS	0.0060	0.0090	NS
GEI, Mcal	34.32	34.49	34.64	34.17	28.30^{b}	26.48^{b}	41.43^{a}	41.42^{a}	NS	NS	<.0001	NS	NS	NS	NS
Energy release in the rumen															
Acetic acid															
Mcal/cow	5.18	5.57	5.14	5.61	4.75^{c}	5.04^{bc}	5.81^{ab}	5.88^{a}	NS	NS	0.0458	NS	0.0121	0.0233	NS
GE, %	15.28	16.59	15.01	16.90	15.88^{ab}	19.53^{a}	14.458^{b}	13.88^{b}	NS	NS	0.0207	NS	NS	NS	NS
DE, %	22.86	24.72	21.93	25.65	25.44a^{b}	30.47^{b}	19.99^{b}	19.26^{b}	NS	NS	0.0116	NS	NS	NS	NS
Propionic acid															
Mcal/cow	2.45	2.26	2.21	2.51	1.83^{b}	2.39^{a}	2.70a	2.51a	NS	NS	0.0022	NS	NS	NS	NS
GE, %	6.95	7.81	6.78	7.98	6.65^{b}	9.49^{a}	6.91^{b}	6.45^{b}	NS	NS	0.0039	NS	NS	NS	NS
DE, %	10.37	11.19	9.85	11.71	10.18^{b}	13.54^{a}	10.38^{ab}	9.02^{b}	NS	NS	0.0119	NS	NS	NS	NS
Butyric acid															
Mcal/cow	2.03	2.00	1.76	2.27	1.33^{b}	2.35^{a}	2.18^{a}	2.20^{a}	NS	0.0484	<.0001	NS	NS	NS	NS
GE, %	5.95	6.06	5.08	6.93	4.66^{b}	8.73^{a}	5.22^{b}	5.41^{b}	NS	0.0220	0.0006	NS	NS	NS	NS
DE, %	9.12	9.55	7.78	10.89	7.53^{b}	14.62^{a}	7.54^{b}	7.65^{b}	NS	0.0110	0.0175	NS	NS	NS	NS
Total SCFA															
Mcal/cow	11.38	12.26	10.98	12.66	9.12^{b}	12.15^{a}	12.90^{a}	13.12^{a}	NS	NS	0.0009	NS	0.0381	NS	NS
GE, %	33.59	37.08	33.18	37.49	31.66^{b}	45.72^{a}	32.07^{b}	31.90^{b}	NS	NS	0.0006	NS	NS	NS	NS
DE, %	49.00	49.97	48.17	50.81	50.01	58.78	44.24	44.93	NS	NS	NS	NS	NS	NS	NS
Methane															
Mcal/cow	1.96	1.95	1.84	2.07	1.42^{b}	2.24^{a}	2.05^{a}	2.10^{a}	NS	NS	<.0001	NS	NS	NS	NS
GE, %	5.75	6.10	5.56	6.30	5.03^{b}	8.31^{a}	5.05^{b}	5.32^{b}	NS	NS	0.0005	NS	NS	NS	NS
DE, %	8.63	9.21	8.16	9.68	8.19^{b}	13.04^{a}	6.99^{b}	7.47^{b}	NS	0.0440	0.0005	NS	NS	NS	NS
Energy release in the intestine															
Mcal/cow	13.40	13.41	13.26	13.55	7.71^{b}	9.45^{b}	17.71^{a}	18.75^{a}	NS	NS	0.0004	NS	NS	NS	NS
GE, %	34.53	35.47	37.19	32.81	27.59^{b}	28.39^{b}	41.33^{a}	42.69^{a}	NS	NS	0.0034	NS	NS	NS	NS
DE, %	49.43	50.32	51.45	48.30	43.08^{b}	44.17^{b}	55.56^{a}	56.67^{a}	NS	NS	0.0396	NS	NS	NS	NS
Energy release in the feces															
Mcal/cow	10.64	10.60	9.91	11.34	10.78^{a}	9.33^{b}	11.31^{a}	11.06^{a}	NS	NS	0.0084	NS	NS	NS	NS
GE, %	31.86	32.66	30.10	34.42	38.50^{a}	36.08^{a}	27.14^{b}	27.39^{b}	NS	NS	<.0001	NS	NS	NS	NS

^{a,b,c} Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. NS: not significant. G: grazing; N: nitrogen source; S: season; G*N: interaction of grazing and nitrogen source G*S: interaction of grazing and season; N*S: interaction of nitrogen source and season; G*N*S: interaction of grazing, nitrogen source and season.
SEM: standard error of mean; SCFA: Short chain fatty acids; GEI: gross energy intake; GE: gross energy; DE: digestible energy.

Discussion

Parameters of ruminal pH

Higher ruminal pH for ruminal content of beef cattle fed nitrate as compared to those fed urea, as the main nitrogen supplementation source, is a trend vastly reported on literature (Tomkins, 2018; Allemu et al., 2019; Rebelo et al., 2019; Granja-Salcedo et al., 2019 and Villar, 2020). The increase in the rumen pH, observed in our study, can be explained by the fact that the reduction of nitrate to nitrite and then to ammonium capitalizes available hydrogen from the rumen environment.

Rumen pH is a clear indicator of an active ruminal fermentation and its whole microbiome, and the different diet regime will directly influence the dynamics of the pH in the rumen since fermented end products are changed (Wanapat et al., 2020). As previously showed in the chapter two, the intake of nutrients (kg/DM) were not affected by treatment and consequently, the observed effect over pH is mainly due the treatments adopted. Animals under rotated grazing had lower ruminal min pH when compared to animals kept in deferred pastures. This find is due to higher amount of digestible carbohydrates available, specifically from the tip of leaves, where it is usually found in higher concentration by animals. When cattle are rotated back to a deferred rested pasture, leaves are supposed to be in the ideal height and maturity stage, which means that it tends to be more digestible and nutritious compared to a more lignified and mature plant. Forages with higher concentration of soluble carbohydrate tend to be more digestible, and that directly affect the ruminal pH, since the intake of high amount of tip of leaves can cause a rapid fermentation, less need of rumination and incorporation of saliva and inorganic compounds into the rumen, higher generation of fermented products. All that associated with lower retention time of the material in the rumen can lead to lower pH values.

Nitrate is a suitable option when it comes to a daily pH adjustment. As it was observed, animals fed ammonium nitrate tend to go through less stress related to lower ruminal pH and the intensity of it when compared to the use of urea. In our findings animals fed urea had more than twice the time (2.37 times) in which its ruminal pH remained below 6.4 (h.pH/day) when compared to animas supplemented with nitrate.

Rumen pH values of Nellore cattle under different grazing systems having different nitrogen sources along different seasons displayed effect for season with higher mean and min pH values during Spring and lower values in Summer and Autumn, as seen on Table 15. In both

season (Summer/Autumn) the forage had lower mean values for ADF and lignin (Table 4), which might have led to an accelerated fermentation of the ruminal content, and faster ruminal passage rate.

Fermented products in the rumen

Rumen works as fermentative chamber in abscess of oxygen, which provides ideal condition to the presence, activity and growth of anaerobic microorganisms that are 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. Short chain fatty acids production, such as acetic, propionic, and butyric, are the main subproducts generated by rumen fermentation and, depending on diet profile and therefore microbiota colonization, the production of SCFA will rely on that.

It was expected higher rumen fermentation and generation of fermented products over the Summer season. In this same season animals under rotated pasture had greater rumen mass (kg), seen in the previous chapter, possibly influenced in greater rumen fermentation activity leading to higher acetic acid (mmol/L) concentrations (before and after incubation), as seen on table 16, and reflected on higher release of energy in the rumen in form of acetic acid (Mcal/cow) within Summer and Autumn season as observed in the decomposition on the Figure 40. Since both grazing methods are prone to influence in higher acetate concentration when compared to propionic and butyric acids, the higher concentration of acetic acid, and thus, the release of energy in the rumen as acetic acid was probably due to the higher nutrient digestibility of the pastures from rotated grazing.

We hypothesize that there was a higher synergistic effect of supplementation over the metabolism of structural carbohydrates (CHOs) in Summer season, moment which forage already has lower concentration of structural CHOs making the plant cell more digestible, and thus higher release of energy as SCFA (Mcal/cow) into the rumen would happen. Under grazing systems, there is a higher prevalence of cellulolytic and hemicellulolytic bacteria in the rumen of beef cattle, such as *Ruminoccocus, Rikenellaceae RC9 gut group, Streptococcus and even Lachnospiraceae* depending on supplementation. However, the number of them can increase even more when protein/energetic supplementation is used, which can lead to better digestibility of the diet and improving the fermentation's outcome. In fact, some of the previous genera and bacteria are known for being able to withstand higher concentration of nitrate and even reduce nitrate up to ammonia.

Despite of no statistical effect for acetic acid production (g/kg.day) when animals were supplemented with nitrate, the release of energy in the rumen in the form of acetic acid (Mcal/cow) was statistically significant and showed a reduction of 23.13% when cows were supplemented with nitrate within Summer season.

For the concentration of propionic acid difference (mmol/L) nitrate caused a reduction of 28.8% when compared to urea within Autumn. Despite of being a very punctual effect, propiogenesis is known for being one of the alternatives to remove H_2 generated by the phosphorylation process (Ungerfeld, 2020) and suppling the ruminant metabolism with glucose by means propionate production. Thus, if molecular hydrogen is being used by nitrate reduction, less precursors for propiogenesis will be available and a reduction of this fermented product might happen, similarly as observed by Duthie et al. (2018) that observed reduction of 12% in propionate concentrations with beef cattle fed diet 55:45 (V:C) with inclusion of 21.5g of nitrate/kg of DM. Capelari (2018), also found a reduction in propionate production by 8% as compared to control treatment. The author worked with beef cattle fed mixed ratio with inclusion of 15g of nitrate/kg of DM.

Butyric acid concentrations (mmol/L) (before and after incubation) also had greater values within Spring and Autumn, when animals were in rotated grazing system. Possibly, an increase in bacteria from Clostridium family could have led that higher concentration of butyrate. Miguel et al. (2019) showed that Clostridium bacteria can use a wide range of carbohydrates such as polymers starch xylan and saccharides such as glucose, arabinose, xylose, and cellobiose. Higher availability of this compound in the rumen might lead to a rumen bacteria shift and thus substrates used for acetate production can be metabolized and generate butyric acid. Looking closely to synthesis of Butyrate, we can understand that it is a four-carbon molecule that to be formed requires two pyruvates, losing one carbon each as CO_2 or formate. From two molecules of acetyl-CoA, Acetoacetil-CoA is generated, which is reduced by means a dehydrogenase-enzyme, generating beta-hydroxybutyril-CoA. This last molecule is then converter into Crotonil-CoA by crotonase, which it is then dehydrated and reduced, eliciting butyril-CoA. This last loses the CoA group and gains a phosphate (Butyril-phosphate), which is then dephosphorylated releasing butyrate and producing ATP (Kozloski, 2009).

Despite of the previous finding and effect of grazing over the butyric synthesis, we also noticed that when animals were supplemented with urea within the Summer season, it was observed an increase of 33% of butyric acid production (g/kg.day) (Figure 32), which lead to greater gross energy (kcal/kg.day) and thus more release of energy into the rumen as butyric

acid (Mcal/cow) (Table 21). According to Wayono et al. (2022) urea supplementation has a significant role in enhancing microbial protein synthesis in the rumen and overall flow of nitrogen to the gastrointestinal tract. The synergistic effect of having a pasture with higher energetic content added by the urea supplement intake over the Summer resulted in our findings.

As observed in our assay, Tomkins et al. (2018) also working with *Bos indicus* steers fed high forage diet with inclusion of nitrate (4.6 and 7.9 g/kg of DM), encountered a reduction of butyrate of 13.2%. Henry (2017), observed a reduction of 11.9% of butyrate production when beef cattle were kept in grazing systems.

Looking at the statements by Natel et al. (2022), Wayono et al. (2022) and specially Ungerfeld, (2020), it is reasonable to understand that when animals are supplemented with nitrate, a hydrogen sinker, greater part of the molecular hydrogen it is used as a precursor to the reduction of nitrate into ammonia (Lan & Yang, 2019) and thus reduce methane production as well. This statement can be supported by the fact that when animals were supplemented by nitrate, methane production (g/kg.day) reduced by 13.1% (Table 18), which led to a reduction of energy release into the rumen (Mcal/cow) by 15.7% (Table 21) when compared to animals supplemented with urea.

Our findings agree with a range of work on literature (Hulsholf et al., 2012; Newbold et al., 2014; Lee et al., 2015; Troy et al., 2015; Duthie et al., 2018; Tomkins et al., 2018; Capelari 2018 and Alemu et al., 2019) in which authors also assessed different inclusion of nitrate in cattle's diet. However, none of the cited paper conducted an assay in intensified grazing systems. On average, the previous cited work had an inclusion of 12.5 g.kg/DM and a methane reduction of 16%.

When it comes to methanogenesis inhibition, the use of nitrate rapidly reduced to nitrate and that significantly contributes to the reduction of enteric methane by consuming available hydrogen in the ruminal environment. This path, is thermodynamically more favorable as compared to CO_2 reduction to methane (Ugerfeld et al., 2020). The idea of incorporating H_2 into electron sinks that are nutritionally beneficial to beef cattle can be an important path that may reduce digestible energy losses from gas production (Lan & Yang, 2019). Corroborating with that, as observed in the decomposition of the triple interaction (Figure 37) for the variable Relative Energy Loss (%), nitrate influenced in a reduction of energy loss of 26.11% when animals were under deferred grazing systems within Spring season.

When nitrate was the sole source of supplementation within Summer, it was possible to see that deferred grazing systems lead to a higher relative loss energy of 25.59% when

compared to rotated grazing system of 17.17%. That shows how important is the adoption of a grazing method that allows animal to access higher forage quality, with higher concentration of nutrients and more digestible as well.

Our findings show that intensified rotated grazing systems led to a reduction of 21.3% of methane yield (g/kg.day) within Summer, and this reduction was mainly attributed to an improved quality of the forage during the Summer season. According to Eugene et al. (2021) when animals are submitted to pastures with great amount of mature cell wall and lower content of soluble carbohydrates, it leads to forages generated higher acetate production in the rumen, which is known to be a pathway that furnish methane production as the acetate synthesis releases molecular hydrogen in the rumen.

On the other hand, when forage-based diet is more digestible, in the case of rotated pastures, especially during Summer season, there is a greater number of new leaves with higher concentration of nutrients, lower concentration of recalcitrant material and greater in vitro digestibility (Table 4), therefore, the diet tend to be more digestible, which lead to lower production of acetate when compared to propionate. This last, a known metabolic route that does not release H_2 into the rumen but in fact it uses it. Moreover, considering all the above, the ruminal content tends to have lower retention time in the rumen, implicating then in less fermentation and, therefore lower production of methane.

Besides methane reduction, the adoption of nitrate can foment the synthesis and growth of bacteria in the rumen by means the incorporation of ammonia nitrogen while nitrate is reduced to ammonia. When nitrate is reduced to ammonia nitrogen, theoretically, it is expected a higher flow of negative Gibbs free energy that is incorporated into the rumen and furnish energy for microorganism's growth (microbial protein synthesis), transport of substrate and mobility (Ungerfeld, 2020). Despite of that, no effect of nitrate over the variables NH_3-N concentration at 0 and 30 min of fermentation were observed (Table 21), neither for the microbial protein synthesis (Table 13).

On our assay, animals had total ammonia concentration above the minimum required to an ideal rumen activity, with an overall concentration average of 22.05 and 23.80 mg/dL at 0 and 30 minutes of fermentation. Animals kept in rotated grazing within Winter season displayed higher ruminal ammonia concentration (mg/dL), at the time 0 and 30 after fermentation, when compared to animals in deferred grazing method. The greater supplement intake in Winter season might have contributed to the higher NH_3-N concentration (mg/dL), especially for

animals under rotated grazing. Deferred pastures have greater forage mass availability and the quality tend to be superior in Winter as well.

