UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EFEITO DE FONTES DE NITRATO *IN VITRO* NA PRODUÇÃO DE METANO E DO NITRATO DE CÁLCIO NA QUALIDADE DO LEITE DE CABRAS E VACAS

Autor: Kleves Vieira de Almeida
Orientador: Prof. Dr. Geraldo Tadeu dos Santos
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Estado do Paraná
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TITULAÇÃO
Doutor em Zootecnia – Área de concentração: Produção Animal

Aprovada em 23 de fevereiro de 2021

Prof. Dr. Paulo Roberto Leme

Prof. Dr. João Alberto Negrão

Prof. Dr. Rodolpho Martin do Prado

Dr. Rafael Canonenco de Araujo

Prof. Dr. Geraldo Tadeu dos Santos
Orientador

"Honra a teu pai e a tua mãe, a fim de que tenhas vida longa na terra que o Senhor, o teu Deus, te dá"

Êxodo 20:12

Aos meus pais,

João e Rosa

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DEDICO

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BIOGRAFIA

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Em março de 2010, ingressou no curso de Zootecnia na Universidade Estadual do Maranhão, São Luís, Maranhão e recebeu o título de bacharel em Zootecnia em novembro de 2014.

Em março de 2015, iniciou no mestrado em Zootecnia na Universidade Estadual do Oeste do Paraná, Marechal Cândido Rondon, Paraná com foco em produção e nutrição de ruminantes sob orientação da Prof.ª Dr.ª Maximiliane Alavarse Zambom e recebeu o título de mestre em Zootecnia em março de 2017.

Em março de 2017, iniciou no doutorado em Zootecnia na Universidade Estadual de Maringá, Maringá, Paraná com foco em produção animal e ênfase na avaliação de aditivos alimentares para cabras e vacas leiteiras sob orientação do Prof. Dr. Geraldo Tadeu dos Santos.

Em 2018, recebeu uma bolsa da CAPES para a realização do doutorado sanduíche no exterior. Em dezembro do mesmo ano, ingressou na *University of Florida* como aluno visitante sob orientação do Dr. José Eduardo Portela Santos.

No dia 7 de fevereiro de 2020, foi aprovado no exame geral de qualificação como requisito do Programa de Pós-Graduação em Zootecnia para obtenção do título de doutor em Zootecnia.