Conclusion

Based on the findings of this study, it is possible to indicated that rotated grazing systems and ammonium nitrate are suitable tools to perform beef cattle production in a more intensified and sustainable way.

None of grazing methods compromised the concentration of ammonia nitrogen in the rumen, however, animals under rotated grazing method improved the overall short chain fatty acids concentration and production, which can then furnish the ruminants' energetic needs with greater amount of energy release in the rumen. Mitigation of enteric methane yield was also observed when animals were subjected to rotated grazing over the Summer season.

Coupled with rotated grazing, ammonium nitrate showed to be an appealing technology to be adopted when it comes to reduction of methane yield in beef cattle production. It can effectively contribute to the reduction of ruminal methane production and partially contribute to mitigate the negative impact from beef cattle production in tropical regions.

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CHAPTER 4 – Effect of intensified grazing systems with adoption of ammonium nitrate or urea on ruminal protozoa counting and microbiome of Nellore beef cattle during different seasons.

Abstract

This study aimed to investigate the rumen microbiota of Nellore beef cattle in intensified grazing systems with adoption of a non-protein nitrogen supplementation across different seasons. Over a two-year trial period, rumen samples were collected from eight cannulated Nellore cattle distributed among eight experimental units with four different treatments, which were formed by the combinations of two grazing methods (rotational or deferred) with supplementation of either ammonium nitrate or urea. They were as follows: 1) Rotational grazing system with urea supplementation; 2) Rotational grazing system with nitrate supplementation; 3) Deferred grazing system with urea supplementation; 4) Deferred grazing system with nitrate supplementation. The samples underwent DNA extraction, library construction, sequencing, and analysis of taxonomical profiles, diversity and richness. Utilizing a metagenomic approach with amplicon sequencing, valuable insights were gained into the independent and combined effects of nitrogen supplementation, grazing methods, and seasons (Winter, Spring, Summer, and Autumn) on the ruminal metabolism of Nellore beef cattle. Data were analyzed using the Mothur, R, and SAS software. The analysis of diversity and richness indicated that the season had the most significant impact, while nitrogen source and grazing method did not influence the Alpha diversity, grazing method did influence the Chao, and animals in rotational grazing displayed greater richness. An interaction effect was observed between the grazing method and nitrogen source, and the deferred grazing plus *nitrate supplementation resulted in higher abundance of the phylum Halobacterota*. Methanosphera exhibited higher relative abundance when nitrate was used as supplementation compared to urea. The family *Methanobactericeace* showed higher relative abundance in the rumen of animals kept in deferred grazing, and nitrate supplementation significantly decreased its abundance. Nitrate supplementation had an impact on specific bacterial and archaeal groups, with *Veillonellaceae-UCG* showing an increase in relative abundance and inhibiting Archaeal microorganisms.

Keywords: beef cattle, GHG emissions, methane sink, 16S rRNA.

Introduction

Facing a variety of external factors, beef production in the tropics deals with many difficulties, and the main one is directly related to low quality of forages and the seasonality, which during dry period, feed sources tend to have lower nutritive value or even be scarce. It can directly influence the ruminal microbiota of beef cattle raised in grazing systems, and ultimately on its performance and contribution to greenhouse gases emission.

Continuous efforts have been done to mitigate GHG emissions caused by beef cattle systems, among them, can be cited: changes in the diet by means the use of ionophores (Beauchemin et al., 2022), tannins (Berça et al., 2023), saponins (Torres et al., 2023), essential oils (Benetel et al., 2022) and lipids (Castañeda-Rodríguez et al., 2023). All that can change ruminal fermentation products by means changing the profile of microorganisms that process the available substrate to furnish useful compounds (Haque, 2018).

The idea of incorporating H_2 into electron sinks that are nutritionally beneficial to beef cattle can be an important path that may reduce digestible energy losses from gas production (Lan & Yang, 2019). Addition of nitrate, for instance, 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.

Studies have demonstrated a notable correlation between the addition of nitrate to the diet and an augmentation in cellulolytic bacteria, leading to improved fiber digestibility (Patra & Yu, 2015). This finding aligns with the research conducted by Zhao et al. (2015), who highlighted the beneficial impact of nitrate on key cellulolytic bacteria from the genus *Ruminococcus*. These results suggest that the presence of nitrate favors the growth and activity of cellulolytic bacteria, which in turn enhances the breakdown and utilization of dietary fiber. In their study, it was observed that the abundance of *Ruminococcus* albus and *Fibrobacter* succinogenes increased linearly with the addition of nitrate, while the abundance of *Ruminococcus flavefaciens* exhibited a quadratic increase. However, contrasting results were reported by Wang et al. (2018) who found no significant effect on fiber digestibility in Holstein cows fed a diet containing 14.6 g of nitrate per kg of dry matter. According to the authors, this finding aligned with the lack of significant changes in the abundance of ruminal fiber-degrading bacteria, as indicated by 16S rRNA gene copies.

There is limited information available on changes in the bacterial community in the rumen of grazing cattle fed with nitrate, as various factors, including the dosage of nitrate,

administration duration, adaptation, and diet composition, can significantly influence the ruminal environment. Certain bacteria in the rumen possess nitrate reductase enzymes, enabling them to utilize nitrate for respiration or incorporate nitrogen into biomass (Besson et al., 2022).

Veillonella and Wolinella bacteria were consistently detected in significant quantities by Iwamoto et al. (2002) in an *in vitro* mixed culture with the addition of nitrate. The authors observed a notable decrease in their presence when nitrate was removed from the media. Additionally, the same research group found that *Selenomonas ruminantium* appears to be tolerant to the toxic effects of nitrate and nitrite, as it primarily derives energy from nitrate reduction. This is also the case for *V. parvula* and *W. succinogenes*, which are recognized as the most efficient nitrate-reducing bacteria. However, the activity of these bacteria declines rapidly in environments with high levels of ruminal fermentation end-products and sugars. Some researchers suggest that *C. fetus* and *M. succiniciproducens* exhibit increased activity when nitrate is added to the diet of ruminants (Lin et al., 2013).

Nitrate in ruminants diet changes the population of microorganisms in the rumen by means serving as substrate to the development of specific species; however, direct inhibition of methanogens may occur as well (Zhao et al., 2018). Zhao et al. (2018), working with steers receiving three different doses of nitrate in diet, observed some changes on the rumen with higher presence of methanogens classes such as *Methanobacteria* and *RCC (Thermoplasmata*). At genera level, *Methanobrevibacter* (*Methanobacteria)* and vadin CA11 from RCC accounted for more than 90% of the total sequences; nevertheless, they also detected prevalent genera in a very less intensity, accounting for 1.25%.

In fact, it is a general sense on literature that more effort must be done to understand the ruminal microbiota from ruminants under grazing supplemented with nitrate to harness more concise explanation regarding nuances on methane emission. Also, up to date microbiota data from beef cattle subjected to grazing systems having different supplementations during different seasons is not available on literature. This sort of data might help us to understand better ruminant metabolism in the tropics over different seasons and thus improve and apply suitable strategies that aims mitigate methane emissions.

In order to understand the effect of specific strategies to modulate ruminal fermentation of Nellore beef cattle and thus, mitigate the negative effect of its activity to the environment, more research is needed within the evaluation of ruminal microbiota of Nellore beef cattle in intensified grazing system in which supplementation is adopted. Obtaining such data would enhance our understanding of ruminant metabolism in the tropics throughout different seasons

and aid in the development and application of effective strategies to mitigate methane emissions.

As already mentioned, changes occurs but, what are the extent of that? How come nitrate modulate ruminal microorganism to favors the uptake of intermediate subproducts towards a valuable product to ruminant's metabolism? A metagenomic approach can be useful to target the microbial community (by means of amplicon sequencing) and provide to us a deep insight of the isolated and combined effect of nitrate, grazing and seasons (Winter, Spring, Summer and Autumn) on Nellore beef cattle's ruminal metabolism.

Thus, the hypothesis is that intensified grazing systems (deferred or rotational grazing systems with nitrate or urea supplementation) changes ruminal microbiota of beef cattle during different seasons (Winter, Spring, Summer and Autumn). It is expected higher prevalence of reducing nitrate and nitrite bacteria, and inhibition of *Archaea* when supplementation with nitrate occurs.

Objective

The objective of this experiment was to investigate the effects of intensified grazing systems (deferred or rotational grazing systems with nitrate or urea supplementation) on the ruminal microbiota of beef cattle during different seasons (Winter, Spring, Summer and Autumn) To assess it, it was investigated the diversity and richness of bacterial and archaeal community, and the relative abundance and differential relative abundance of them.

Material and Methods

Location

The experiment was carried out at College of Veterinary Medicine and Animal Science (FMVZ/USP), Pirassununga, Sao Paulo State, Brazil, for two years, in between June of 2019 and April of 2021. The experimental animals were handled and managed according to the Ethic Committee on Animal Use on Research (FMVZ/USP). A total of 8 Nellore female cows, of approximately 551 ± 7.01 kg of BW were used as experimental animals for rumen fermentation data (cannulated animals).

Experimental design, pasture system and treatments

The experimental animals were randomly allotted to 8 paddocks. Each treatment was allotted to a paddock in a randomized block design (blocks were formed as a function of terrain location) for two years (total of four replicates). Treatments is composed by combination of two different grazing systems with supplementation of ammonium nitrate (NH_4NO_3) or urea within four seasons, they are as follows: 1) Deferred grazing system with nitratesupplementation, 2) Deferred grazing system with urea supplementation, 3) Rotational grazing system with nitrate supplementation, and 4) Rotational grazing system with urea supplementation.

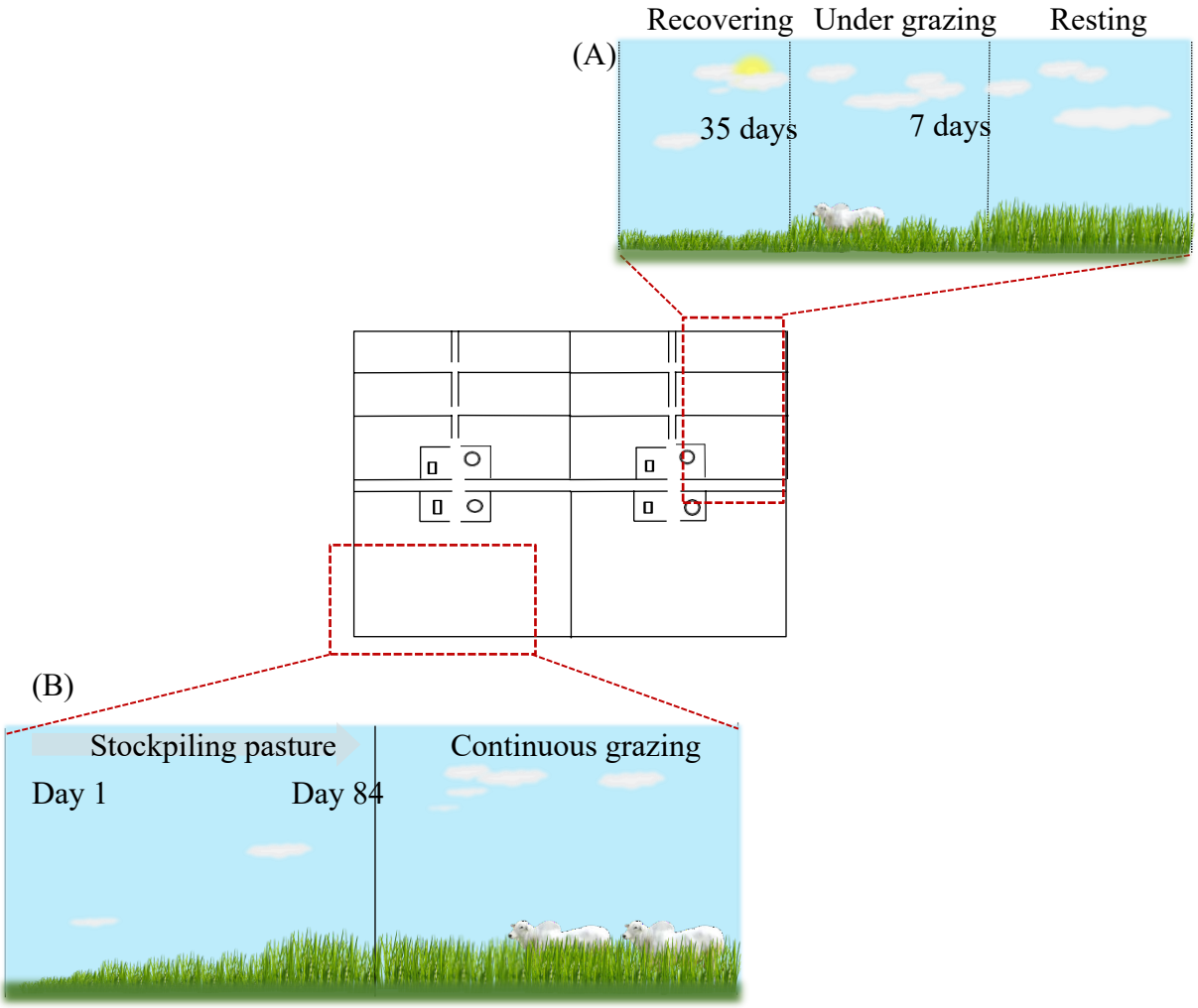
The experimental area as seen on Figure 39, has 26.5 ha^{-1} divided by management corridors. The area was established in 1999 with Urochloa (syn. Urochloa brizantha) brizantha. Additionally, 13.1 ha^{-1} was used for allocation of extra animals used to adjust stocking rate. Fertilization procedures were adopted along the experimental period following soil's recommendation.

In January of 2019, previously to the beginning, experimental area was fertilized with 50 kg ha^{-1} of nitrogen and 50 kg ha^{-1} of potassium. In November of 2019, the pastures were fertilized with 53 kg ha^{-1} of nitrogen and 57.5 kg ha^{-1} of sulfur, using ammonium sulfate. In January and March of 2020, ammonium nitrate fertilizer was applied in the amount of 56.7 kg ha^{-1} of nitrogen in each post-grazing rotated paddocks, and in the deferred pastures it was carried at once, on the same day when the last paddock of the rotated pastures was fertilized.

The deferred pastures, where stockpiling was adopted, were locked for 84 days at the end of rainy season in the first and second year (at the end of March). The management of

grazing in the deferred systems was simple and animals were introduced into the systems when stockpiling was ceased, then animals were left to continuously grazing. Each rotational system was comprised by 6 even paddocks, where animals were left under grazing in each paddock for seven defoliation days with a resting period of 35 days.

Figure 39 - Schematic representation of the rotational and deferred grazing systems with indication of recovering period, under grazing, resting period and deferred period.



Independently of the systems, animals had easy access to fresh water and to the formulated supplement, which has its composition described on table 22.

Table 22 - Composition and proportion of each ingredient used to prepare supplement for the adaptation, rainy and dry season using urea or nitrate as nitrogen source.

Ingredients		Adaptation		(Dry season)		Rainy (Season)	
		Urea (%)	Nitrate (%)	Urea (%)	Nitrate (%)	Urea (%)	Nitrate (%)
Ground corn		55	55	48	45	72	69
Urea		10		22		13	
Salt		20	15	15	10	7	5
Mineral mixture[1]		15	15	15	15	8	8
Ammonium nitrate			15		30		18
Nutritional composition							
CP	(%)	33.14	33.49	66.34	61.13	43.01	43.34
TDN	(%)	48.22	48.22	42.02	39.46	63.13	60.5
EE	(%)	1.60	1.60	1.39	1.31	2.09	2.00
NDF	(%)	4.35	4.35	3.79	3.56	5.69	5.45
ADF	(%)	1.43	1.43	1.25	1.17	1.87	1.79
Ca	(%)	2.70	2.70	2.69	2.69	1.45	1.45
P	(%)	2.54	2.54	2.52	2.52	1.47	1.46
Na	(%)	7.81	5.86	5.86	3.91	2.74	1.96

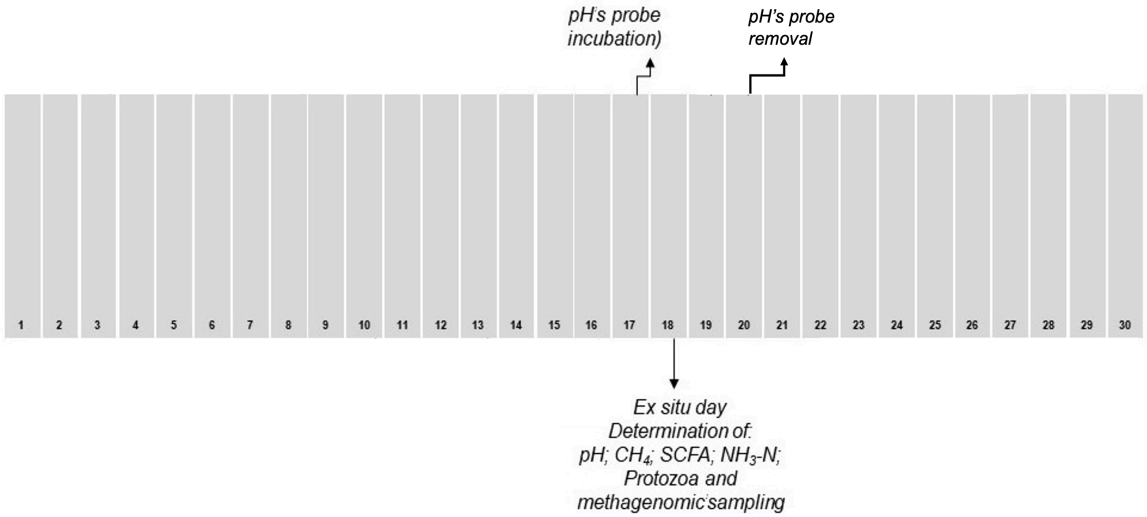
[1]Minerthal®**Estimated Macro and micromineral composition for the urea and nitrate supplement adopted in adaptation period and dry season:** 1.93 g/kg of potassium, 0.77 g/kg of magnesium, 3.29 g/kg of sulfur, 12.30 mg/kg of cobalt, 342.45 mg/kg of copper, 16.79 mg/kg of iodine, 402.90 mg/kg of Iron, 291.00 mg/kg of molybdenum, 3.36 mg/kg of selenium, 812.70 mg/kg of zinc. **Estimated Macro and micromineral composition for the urea supplement adopted in rainy season:** 2.52 g/kg of potassium, 1.01 g/kg of magnesium, 2.22 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. **Estimated Macro and micromineral composition for the ammonium nitrate supplement adopted in rainy season**: 2.42 g/kg of potassium, 0.97 g/kg of magnesium, 2.19 g/kg of sulfur, 6.56 mg/kg of cobalt, 182.64 mg/kg of copper, 8.96 mg/kg of iodine, 214.88 mg/kg of Iron, 155.20 mg/kg of molybdenum, 1.79 mg/kg of selenium, 433.44 mg/kg of zinc. CP: crude protein; TDN: total digestible energy, EE: ether extract, NDF: neutral detergent fiber; ADF: acid detergent fiber; Lig: lignin; EE: ether extract; Ca: Calcium, P: phosphorous, Na: sodium.

It is important to point out that animals were adapted to the supplementation of ammonium nitrate and previously to this experiment other studies were carried out evaluating the inclusion of different dosages of nitrate into the diet and no intoxication was detected. Despite of that, Methylene blue antidote was readily available in any case of intoxication sign.

Experimental period

All variables were collected during four periods of two years (Winter, Spring, Summer and Autumn). In the following schematic representation, it is shown all the activities scheduled in each month (the second month of the season) of each season.

Figure 40 - Schematic representation of the activities scheduled in each sampling month of each season.



In the first month of the season all animals were under adaptation on its respective experimental units.

Rumen content Sampling

To assess ruminal protozoa counting, ruminal digesta was samples at 6 and 10 a.m, and 2 and 6 p.m of the day 18, in which happened the *ex-situ* essay, as depicted in the schematic representation in the figure 40. While sampling for assessing bacterial community was performed only at 10 p.m.

Sampling was performed in all rumen's compartment by means manual procedure and, after that, collected material used for protozoa determination was rapidly processed through addition of 20 mL od Formaldehyde into flask with 10 mL of liquid ruminal sample, and then stored for further analysis.

Samples for determination of bacterial and archaeal community, as previously mentioned, occurred only at 10 am, and it was performed as liquid and solid material without fractionating them. Sterilized gloves were used to avoid contamination and, the individual collecting the material straight from the rumen was a different person from the individual that handled the animal and opened the cannula. All that was performed to mitigate as much as possible of cross contamination. After sampled, the rumen content was immediately frozen and lyophilized for further analysis.

Protozoa counting

Equal portions of the solid and liquid fractions (of rumen content) of each cow were mixed and homogenized; then about 10 mL of this mixture were inserted into flasks containing 20 mL of formaldehyde at 18.5%. Then, 1 mL of this content was stained for 4 hours with 2 drops of 2% brilliant green. Afterwards, 9 mL of glycerol at 30% was added and homogenized, making the aliquot diluted 30 times. Afterwards, the counting chamber (1 mL capacity) was filled with the diluted sample and coupled to microscope; 100 optical fields were counted through the reticulum with the magnification of 100X.

The identification and counting of ciliate protozoa were performed by using a Neubauer Enhanced Bright-Line counting chamber (Hausser Scientific Partnership®, Horsham, PA, USA) by optical microscopy (Olympus CH-2®, Japan), according to Dehority (1993). Three genera of protozoa were distinguished: *Isotricha, Dasytricha* and *Entodinium* as well as the subfamily *Diplodiniinae*, which included *Diplodinium, Eudiplodinium, Ostracodinium, Metadinium* and *Polysplatron*.

Samples' stomaching and DNA extraction.

DNA of all samples were extracted by means Phenol: Chloroform technique. Approximately, 10 grams of lyophilized rumen samples were placed in Stomach filter bags with 40 mL of DNA buffer extraction solution. Each sample were individually stomached for 5 minutes, and then liquid fraction was strained to a falcon tube. Samples were then centrifuged for 1 hour and supernatant were poured off. The formed Pellet after centrifugation was resuspended with 6mL of DNA buffer solution, and 1mL was aliquoted to bead-beading tubes with 0.5g of beads.

To proceed with the bead-beading process, 700 µL of equilibrated phenol and 50 µL of SDS at 20% were added in the tubes and it was bead-beaded for 2 minutes, followed by incubation time of 10 min at 60°C, and a second bead beating procedure of 2 minutes. Samples were then centrifuged and a fraction of 850µL were collected from aqueous portion.

Following the extraction procedure, phenol: chloroform: isoamyl alcohol at 25:24:1 was added to the aqueous fraction and centrifuged for 10 minutes. This procedure was repeated using the aqueous fraction and, after that, 500 µL of it was precipitated by inversion overnight at -20°C using 50 µL of Sodium Acetate (2 mmol L^{-1}) and 300 µL of isopropyl alcohol.

After precipitation, samples were then centrifuged for 20 min at 4°C, and supernatant was poured off. Ice-cold ethanol was used to wash the pellet by centrifuging sample for 20 min at maximum rotation (rpm). This procedure was repeated twice but with 2 min of centrifuging. Pellet was dried in the hood overnight and resuspended with 50 mL of elution buffer. Extracted DNA was quantified and, if needed, diluted to 10 ng μL^{-1} to be used for the amplification of the region of interest.

Bacterial V4 hypervariable region amplification

For bacteria, the fourth hypervariable (V4) region of the bacterial 16S rRNA gene was amplified using the one-step polymerase chain reaction (PCR) approach with universal bacterial barcoded V4 primers (515F: GTGCCAGCMGCCGCGGTAA, 806R: GGACTACHVGGGTWTCTAAT), which had adapters compatible to Illumina sequencing technology (Illumina, San Diego, CA, USA).

The reaction was performed using 12.5 µL of KAPA 2x HiFi Master Mix, 6.5 µL of water, 5 µL of the diluted DNA at 10 ng/µL and 0.5 µL of forward and reverse primer. PCR procedure was then carried using the following cycling conditions: initial denaturation at 95°C for 3min, then 24 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C for 5min.

The amplicons with the region of interest were obtained after running 1% low-melt agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Waltham, CA) at 100 mV for 50 minutes. Collected amplicons displayed a DNA band of approximately 380 base pairs (bp), which was subsequently extracted, purified, and recovered using a Zymo clean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA was quantified using a Qubit Fluorometer, and then equimolar pooled with 10% PhiX control DNA to be sequenced using an Illumina MiSeq 2 x 250 kit.

Archaeal V6-V8 hypervariable region amplification

For Archaea amplicons, the sixth and eight hypervariable (V6-V8) regions of the bacterial 16S rRNA gene was amplified using the two-step polymerase chain reaction (PCR) approach.

In the first step PCR used universal primers flanking the V6-8 from 16S rRNA gene region for archaea, generating the first complementary strand (cDNA). It was used the following

cycling conditions: initial denaturation at 95°C for 3min, then 34 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C for 5min.

Products (5 µl) of the first-step PCR were ran in a 1% agarose Ethidium bromide Gel with 6x loading dye at 90 V for approximately 30 minutes for the amplification. Successful amplified samples went through purification using the Pure Link Pro 96 PCR purification kit (Invitrogen).

Then, DNA samples were used in the second step PCR, which was performed using 5 µl of cleaned PCR product with primers to add Illumina adapters and unique indices for each sample. The cycling conditions had initial denaturation at 95°C for 3min, then 8 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C for 5min.

The amplicons with the region of interest were obtained after running 1% low-melt agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Waltham, CA) at 100 mV for 50 minutes. Collected amplicons displayed a band of approximately 610 base pairs (bp), which was subsequently extracted, purified, and recovered using a Zymo clean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA was quantified using a Qubit Fluorometer, and then equimolar pooled with 10% PhiX control DNA to be sequenced using an Illumina MiSeq 2×300bp v3 kit.

Sequence data clean-up in mothur

Sequenced samples were demultiplexed to their sample-specific indexes on the Illumina MiSeq. Data was cleaned up using the software. Mothur in the version 1.44.2. First, paired end reads were assembled to form contigs. However, it was removed sequences with ambiguous bases, long polymers and that were bigger or smaller than 270bp or 600bp for bacteria or Archaea, respectively. Those represented a poor-quality sequence that only increases noise in the data set. Since many of the sequences are duplicates, a command was used to select only unique sequences. With the first cleaning step done, sequences were then aligned to the SILVA database for the 16S rRNA. In this procedure, the alignment was done by checking both forward and revers complement.

Pre-clustering was performed to reduce sequencing errors with a defect value of 2. Data was assessed for chimera and the identified ones were removed before conducting sequence operational taxonomic unit clustering. High-quality sequences were clustered into OTUs with 97% similarity threshold.

Bacterial and Archaeal sequences were classified against the SILVA 16S rRNA gene reference database with a bootstrap cutoff of 80. Sequence coverage was calculated in mothur with Good's coverage index. After that, samples were normalized to reads count of 5000 for bacteria and 500 reads for archaea per sample, which was the lowest number of sequences that ensures reasonable coverage for all samples. After normalization, samples displayed a Good's coverage of 91% for Bacterial and 70% for Archaea.

For both, Alpha and Beta diversity metrics were calculated in mothur, and further analyzed using R (Version 3.6.1)

Data analysis using R

Alpha diversity (Shannon diversity, and Chao richness) was tested for normality using the Shapiro-Wilk test and threshold normalcy was considered when p-value was equal or >0.05. Alpha diversity parameters were assessed by analysis of variance (ANOVA) and differences between groups were confirmed by Tukey's hones significant difference (HSD). For Chao richness was first assessed by means Kruscall-Wallis's test, and differences were found by using Wilcoxon rank sum test with false discovery rate (fdr) p-value correction to confirm differential abundance in between treatments.

Beta diversity was visualized by means Venn Diagram of OTUs and using the Bray-Curtis dissimilarity metric using non-metric multidimensional scaling (nMDS) plots of square root transformed data (Bray & Curtis, 1957). Beta diversity was statistically assessed by permutational multivariate analysis of variance (PERMANOVA).

Data was further analyzed to identify the OTUs that differs, and to do that a DESeq2 (Love et al., 2014) command was used to find specific OTUs of interested which differ between the categorical explanatory variables of the model. Fixed and interaction effect among categorical variables were considered to assess OTUs that differs when p-adjusted value was <0.10, the specific OTU was plotted and differences between groups were tested using Kruskal-Wallis's rank sum tests with false discovery rate (fdr) p-value correction to confirm differential abundance. To better visualize the OTUs that would differ, we also performed a Volcano scatterplot, in which we plotted the OTUs to show statistical significance and the magnitude of change according to each fixed effect. With that plot we were able to identify OTUs that were most likely to be significantly different.

Data analysis using SAS

Data was also used to generate the overall Relative abundance based on the taxonomic classification. To do that, the taxonomic data generate in Mothur, after sequences were clustered into OTUs with 97% similarity threshold, was used to generate the relative abundance by phylum and genus. To do that, the five most abundant phylum and the top ten genus with the highest relative abundance were selected to be grouped into the metadata and statistically analyzed using the online version of the software Statistical Analysis Systems – OnDemand for Academics SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Previously to the statistical analysis, the data was assessed for the presence of disparate information ("outliers") and the normality assumption of the residuals was assessed by means the Shapiro-Wilk test. When the normality assumption was not accepted, the logarithmic or the square root transformation was applied.

Data was analyzed according to the mixed procedure (PROC MIXED), in which season was considered as repeated variable (split-plot in time). A total of 15 different covariance structures were tested, and the chosen one was based on the lower value of Corrected Akaike Information Criterion (AICC) (Wang and Goonewardene, 2004).

The model includes the effect of treatment, period of the year (Winter, Spring, Summer and Autumn) and the interaction between treatments and season as fixed factors. The effects of block were considered as random factor.

$$Y_{ijkl} = u + b_i + g_j + n_k + (gn)_{jk}\, e_{(1)ijk} + s_l + (sg)_{lj} + (sn)_{lk} + (sgn)_{ljk}\, e_{(2)ljk}$$

Where:

Y_{ijkl}: experimental answer

u: Constant

b_i: Effect of the block

g_j: Effect of grazing

n_k: Effect of nitrogen source

$(gn)_{jk}$: Interaction effect of grazing and nitrogen source

$e(1)_{ijk}$: Random error

s_l: Effect of season

$(sg)_{lj}$: Interaction effect of season and grazing

$(sn)_{lk}$: Interaction effect of season and nitrogen source

$(sgn)_{ljk}$: Interaction effect of season, grazing and nitrogen source.

$e(2)_{ljk}$: Random error

The model includes the effect of grazing method, nitrogen source, period of the year (Winter, Spring, Summer and Autumn) and the interaction between grazing method, nitrogen source and season of the year. The effects of block were considered as random factor.

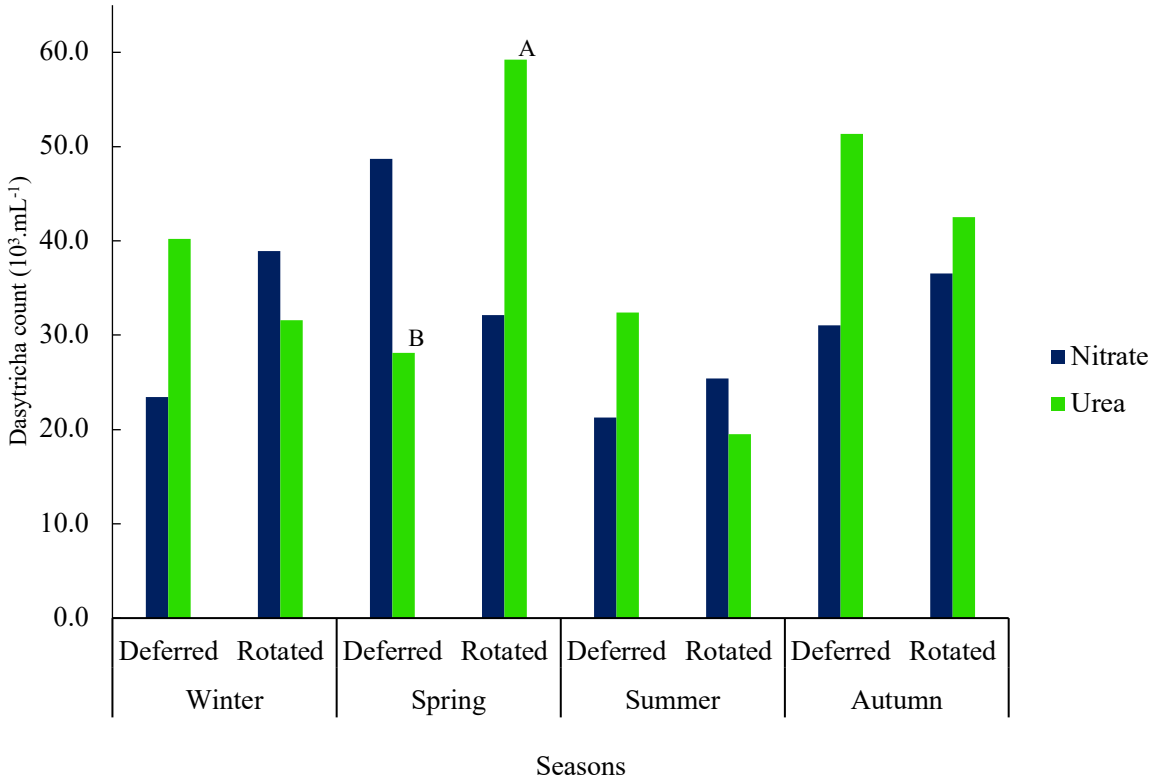
In the presence of interaction, effects of one factor inside the other were evaluated using the SLICE command of Mixed Procedure. All means were presented as least squares means and statistical differences by treatment effects were obtained by means pairwise difference test (PDIFF lines) using the Fisher test considering a significance of $P \leq 0.05$.

Results

Protozoa and relative count

Total count of protozoa and the relative count were performed considering the total count of the genera *Dasytricha Diplodiniina, Entodinium* and *Isotricha*, as showed in the Table 23. Surprisingly, no effect was detected for any of the variables assessed. However, it was observed a triple interaction for the counting of the genus *Dasytricha*. The interaction was unfolded and depicted on the Figure 41. It was assessed that, when slices were studied to better understand the treatment effect over the count of *Dasytricha*, there was only grazing system effect within urea during Spring, in which animals kept in rotated grazing systems had approximately 52% greater count of *Dasytricha*.

Figure 41 - Interaction effect of grazing, nitrogen source and season on Dasytricha count (10^3.mL^{-1}) from rumen content of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Different capital letters within nitrogen source systems differs at P<0.05;

Entodinium and total protozoa count also displayed interaction effect of grazing and season, as seen in the table 23, but despite of that, when interaction was unfolded no treatment effect was revealed.

Table 23 - Total count and relative count of protozoa from the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Fixed effects			Protozoa counting					Relative counting			
Grazing	N Source	Season	*Dasytricha* (10^3/mL)	*Diplodiniinae* (10^3/mL)	*Entodinium* (10^3/mL)	*Isotricha* (10^3/mL)	Total (10^3/mL)	*Dasytricha* (%)	*Diplodiniinae* (%)	*Entodinium* (%)	*Isotricha* (%)
Deferred			34.58	3.86	272.95	6.67	318.06	10.82	1.35	85.80	1.87
Rotated			35.72	4.55	250.28	5.82	300.91	12.68	1.58	83.89	1.91
	Nitrate		32.18	4.22	270.08	6.32	318.57	11.32	1.37	85.44	1.70
	Urea		38.11	4.19	253.15	6.17	300.39	12.17	1.55	84.25	2.08
		Winter	33.54	4.65[a]	216.39	4.80[b]	259.39	11.64	1.99[a]	84.57	1.77
		Spring	42.03	5.31[a]	322.20	8.59[a]	387.19	12.25	1.49[a]	84.00	1.94
		Summer	24.66	2.59[b]	239.01	3.98[b]	270.22	9.73	0.92[b]	87.82	1.51
		Autumn	40.37	4.28[a]	268.88	7.61[a]	321.13	13.37	1.43[a]	82.99	2.34
Mean data											
	Mean		34.81	4.20	261.61	6.24	303.35	11.87	1.46	84.72	1.88
	SEM		2.498	0.353	12.115	0.636	13.096	0.820	0.129	0.909	0.150
Statistic Probabilities											
Grazing			NS	NS	NS	NS	NS	NS	NS	NS	NS
N source			NS	NS	NS	NS	NS	NS	NS	NS	NS
Season			0.0004	0.0424	0.0001	0.0131	0.0041	NS	0.0499	NS	NS
Grazing x **N source**			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x **Season**			NS	NS	0.0255	NS	0.0139	NS	NS	NS	NS
N source x **Season**			NS	NS	NS	NS	NS	NS	NS	NS	NS
Grazing x **N Source** x **Season**			0.0032	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant.

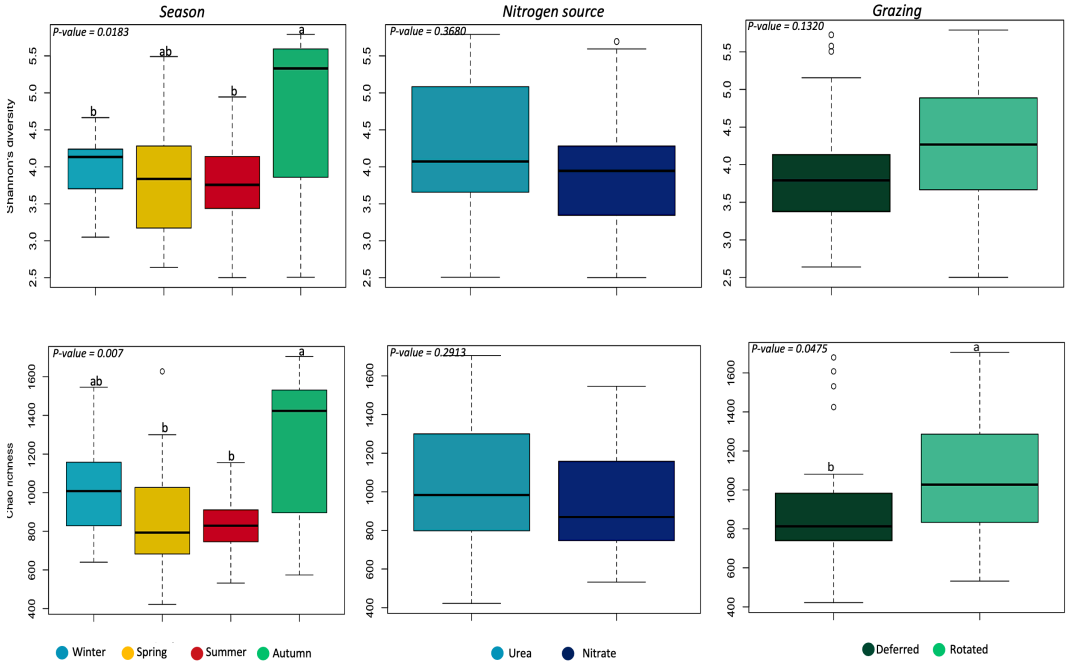
Bacteria – Diversity and richness of bacterial community

After processing the data, it was found a total of 1572654 raw sequences, resulting in an average of 24573 sequences per samples that went through filtering process. Prior to data normalization the overall average Good's coverage was ≥0.97.

Assessing the data by the main indexes that shows richness and diversity it was possible to understand that the main effect for Shannon's diversity and Chao richness estimator was observed for season, while nitrogen source and grazing did not influence in the diversity and richness of the microbial community. To better understand the indexes were depicted in a boxplot, as demonstrated in the figure 42.

Bacterial community had higher diversity over Autumn, while no statistical difference was detected among Winter, Autumn, and Spring seasons, which displayed lower variability when compared to Autumn. When it comes to Chao richness index, rumen samples collected over the Autumn showed higher richness as compared to Summer and Spring. Winter was statistically similar to Autumn; however, it had lower variability as seen in the Figure 42.

Figure 42 - Shannon's diversity and Chao's richness estimator for bacteria communities in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Data are expressed as standard boxplots with medians. Outliers are shown as dots. Groups with different letters above the same boxplot are significantly different ($P < 0.05$).

Animals were subjected to two different sources of non-protein nitrogen, nitrate and urea, associated with two grazing methods, deferred and rotated. As observed on the boxplot

for nitrogen source and grazing method, no statistical effect was detected for the variable Shannon diversity. However, it was possible to notice a slight statistical effect of grazing method over the Chao richness, in which animals under rotated grazing method had greater richness when compared to the microbiota of animals kept in deferred grazing.

Beta diversity parameters for Bacteria

A non-metric multidimensional scaling approach was used to better understand and represent the beta diversity index of the bacterial community from our study. To do that, it was used two indexes, Bray-Curti's dissimilarity and Jaccard similarity.

It was possible to visualize that after Bray-Curti's dissimilarity analysis that sources of nitrogen and grazing method did not had influence over the bacterial community. This same trend can be further visualized by means the Venn diagram (Figure 44), which showed a considerable amount of shared OTUs in between nitrogen source and grazing method. However, a clear effect of season was detected indicating a distinct cluster of Bacteria for the Autumn, when compared to Spring and Summer. A similar trend was detected for the season Winter, which was statistically different from Spring and Summer as seen on the Figure 43.

Table 24 - Pairwise test, using Bray-Curtis and Jaccard indexes, used to assess the variability between ruminal samples content of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons for two years

Fixed effects	Bray-Curtis	Jaccard
	P-value	P-value
Season	0.0001***	0.0001***
Grazing	0.4866	0.4801
Nitrogen Source	0.3005	0.3047
Interactions effects		
Grazing x Season	0.6506	0.6408
Grazing x Nitrogen Source	0.6544	0.1750
Nitrogen Source x Season	0.1761	0.6751
Season x Grazing x Nitrogen Source	0.2586	0.2578

***$P \leq 0.0001$ denote significant effect.

Interaction between the factors were also tested to check if there was any influence of one of the categorical variables over the studied index, Bray Curtis and Jaccard. No statistical effect was detected for interactions as seen on table 24.

Figure 43 - Non-metric multidimensional scaling (NMDS) representation of the Bray–Curti's dissimilarity metric for bacterial communities in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years. Ellipses represent 95% confidence intervals of individual samples.

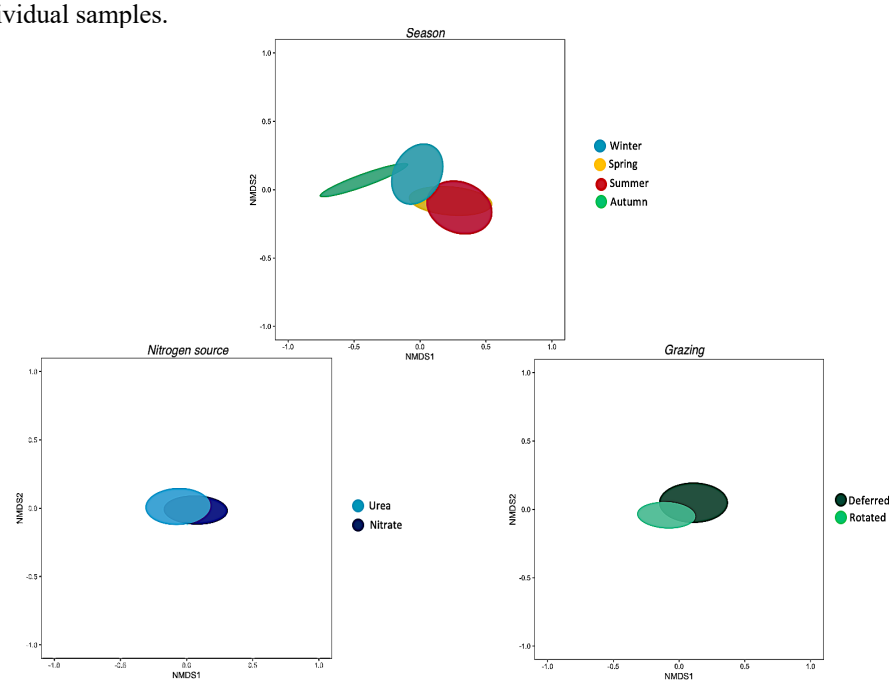
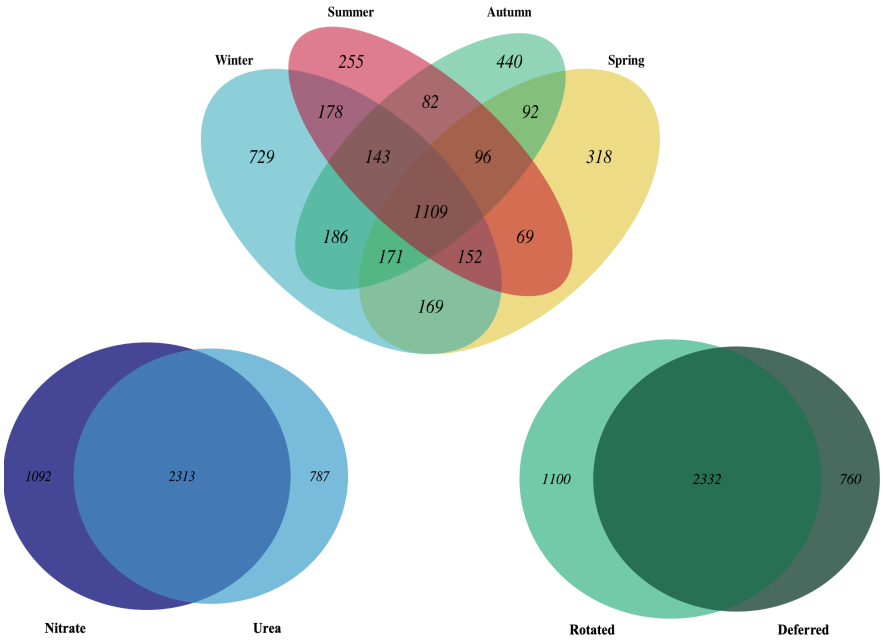


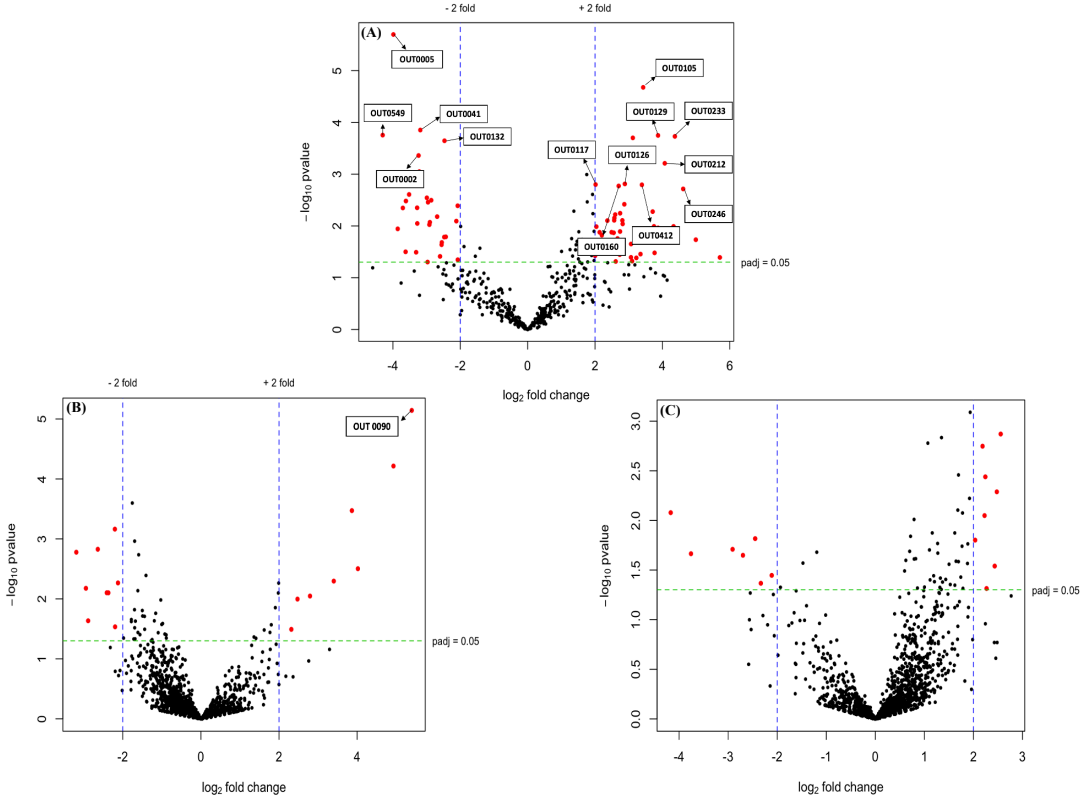
Figure 44 - Shared OTUs of bacterial community from rumen samples of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Before running the Desq2 (differential gene expression analysis based on the negative binomial distribution) command and trying to find differences, it was plotted a volcano scatterplot graphics that help to visualize and understand the trend of the data according to each

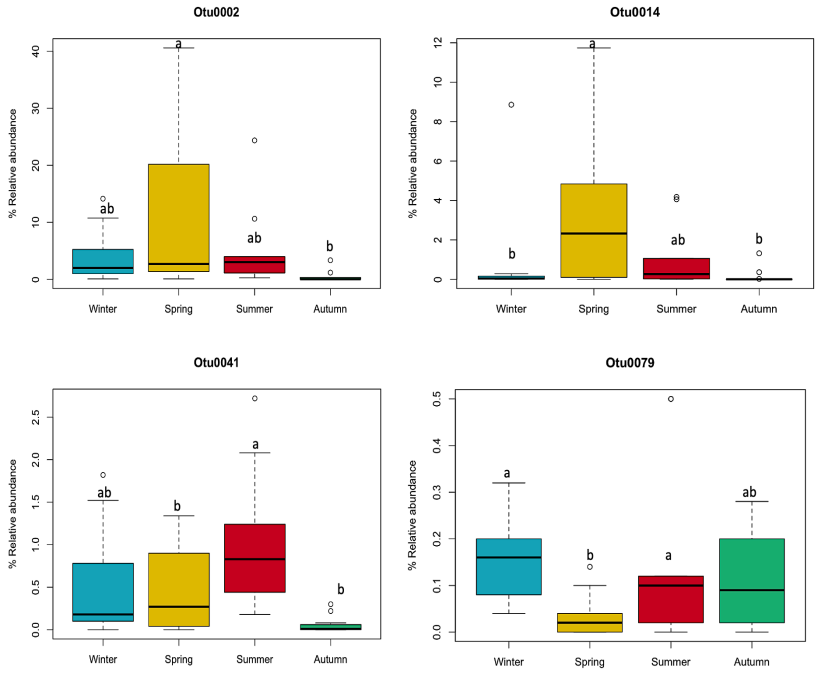
effect assessed upon the variable relative abundance. As it can be seen in the figure 45, (A), (B) and (C), for Season, Nitrogen Source and Grazing effect, respectively, we found that most of the effect over OTUs that differs comes from season effect, while no major effect of nitrogen source or grazing were found.

Figure 45 - Volcano scatterplot showing the statistical significance line (padj=0.05) and the magnitude change (log fold change) of OTUs according to season (A), nitrogen source (B) and grazing system (C) of rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation.



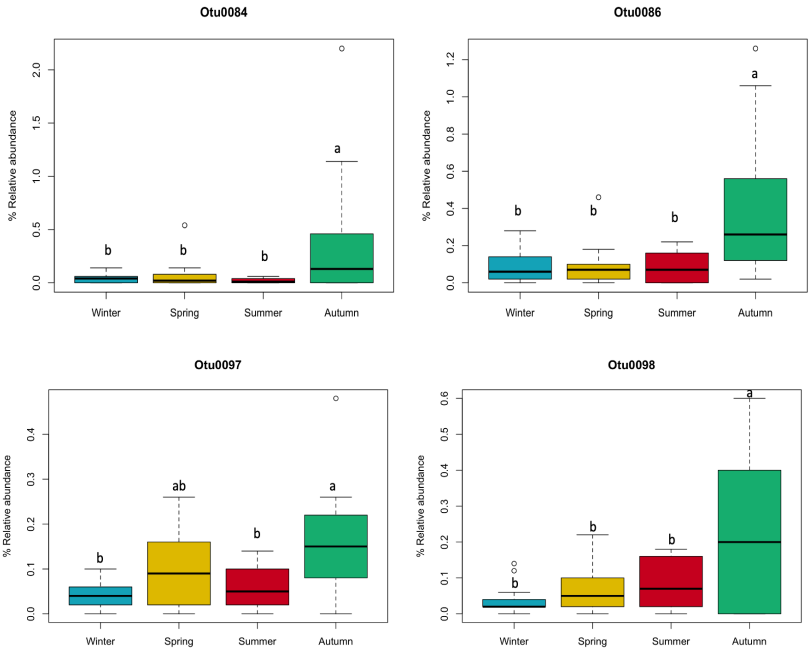
Despite of the previous results, it is still not possible to understand if there is statistical effect of treatment over the OTUs and the phylum and genus assigned to them. Understanding that, it was used a differential gene expression analysis based on the negative binomial distribution. This analysis was performed for effect of season, treatments, and interactions of fixed effects.

Figure 46 - Effect of season of the year for relative abundances (%) of OTUs found to be significantly different in the rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation.



For season effect, different superscript letter differs at $P < 0.10$.

Figure 47 - Effect of season of the year for relative abundances of OTUs found to be significantly different in the rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation.



For season effect, different superscript letter differs at $P < 0.10$.

The box plot displayed in the figure 16 shows the OTU0002, OTU0014, OTU0041 and OTU0079, which are assigned to the genus of *Kurthia* (*Firmicutes*), *Pseudmonadales*

(*Proteobacteria*), *Streptococcus* (*Firmicutes*) and *Succiniclasticum* (*Firmicutes*), respectively. The first, OTU0002 (*Kurthia*) showed higher relative abundance on Spring and lowered towards the other seasons, especially on Autumn. Similar trend was detected for OTU0014 (*Pseudomonadales*) with higher relative abundance over Spring and lower in Winter and Autumn. The OTU0041 (*Streptococcus*) appeared to have higher relative abundance over warmer season and the lowest on Autumn, while OTU0079 (*Succiniclasticum*) had the lowest relative abundance on Spring as seen in the figure 46. For the OTU0084 (*Prevotellacea* UCG-003), OTU0086 (*Prevotella*) and OTU0098 (*Christencellacea* R-7 gut group), it was noticed a similar trend with an increased relative abundance in Autumn and lower in the other seasons.

No significant effect was observed for grazing or nitrogen source. When the interactions were assessed using the same command (DEseq2) the observed statistical effect among those OTUs that differed was limited to differences in between season while no effect of treatment was detected within season. Knowing that, we decided to take a different approach to assess the bacterial data. The taxonomic data generated on Mothur, after being filtered and normalized, was used on SAS to assess if there was a possible difference among phylum or genus of bacteria according to treatments that were used.

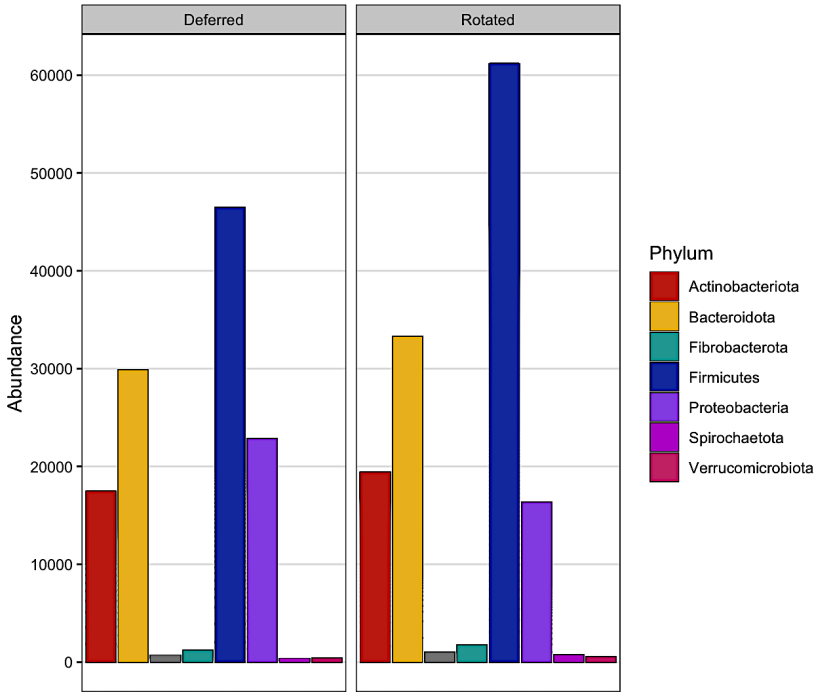
Relative abundance of phylum and genera

As observed in the table 25, the relative abundance of the main phylum detected in the assessed samples did have effect of season. Though, it was observed that animals kept in rotated pastures had higher relative abundance of the bacteria from the phylum *Firmicutes* when compared to animas under deferred grazing system, which is also observed in the figure 48.

Actinobacteiota, *Verrrumcomicrobiota* and *Spirochaetota* were all affected by season. Actinobacteria had lower relative abundance over Autumn while no significant effect was detected among the other seasons. For the phylum *Verrucomicrobiota* and *Spirochaetota*, higher relative abundance was observed in Summer and Autumn, and lower during Winter and Spring, as seen on the Table 25.

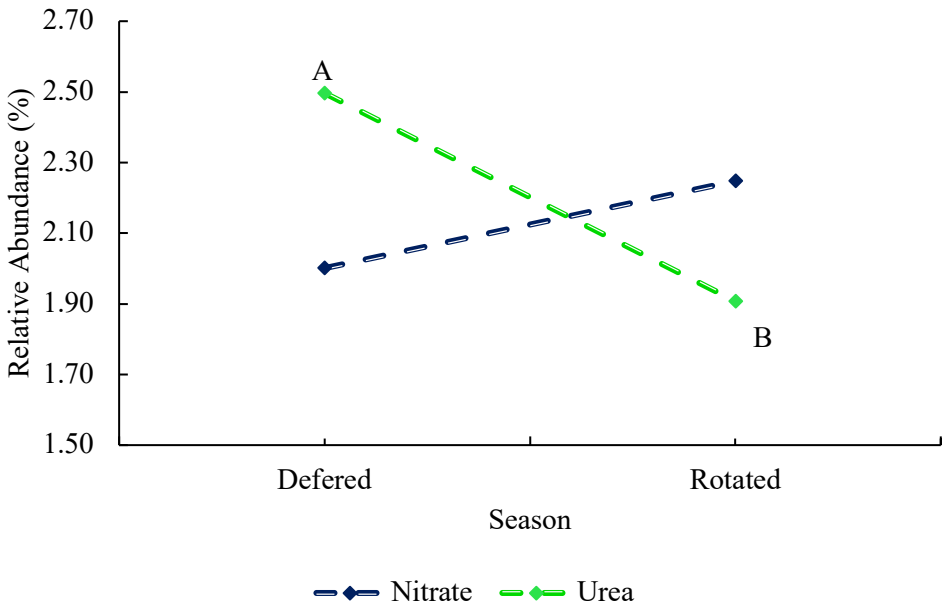
When it come to the relative abundance of the main genus, it is possible to notice only one treatment effect over the relative abundance of a bacteria. As seen on Table 26 grazing system had effect over the genus *Rikenellaceae RC9 gut group*, in which rumen content of animals under deferred grazing systems had higher relative abundance (%) when compared to those in rotted grazing systems.

Figure 48 - Relative abundance of the top seven phylum of ruminal bacterial communities observed in the rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation.



Prevotella had lower relative abundance during Summer and Autumn and higher during Winter and Spring. No treatment effect was detected for that genus. As observed in the Table 26, the bacteria from the family *Lachnospiraceae (unclassified)* showed interaction effect for grazing and nitrogen source, however, when the interaction was unfolded, no effect was revealed. Same trend was observed for bacteria from the genus *Muribaculaceae*_gu, and for the triple interaction observed for the *Clostridia unclassified* as well.

Figure 49 - Interaction effect of grazing and nitrogen source on relative abundance (%) of *Bacilli (unclassified)* from rumen content of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within grazing system at P<0.05.

Bacilli (unclassified) had interaction effect for grazing and nitrogen source, as seen in the Table 26, and its interaction's decomposition is unfolded on the Figure 52. Animals kept in deferred grazing pastures supplemented with urea as the main nitrogen source had higher relative abundance (%) of *Bacilli (unclassified)* when compared to animals kept in rotated grazing systems.

Table 25 - Relative abundance of the main phylum observed in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Phylum	Grazing		Nitrogen source		Season				SEM	Statistics Probabilities (*p*-values)						
	Deferred	Rotated	Nitrate	Urea	Winter	Spring	Summer	Autumn		G	N	S	GxN	GxS	NxS	GxNxS
Firmicutes	45.73	48.94	47.21	47.46	46.59	46.25	48.37	48.13	0.730	0.0220	NS	NS	NS	NS	NS	NS
Bacteroidota	32.14	33.54	32.57	33.11	33.74	34.27	30.74	32.60	0.696	NS	NS	NS	NS	NS	NS	NS
Actinobacteriota	8.56	7.40	8.04	7.93	9.24	8.55	8.46	5.70	0.350	NS	NS	0.0011	NS	NS	NS	NS
Proteobacteria	6.61	6.54	6.39	6.76	6.32	8.31	5.46	6.21	0.460	NS	NS	NS	NS	NS	NS	NS
Bacteria unclass.	1.17	1.21	1.34	1.04	1.03	0.72	1.18	1.83	0.121	NS	NS	0.0088	NS	NS	NS	NS
Verrucomicrobiota	1.01	1.39	1.15	1.25	0.81	0.75	1.41	1.84	0.132	NS	NS	0.0387	NS	NS	NS	NS
Spirochaetota	1.01	1.39	1.15	1.25	0.80	0.75	1.41	1.84	0.132	NS	NS	0.0290	NS	NS	NS	NS
Chloroflexi	1.35	1.40	1.39	1.36	1.44	1.50	1.22	1.35	0.054	NS	NS	NS	NS	NS	NS	NS
Patescibacteria	0.32	0.31	0.35	0.29	0.41	0.20	0.33	0.33	0.029	NS	NS	NS	NS	NS	NS	NS
Planctomycetota	0.53	0.59	0.57	0.55	0.52	0.64	0.53	0.56	0.030	NS	NS	NS	NS	NS	NS	NS
Fibrobacterota	0.34	0.45	0.42	0.38	0.18	0.48	0.32	0.60	0.058	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Different lowercase letters in the same row indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant; G:grazing; N: nitrogen source; S: season; *Interaction effect between fixed factors; NS: non-significant.

Table 26 - Relative abundance (%) of the top 18 genus assigned to the main OTUs observed in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Genus	Grazing		Nitrogen source		Season				SEM	Statistics Probabilities (*p*-values)						
	Deferred	Rotated	Nitrate	Urea	Winter	Spring	Summer	Autumn		G	N	S	GxN	GxS	NxS	GxNxS
Prevotella	18.88	19.31	18.6	19.6	21.14	20.88	17.51	16.86	0.9729	NS	NS	0.0045	NS	NS	NS	NS
Christensenellaceae_R-7	13.88	13.74	13.91	13.72	13.88	15.2	13.31	12.86	0.9135	NS	NS	NS	NS	NS	NS	NS
Rikenellaceae_RC9_gut	10.34	9.07	9.58	9.83	10.01	9.42	9.80	9.58	0.3483	0.031	NS	NS	NS	NS	NS	NS
RF39_ge	9.10	8.63	8.86	8.88	9.42	8.22	8.90	8.93	0.3684	NS	NS	NS	NS	NS	NS	NS
Lachnospiraceae[1]	7.59	7.94	7.93	7.60	6.70	6.92	8.47	8.98	0.4168	NS	NS	NS	0.0297	NS	NS	NS
F082_ge	6.23	6.66	6.26	6.63	6.50	5.81	6.50	6.97	0.3177	NS	NS	NS	NS	NS	NS	NS
Clostridia_UCG-014_ge	3.9	4.17	4.16	3.91	3.81	3.77	4.75	3.82	0.2159	NS	NS	NS	NS	NS	NS	NS
Prevotellaceae_UCG-003	3.55	4.15	3.80	3.90	3.75	3.78	3.56	4.30	0.1727	NS	NS	NS	NS	NS	NS	NS
Muribaculaceae_ge	3.57	3.34	3.38	3.53	3.77	3.62	3.23	3.19	0.1921	NS	NS	NS	0.0314	NS	NS	NS
Prevotellaceae_UCG-001	2.78	2.98	2.9	2.87	2.97	3.27	2.48	2.82	0.1065	NS	NS	NS	NS	NS	NS	NS
Clostridia[1]	2.52	2.32	2.58	2.26	2.59	2.37	2.59	2.12	0.1174	NS	NS	NS	NS	NS	NS	0.0432
Bacillales[1]	2.26	2.36	2.22	2.39	2.41	2.41	2.20	2.22	0.1796	NS	NS	NS	NS	NS	NS	NS
UCG-010_ge	2.36	1.99	2.27	2.07	1.94	1.6	2.47	2.68	0.1735	NS	NS	NS	NS	NS	NS	NS
Planococcaceae_unc	2.18	2.19	1.99	2.38	2.25	2.82	1.91	1.76	0.2036	NS	NS	NS	NS	NS	NS	NS
NK4A214_group	2.25	2.08	2.12	2.20	2.14	2.50	2.07	1.95	0.1018	NS	NS	NS	0.0095	NS	NS	NS
Prevotellaceae[1]	2.01	2.10	2.12	1.99	2.32	2.05	2.13	1.72	0.0885	NS	NS	NS	NS	NS	NS	NS
Treponema	1.77	1.69	1.6	1.86	1.09^{b}	1.25^{b}	2.00^{ab}	2.58^{a}	0.2067	NS	NS	0.0345	NS	NS	NS	NS
Bacilli*	1.40	1.74	1.45	1.68	1.44	2.18	1.39	1.25	0.1726	NS	NS	0.5377	NS	0.0333	NS	NS
WCHB1-41_ge	1.45	1.41	1.49	1.38	0.63^{b}	0.60^{b}	1.88^{ab}	2.62^{a}	0.2402	NS	NS	0.0191	NS	NS	NS	NS

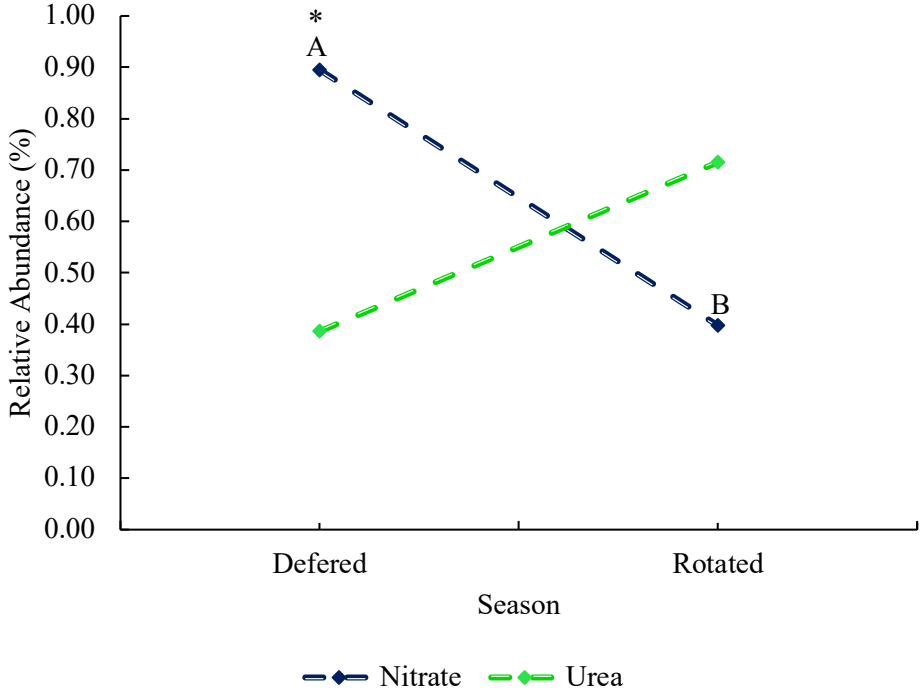
^{a,b,c} Different lowercase letters in the same row indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant; G:grazing; N: nitrogen source; S:season; *Interaction effect between fixed factors; NS: non-significant.

Bacteria from the genus *Treponema* and from the group *WCHB*-41_ge displayed effect for season. It showed an increased linear trend, in which higher relative abundance was observed on Autumn and lower during Winter and Spring.

We selected six genera of bacteria from the entire taxonomic data, as seen on Table 26, that are known in the literature as bacteria able to reduce nitrate and nitrite. As shown in the table 26, they are *Ruminococcaceae unclassified, Ruminococcus, Fribrobacter, Selenomonadaceae unclassified, Selenomonas* and *Veillonellaceae-UCG*. Among all of them it was found treatment effect only for bacteria from the family *Veillonellaceae-UCG*, which had interaction effect for grazing and nitrogen source. Its interaction was unfolded as it shows in the figure 50.

It is possible to identify that when animals were under deferred grazing and having nitrate as the mains non-protein nitrogen source, that the relative abundance of bacteria from the genera *Veillonellaceae-UCG* increases 56.8%, when compared to animals supplemented with urea within the same grazing system.

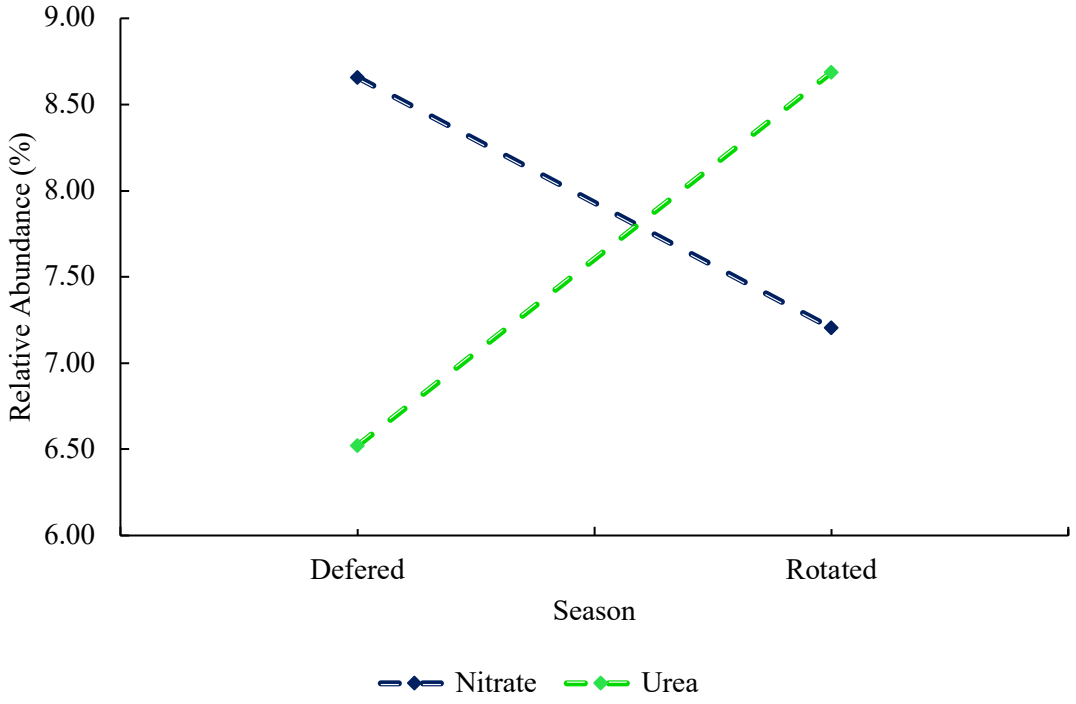
Figure 50 - Interaction effect of grazing and nitrogen source on relative abundance (%) of *Veillonellaceae_UCG* from rumen content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within grazing system at P<0.05

Genus of the bacteria *Veillonellaceae - UCG* reduces its relative abundance by 55.6% when animals are kept in rotated grazing systems within nitrate supplementation.

Figure 51 - Interaction effect of grazing and nitrogen source on relative abundance (%) of *Lachnospiraceae* from rumen content of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years.



Capital letters within nitrogen source systems differs at P<0.05; * indicates statistical difference within grazing system at P<0.05

Table 27 - Relative abundance (%) of the selected genus, known in the literature as nitrate/nitrite reducers, observed in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

Genus	Grazing		Nitrogen source		Season				SEM	Statistics Probabilities (*p*-values)						
	Deferred	Rotated	Nitrate	Urea	Winter	Spring	Summer	Autumn		G	N	S	GxN	GxS	NxS	GxNxS
***Ruminococcaceae*[1]**	1.60	1.70	1.63	1.67	1.72	1.23	1.71	1.95	0.101	NS	NS	NS	NS	NS	NS	NS
Ruminococcus	1.12	1.36	1.34	1.14	0.95	0.84	1.49	1.68	0.134	NS	NS	0.0106	NS	NS	NS	NS
Fibrobacter	0.88	0.84	0.93	0.79	0.57	0.35	1.14	1.37	0.112	NS	NS	0.0105	NS	NS	NS	NS
***Selenomonadaceae*[1]**	0.95	0.87	0.92	0.90	0.80	0.79	0.96	1.09	0.052	NS	NS	0.0222	NS	NS	NS	NS
Selenomonas	0.51	0.36	0.47	0.41	0.26	0.17	0.76	0.56	0.063	NS	NS	0.0039	NS	NS	NS	NS
Veillonellaceae_UCG	0.64	0.56	0.65	0.55	0.53	0.39	0.66	0.81	0.072	NS	NS	NS	0.0035	NS	NS	NS

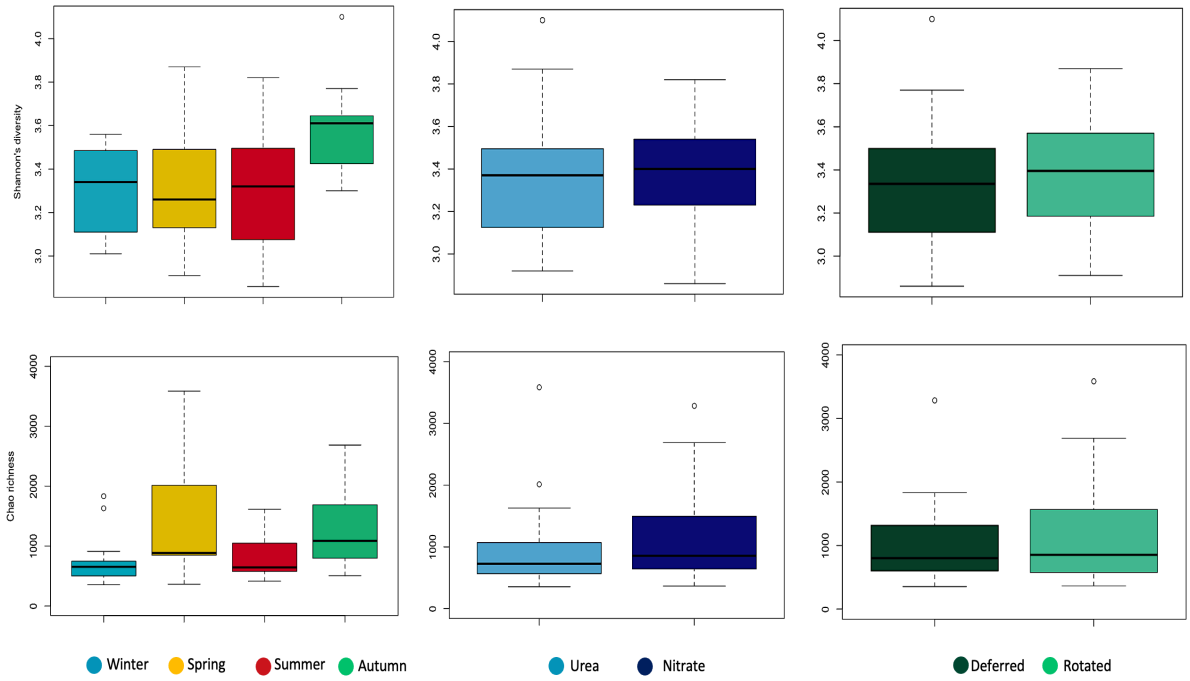
[a,b,c] Different lowercase letters in the same row indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant; G: grazing; N: nitrogen source; S: season; *Interaction effect between fixed factors; NS: non-significant. [1]unclassified at genus level

Archaea – Diversity and richness of archaea community

As observed in the Figure 52, by means Shannon's diversity it was possible to detected only season effect for Archaeal community diversity, and that same trend was observed for Chao richness estimator as well.

Bacterial community had higher diversity over Autumn, while no statistical difference was detected among Winter, Autumn, and Spring seasons, which displayed greater variability when compared to Autumn. When it comes to Chao richness index, rumen samples collected over the Winter showed higher richness as compared to Spring and Autumn. Winter showed the lower richness and less variability as well when compared to the other seasons, as seen in the Figure 51.

Figure 52 - Shannon's diversity and Chao's richness estimator for *Archaea* communities in the rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.



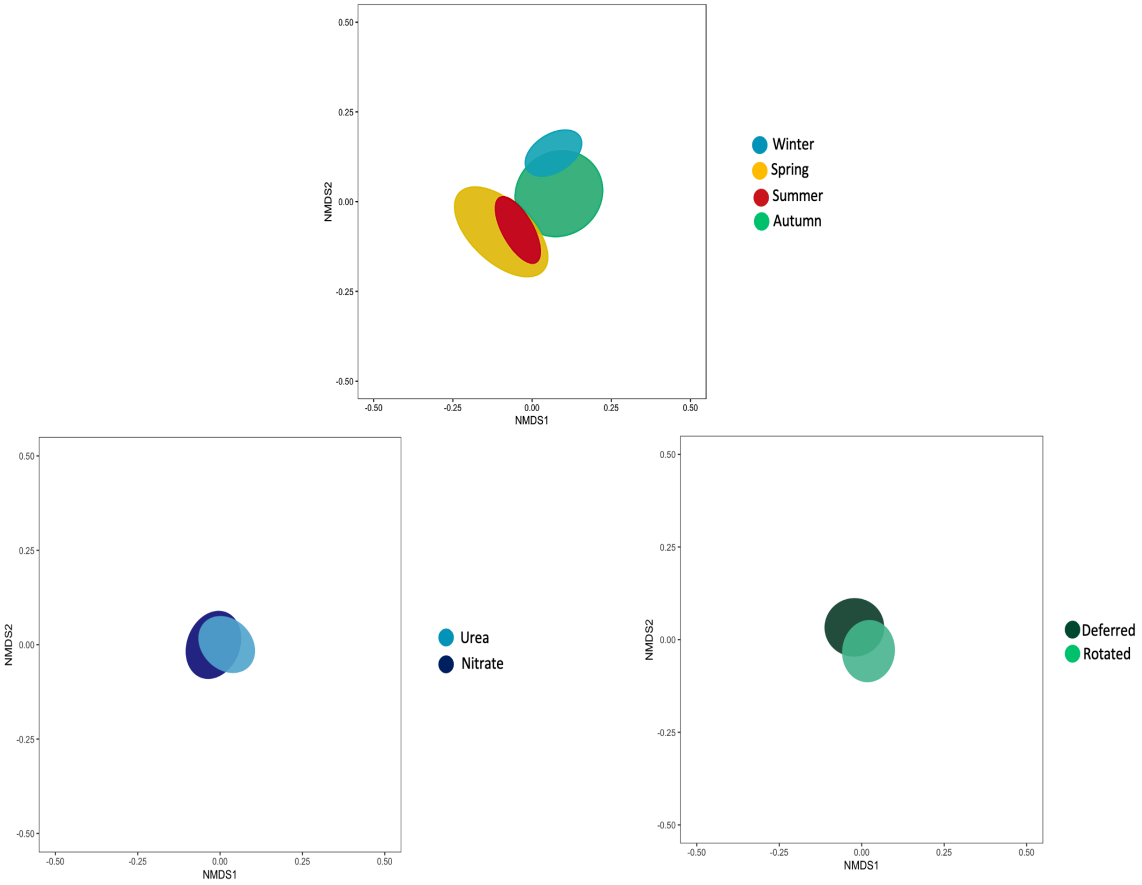
Data are expressed as standard boxplots with medians. Outliers are shown as dots. Groups with different letters above the same boxplot are significantly different ($P < 0.05$).

As observed for bacterial community, we also found that the different sources of non-protein nitrogen (nitrate or urea) associated with two grazing methods (deferred or rotated) did not affect the diversity and richness of the rumen samples using the estimators of Shannon and Chao, respectively.

Beta diversity parameters for Archaea

It was possible to visualize that after Bray-Curti's dissimilarity analysis, represented as NMDS, that sources of nitrogen and grazing method did not had influence over the bacterial community as it is clear the overlap of ellipses (Figure 53). However, a clear effect of season was detected indicating that Archaea community were distinct among seasons. As seen in the plotted NMDS, Winter overlap Autumn but it is far away from Spring and Summer.

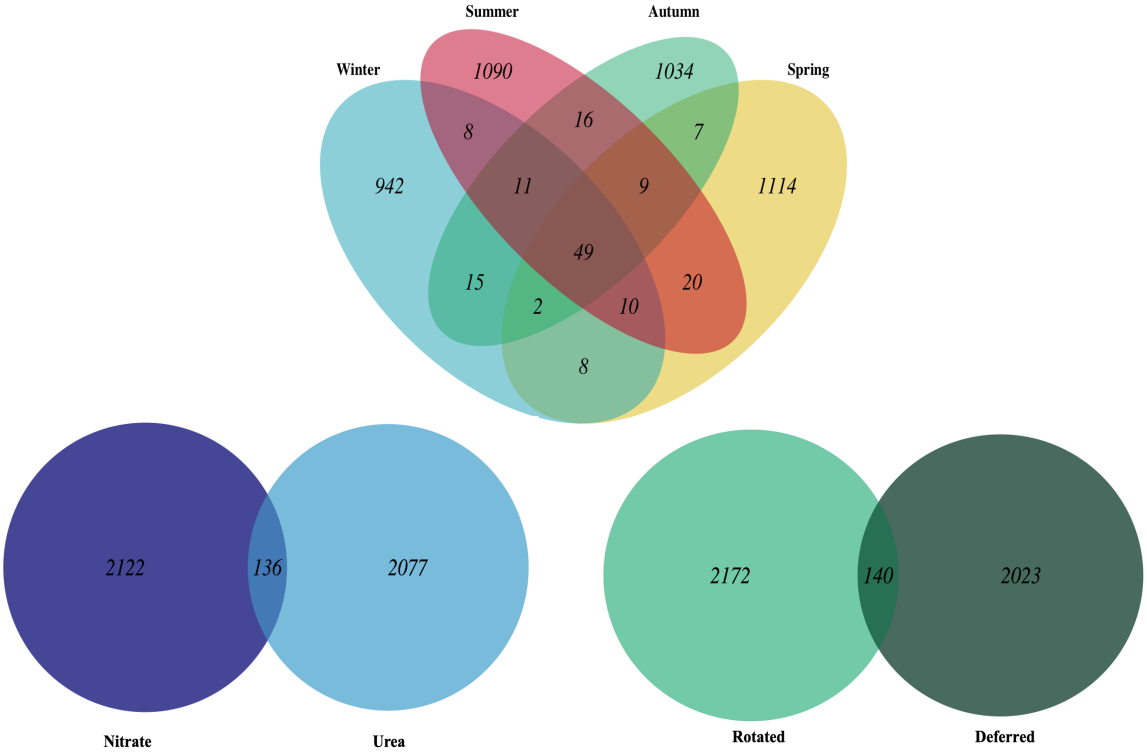
Figure 53 - Non-metric multidimensional scaling (NMDS) representation of the Bray–Curtis dissimilarity metric for Archaeal communities in the rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years. Ellipses represent 95% confidence intervals of individual samples.



Despite of no statistical effect over beta diversity for nitrogen sources and grazing methods, by means the Venn diagram we could see that when it comes to amount of shared OTUs nitrogen sources showed to share 136 while grazing method had 140 shared OTUs as seen in the Figure 54.

Interaction between the factors were also tested to check if there was any influence of treatment over the studied index, Bray Curtis and Jaccard, but no statistical effect was detected for interactions.

Figure 54 - Shared OTUs of *Archaeal* community from rumen samples of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.

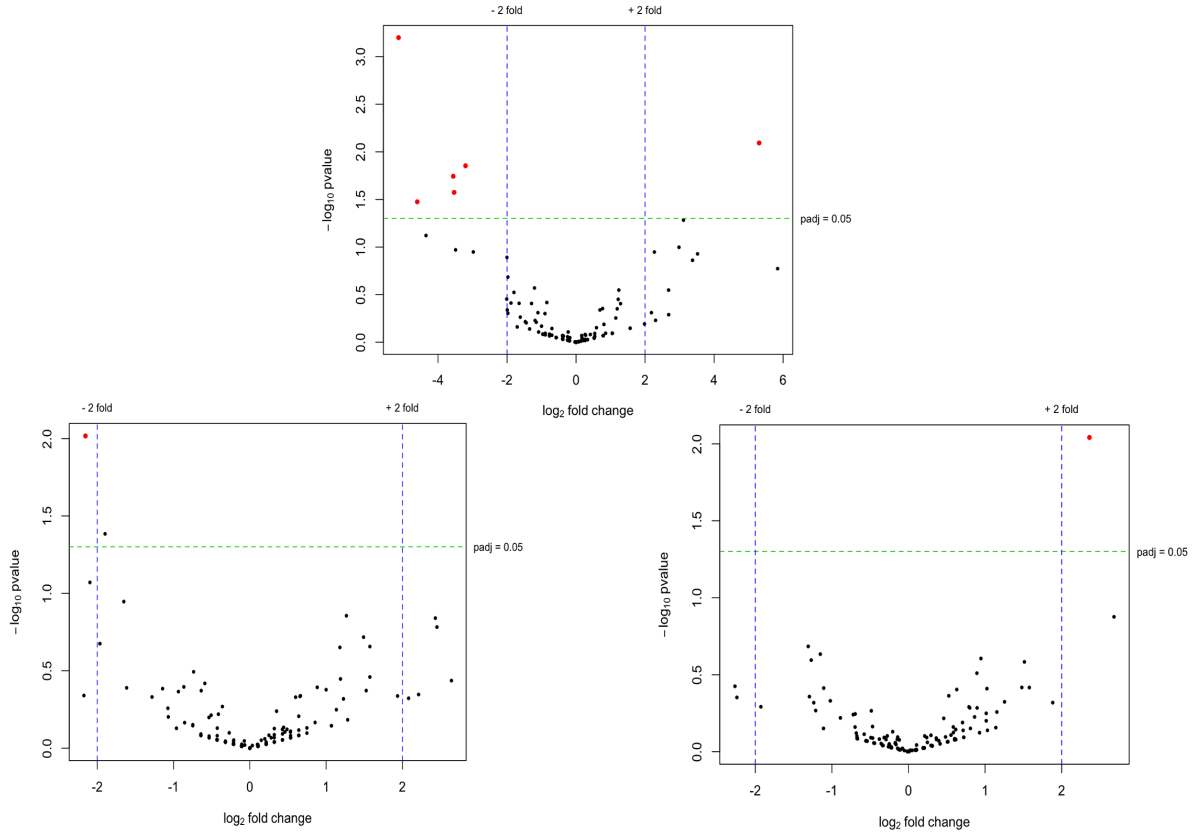


Despite of the previous results, and even using the Deseq command to assess OTUs that differ according to treatment, we did not find any difference, and the differences found did not showed to be statistically different when means were compared using the Tukey test at *P-value* of 0.05 and 0.10.

A volcano scatterplot was also plotted to visualize and understand the trend of the data according to each effect assessed upon the variable relative abundance of OTUs and its statistical significance. As it can be seen in the figure 55, (A), (B) and (C), for season, nitrogen source and Grazing effect, respectively, we found fewer OTUs that differs through the seasons; however, most of them were labeled as unclassified OTUs. When it comes to treatment effect it can be also seen most if not all OTUs plotted on the graphic B were below the threshold adjusted p-value of 0.05. Similar trend was detected for graphic C where was plotted OTUs according to the grazing method.

Knowing that, a different approach to assess the date was taken. We used the taxonomic classification file from Mothur, which were clustered into OTUs with 97% similarity threshold. Using that file, we generated a relative abundance by phylum and genus, taking in to account the most abundant phylum and genus. This data was grouped into the metadata and statistically analyzed using the online version of the software Statistical Analysis Systems.

Figure 55 - Volcano scatterplot showing the statistical significance line (padj=0.05) and the magnitude change (log fold change) of Archaeal OTUs according to season (A), nitrogen source (B) and grazing system (C) of rumen of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation.



Relative abundance for Archaea of the main phylum and genera

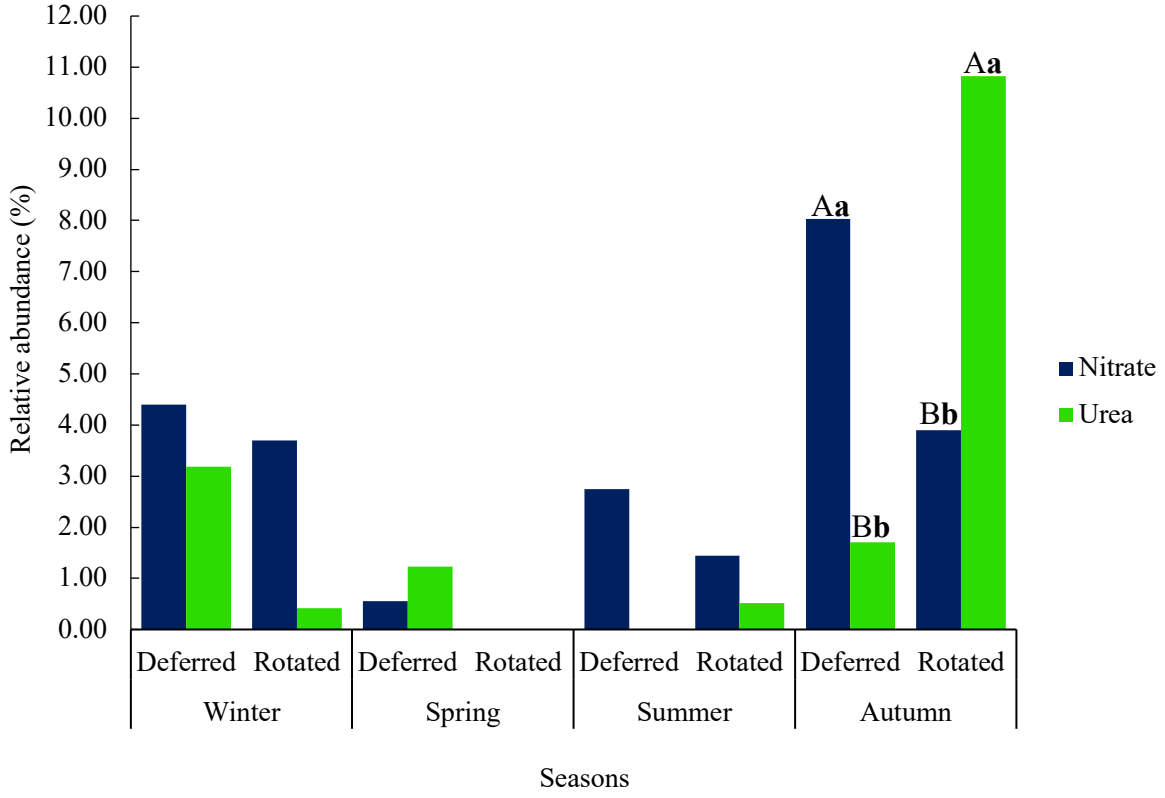
The phylum *Euryarchaeota* had the highest relative abundance among all with average of 79% of all sequences. It is followed by the phylum *Thermoplasmatota* (18%) Archaea unclassified (2%). While with less than 1% of all total sequences there was *Halobacteorata* (0.89%) and *Crenarchaeta* (0.04).

Euryarchaeota showed lower abundance over Autumn but an increased relative abundance (%) of *Thermoplasmatota* in the same season. Within that phylum we were able to

detect the following genera,*Candidatus_Methanogranum, Candidatus_Methanomethylophilus, Methanomethylophilaceae_unclassified, Thermoplasmata_unclassified*, while within Euryachaetota, it was possible to see that the Archaea genus with higher relative abundance were *Methanobrevibacter*, with an average abundance of 73%. There were also another two important Archaea genera detected, which were the *Methanosphera*, *Methanobacteraceae*.

There was triple interaction effect for the relative abundance (%) of the phylum Halobacterota, which is depicted in the Figure 56. Analyses that unfolded interaction we can see that contrasting grazing methods when animals are feed nitrate as the main source of non-protein nitrogen within Autumn season, there is a higher relative abundance of Halobacterota in samples from animals under deferred grazing. On the other hand, when the main supplementation is urea, the higher relative abundance shifts to rotated grazing method.

Figure 56 - Interaction effect of season, nitrogen source and grazing method on Halobacterota relative abundance (%) of Nellore beef cattle subjected to deferred or rotated grazing having nitrate or urea as supplementation during different seasons over two years.

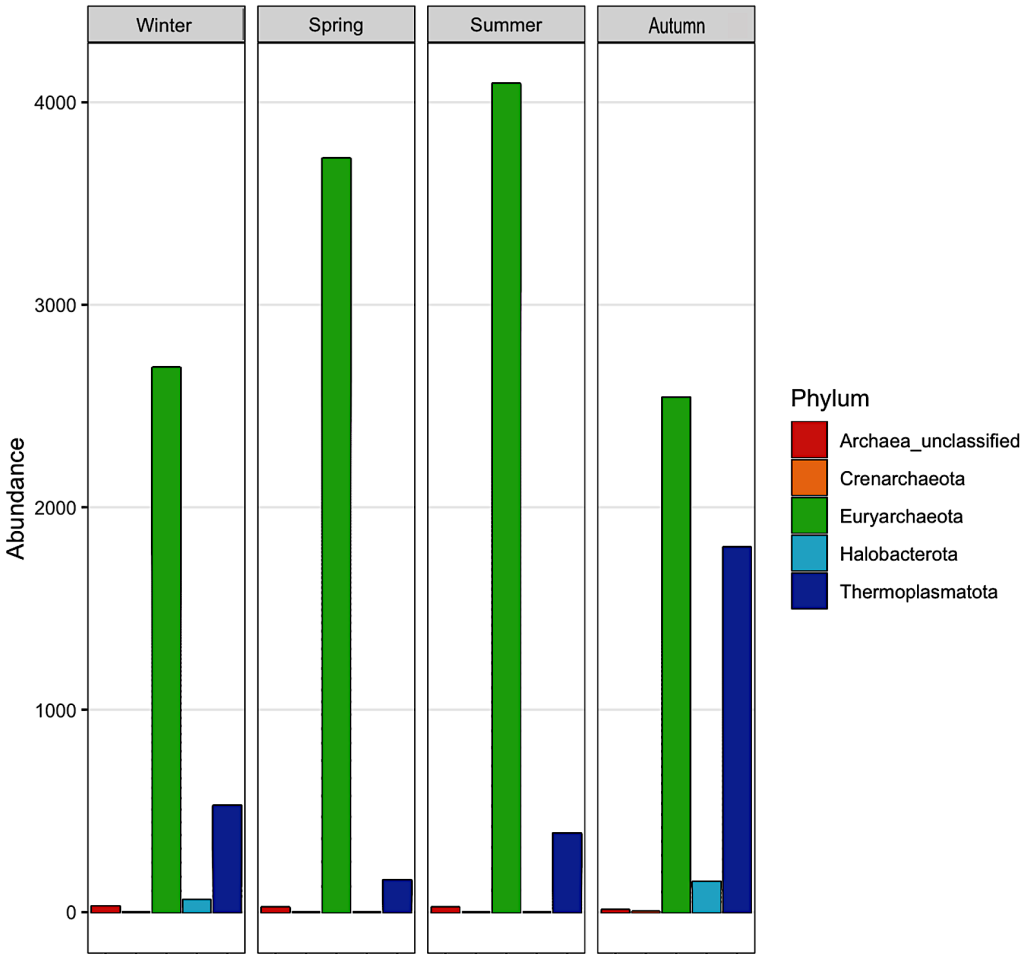


Different capital letters (A) within nitrogen source indicate difference between grazing method at P<0.05; Different bold lowercase letter (**a**) within grazing method indicates difference between nitrogen source within season at P<0.05.

It was also possible to see, within the same season, that when animals were in deferred grazing, the supplementation of nitrate positively influence in a higher relative abundance of Halobacterota, but the opposite trend was observed within rotated grazing method (Figure 56).

Methanobrevibacter did show a statistical effect for interaction, however, when it was unfolded the treatment effect was not found (Table 28). Nitrogen source effect was found for the genus *Methanosphera* which showed higher relative abundance in samples of animals feed nitrate when compared to urea (Table 28).

Figure 57 - Relative abundance (sequences number) of the main phylum of ruminal Archaeal communities observed in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation.



An important family from the *Euryarchaetota* phylum, *Methanobactericeace*, also showed significant effect for nitrogen source and grazing method as well. Samples from animals under deferred grazing 38.7% more *Methanobactericeace* as opposed to animals kept in rotated grazing. Nitrate showed to strongly affect the relative abundance of this genus with a decrease

of 39.8% in total relative abundance (%) for *Methanobactericeace*, when compared to animals fed urea as the main non-protein nitrogen source.

Archaea from the genus *Methanomethylophilus* was only affected by season with higher relative abundance of this genus over Autumn and the lowest in Spring season.

Table 28 - Relative abundance (%) of the Archaea phylum *and* genus (>1%), observed in the rumen of Nellore beef cattle subjected to deferred and rotated grazing having nitrate or urea as supplementation during different seasons over two years

	Grazing		Nitrogen source		Season				SEM	Statistics Probabilities (*p*-values)						
	Deferred	Rotated	Nitrate	Urea	Winter	Spring	Summer	Autumn		G	N	S	GxN	GxS	NxS	GxNxS
Phylum																
Euryarchaeota	78.38	79.14	78.77	78.75	81.97^{a}	88.32^{a}	84.25^{a}	60.50^{b}	2.470	NS	NS	0.0053	NS	NS	NS	NS
Thermoplasmatota	18.57	18.49	19.28	17.79	16.63^{b}	8.30^{c}	14.67^{bc}	34.53^{a}	2.390	NS	NS	<.0001	NS	NS	NS	NS
Archaea_unclassified	1.76	2.27	1.90	2.13	2.46^{ab}	3.35^{a}	1.21^{b}	1.05^{b}	0.380	NS	NS	0.0461	NS	NS	NS	NS
Halobacterota	0.65	1.12	0.76	1.01	0.732	0.071	0.13	2.61	0.310	NS	NS	<.0001	0.0243	NS	NS	0.0013
Crenarchaeota	0.04	0.04	0.06	0.03	0.029^{b}	0.005^{b}	0.071^{a}	0.06^{a}	0.020	NS	NS	0.047	NS	NS	NS	NS
Genus																
Methanobrevibacter	73.14	72.36	71.80	73.70	73.63	84.21	80.48	52.66	2.310	NS	NS	<.0001	NS	NS	0	0.0414
Methanosphera	4.16	3.62	4.44	3.34	4.90	4.10	3.58	2.97	0.290	NS	0	NS	NS	NS	NS	NS
Methanomethylophilus[1]	2.82	3.47	3.24	3.05	0.49	0.18	0.48	11.65	1.060	NS	NS	0.0001	NS	NS	NS	NS
Methanobacteriaceae	1.42	0.87	0.86	1.43	1.23	1.15	1.66	0.55	0.120	0.012	0	0.007	NS	NS	NS	NS
Archaea unclassified	1.76	1.35	1.29	1.82	2.06	2.36	0.96	0.85	0.230	NS	NS	0.0022	NS	NS	NS	NS

^{a,b,c} Different lowercase letters in the same row indicate statistical difference ($p < 0.05$) by Fisher's test. N Source: nitrogen source; SEM: Standard error of mean; NS: not significant; G: grazing; N: nitrogen source; S: season; *Interaction effect between fixed factors.

Discussion

Digestion of feed's components such as fibers, starches, sugars, organic acids, and proteins furnish the ruminant's metabolism, leading to short chain fatty acids production, such as acetic, propionic, and butyric. The amount of each of them varies according to the diet which influences the microbiota colonization, and thus differences in the diet cause results in changes on microbiota.

Grazing and nitrogen source did not have a statistically significant effect on the overall Alpha and Beta diversity parameters. However, when examining Chao richness, grazing did show an impact on richness, indicating a higher bacterial community richness in the rumen of animals under rotated grazing systems. This finding is further supported by the analysis of relative abundance, which revealed a higher abundance of Firmicutes in the rumen of animals under rotated grazing compared to those in deferred pastures. The slightly higher digestibility of nutrients, such as EE (%), NDF (%), and GE (%), in the rotated pastures suggests that these nutrients are more readily available to the rumen microbiota for processing. This, in turn, can provide a greater variety of fermentation byproducts, promoting the development and growth of a wider range of bacteria in the rumen.

In our assay, relative abundance (%) of the *Prevotella* was 17.17% higher on rumen of animals during Winter when compared to Summer, for instance. Findings on literature corroborate our findings (Pandit et al., 2018; Xie et al., 2019; Daghio et al., 2021; Wei et al., 2022) which shows that not only *Prevotella,* but also *Rikenellaceae RC9 gut group* were in between the most abundant genera in rumen of grazing animals.

Most, if not all, members of the Bacteroidetes phylum possess a polysaccharide utilization locus responsible for regulating the enzymatic digestion and transport of complex carbohydrates (Liu et al., 2021). Among these, *Prevotella* is a prominent and crucial genus of bacteria in the rumen of cattle. As mentioned earlier, *Prevotella* is endowed with a specialized polysaccharide locus that enables it to efficiently utilize complex carbohydrates (Betancur-Murillo et al., 2022). This genus is adept at binding and degrading various types of glycans (Acceto & Avgustin, 2019) and exhibits significant metabolic diversity (Tett et al., 2021). Prevotella plays a vital role not only in the metabolism of carbohydrates such as hemicellulose, starch, xylan, and pectin but also in nitrogen metabolism (Aakko et al., 2020).

Rikenellaceae RC9 gut group genus represented about on average about 10% of the sequences, and as it is shown on Table 3, *Rikenellaceae RC9 gut group* had higher relative abundance in the rumen of animals under deferred grazing. As mentioned by Zhang (2018) this genus has an important role on crude fiber digestions, and that is confirmed by Zened et al. (2013) who observed that when NDF (%) of the diet is reduced from 39.7 to 28.6% the relative abundance of *Rikenellaceae RC9 gut group* also decreases by 93.7%. Similar results were found by Huang et al. (2022) who were assessing rumen samples of yaks managed in two feeding regimes, under grazing and total mixed ration. The author noticed a decrease of *Rikenellaceae RC9 gut group* affected mainly by lack of fiber and higher concentration of energy in the diet.

The findings give us evidence that *Rikenellaceae RC9 gut group* is mostly related to the production of important short chain fatty acids in the rumen, such as acetic acid (Su et al., 2014), and possibly playing an important role in the sink of H_2 as well (Daghio et al., 2021).

Bacteria from the genus *Lachnospiraceae* showed to have higher relative abundance in rumen of cattle under deferred grazing being supplemented with nitrate (Figure 54), and that gives us an indication that these sequences may play an important role on the fermentation of a more cellulosic fiber. As mentioned by Ren et al. (2019) *Lachnospiraceae Lachnospiraceae* are enriched in solid fraction of rumen samples, especially when there is availability of fiber. *Lacnospiraceace* is known for having bacteria specialized on the metabolism of the hemicellulose. According to Dworkin (2006) they are specialized on metabolizing xylan polysaccharide complex, which makes part of forage cell-wall, and it is mainly composed by β 1,4-linked xylose rich polysaccharides. Bacteria from this family are known for endowed and release enzymes capable to act hydrolyzing the polysaccharides to monosaccharides. Despite of that, they also metabolize simple sugars (Ricci et al., 2022).

The higher relative abundance of *Bacilli unclassified* in the rumen content of cattle kept under deferred grazing corroborates with findings on the literature, which shows that classified genus from the class *Bacilli* is known for not only being able to use simples and promptly available nutrients in the rumen, but also, they make part of a more complex microbial interaction that degrades plants complex carbohydrates. As mentioned by Malik & Javed (2021) who identified a potential cellulose degrading enzyme from *B. subtillis*, a specie isolated from cow's rumen, with high capability to disintegrated cellulosic biomass.

As seen in Table 6, a group of nitrate-reducing bacteria were assessed, as it is shows they are bacteria from the genera *Ruminococcaceae, Ruminococcus, Fibrobacter, Selenomonas, and Veillonellaceae-UCG*. According to the literature, they are bacteria that possessed

reductase binding sites to allows the reduction of nitrate and nitrite to ammonia, and thus overcome the toxicity caused by both components. *Veillonellaceae-UCG* was the only unclassified genus found on our assay that statistically shows increase of its relative abundance when nitrate is in the diet.

As showed previously, the unfolded interaction effect of grazing and nitrogen source displayed that *Veillonellaceae-UCG* increased by approximately 55% when animals were fed with nitrate as opposed to urea withing deferred grazing. As extensively mentioned in the literature, *Veillonellaceae* are not only known to be nitrate-reducing bacteria since it has the reductase binding sites, but they also produce acetic, propionic acid and release H_2O and CO_2 in the rumen environment (Rosenberg et al., 2014).

It is noteworthy mention that *Ruminococcus,* which plays an important role in nitrate-rich environment that may vary according to the e specie. As observed in the literature (Zhao et al., 2015; Natel et al., 2022), *Rumminococus* is one of the rumen's bacterial able to reduce nitrate to nitrite and alleviate the possible scenario of toxicity. As observed on the table 6, nitrate increases *Ruminoccocus* relative abundance by 14.9%, however, no statistical effect was detected for nitrogen sources.

When it comes to findings regarding the Archaeal microbial community, we understand that season was again the main effect when it comes Archaeal microbial community diversity and richness, and fewer differences for treatment were found for the relative abundances of the Archaeal microbial community.

Methanogens within *Halobacteria* phylum are known for being very diverse when it comes to the substrate used within the rumen environment. As mentioned by Lyu et al. (2018) they can be hydrogenotrophic, aceticlastic and methylotrophic members, and that diversity in terms of substrate uptake for living might be one of the reasons that the triple interaction found on our research shows the inverse trend according to nitrogen sources as well as for grazing method.

Nitrogen source also influenced the relative abundance of the *Methanosphera*, an Archaea that is constantly correlated to methane reduction. Alemu et al. (2018) showed that when beef cattle were supplemented with 2% of nitrate in the diet (DM basis), *Methanosphera* relative abundance showed an increase of 40%, and the authors justified that the reason of the increases of this genus are unknow. In our study we also found an increase of *Methanosphera* by 24.7% in samples from animals supplemented with nitrate. One of the main products that *Methanosphera* uses to acquire energy is methanol, which is usually a product generated by

bacteria from genus *Prevotella* by means the degradation of pectin, an important compound of forage cell wall (Kelly et al., 2019).

To confirm the previous finding, we encounter that *Methanobacteriaceae unclassified*, from the same family of *Methanobrevibacter* and *Methanophera*, had greater relative abundance in samples from animals kept in deferred grazing as opposed to rotated grazing method.

Nitrate also negatively influenced that relative abundance of *Methanobacteriaceae unclassified*, which corroborates with findings from Granja-Salcedo et al. (2019), who worked with cannulated Nellore steers and found out that the addition of nitrate in the diet does reduces *Methanobrevibacter*, which is a genus within the family *Methanobacteriaceae*.

This finding is in accordance with the literature and with the expected effect of nitrate in the ruminant metabolism. Nitrate leads to a competition for the available hydrogen in the rumen, which directly reduces the availability of intermediates that methanogenic microorganism requires to reduced CO_2 or methanol to methane, and thus it implies and reduction of availability of energy to the Archaea metabolism. That might be one of the ways of Archaeal inhibition when nitrate is added to the diet of ruminants.

Conclusion

Based on the findings of our study we understand that grazing method and nitrogen source did not affect the rumen bacterial and archaeal diversity; however, the richness seems to be higher within rumen of animals in rotated stocking, which was a grazing method that displayed overall greater digestibility of feed, and also intake of NPN (kg/gay) which might have furnished the bacterial growth in the rumen, specially the nitrate-reducer.

Overall, the relative abundance of important nitrate reducing bacteria was not affected by nitrate supplementation, except for *Veillonellaceae-UCG*, which is a genus from the family *Veillonellaceae* where there are important bacteria that act as nitrate consumer. We also found indication of inhibition of archaeal microorganisms within the family *Methanobacteriaceae*.

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