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LISTA DE ABREVIAÇÕES

AGV - Ácidos graxos voláteis

CH_4 - Metano

CO_2 - Dióxido de carbono

FDN - Fibra em detergente neutro

Fe^{2+} - Ferro no estado ferroso

Fe^{3+} - Ferro no estado férrico

[H] - Hidrogênio metabolizável

H_2 - Hidrogênio

MetHb - Metemoglobina

MS - Matéria seca

N_2O - Óxido nitroso

NH_3 - Amônia

NNP - Nitrogênio não proteico

NO - Óxido nítrico

NO_2^- - Nitrito

NO_3^- - Nitrato

O_2 - Oxigênio

PB - Proteína bruta

SO_4^{2-} - Sulfato

AA - Amino acids

ADF - Acid detergent fiber

BW - Body weight

CD - Conjugated dienes

CH_4 - Methane

CO_2 - Carbon dioxide

CP - Crude protein

DIM - Days in milk

DM - Dry matter

DMI - Dry matter intake

DRC - Dry rolled corn

ECM - Energy corrected milk

EE - Ether extract

FA - Fatty acids

FCM - Fat-corrected milk

Fe^{2+} - Ferrous iron

Fe^{3+} - Ferric iron

[H] - Metabolizable hydrogen

H_2 - Hydrogen

HMC - High moisture corn

iNDF - Indigestible neutral detergent fiber

LSM - Least square means

MetHb - Methemoglobin

MUN - Milk urea nitrogen

N_2O - Nitrous oxide

NDF - Neutral detergent fiber

NFC - Non-fibrous carbohydrates

NH_3 - Ammonia

NH_3-N – Ammonia-nitrogen

NO_2^- - Nitrite

NO_3^- - Nitrate

NPN - Non-protein nitrogen

PUN - Plasma urea nitrogen

SD - Standard deviation

SEM - Standard error of the mean

TAC - Total antioxidant capacity

TBARS - Thiobarbituric acid reactive substances

TMR - Total mixed ration

VFA - Volatile fatty acids

RESUMO

O objetivo deste trabalho foi avaliar o nitrato (NO_3^-) de cálcio e fontes adicionais de NO_3^- na produção *in vitro* de metano (CH_4) e a suplementação do NO_3^- de cálcio para cabras e vacas em lactação. No primeiro estudo, foram avaliadas fontes de NO_3^- associadas a dietas com milho seco ou silagem de grão úmido como fonte principal de amido. Adotou-se o delineamento experimental em blocos ao acaso em um sistema fatorial 5 × 2, com cinco fontes de nitrogênio não proteico (NNP) denominados: URE, ureia (grupo controle); PON, nitrato de potássio; CAN, nitrato de cálcio; DON, nitrato dolomítico e AMN, nitrato de amônia, combinados a milho seco (MS) ou silagem de grão úmido de milho (SG). A degradabilidade da matéria seca (MS) e da fibra em detergente neutro (FDN) não foram afetadas pelas fontes de NO_3^-. A SG aumentou a proporção de propionato, reduziu a razão acetato:propionato e diminuiu a produção de CH_4. A suplementação de NO_3^- reduziu a produção de CH_4 em comparação a URE, mas sem diferenças entre as fontes utilizadas. Ao contrário da hipótese principal, não houve interação entre as fontes de NO_3^- e de amido para os parâmetros avaliados. Todas as fontes de NO_3^- foram eficazes em mitigar a produção de CH_4 independentemente da taxa de degradação ruminal do amido. No segundo estudo, foram utilizadas 12 cabras Saanen em lactação, distribuídas em quatro quadrados latinos 3 × 3, com períodos de 21 dias, nos quais 14 dias foram destinados à adaptação dos animais as dietas e os últimos 7 dias para coleta de amostras e dados. Os tratamentos foram denominados: UREA, ureia (grupo controle); CAN10, 10 g de nitrato de cálcio (7.65 g/kg de NO_3^- na MS) e CAN20, 20 g de nitrato de cálcio (15.3 g/kg de NO_3^- na MS). A ingestão de MS e a digestibilidade dos nutrientes não foram afetadas pela suplementação de CAN. Não houve

efeito do NO_3^- na produção, composição e perfil de ácidos graxos do leite. A capacidade total antioxidante do leite não foi afetada pelos tratamentos, enquanto a concentração dos dienos conjugados reduziram e os TBARS aumentaram. As concentrações de NO_3^- e nitrito (NO_2^-) residuais no leite foram aumentadas em função dos tratamentos. Os tratamentos não afetaram as proporções e o total de ácidos graxos voláteis (AGV) no rúmen, assim como a concentração de nitrogênio amoniacal (NH_3-N). O nitrato de cálcio pode ser suplementado para cabras em lactação em até 20 g/kg de MS sem afetar a fermentação ruminal e a qualidade do leite. No terceiro estudo, avaliou-se a suplementação de CAN na dieta de vacas em lactação. Foram utilizadas seis vacas da raça Holandês distribuídas em dois quadrados latinos 3 × 3, com períodos de 21 dias cada, nos quais 14 dias foram destinados à adaptação dos animais e os demais para coleta de amostras e dados. Os tratamentos foram denominados UREA, grupo controle; CAN15, 15 g de nitrato de cálcio (11.5 g/kg de NO_3^- na MS) e CAN30: 30 g de nitrato de cálcio (23 g/kg de NO_3^- na MS). A suplementação de nitrato de cálcio reduziu o consumo de matéria seca, mas não afetou a digestibilidade dos nutrientes. Não houve efeito dos tratamentos na produção de leite, no entanto, houve redução na produção de leite corrigido para energia e gordura. Foram observados níveis residuais de NO_3^- e NO_2^- no leite, assim como baixos níveis de metemoglobina no sangue. A quantidade de gordura no leite foi reduzida pelos tratamentos, assim como a proporção dos ácidos graxos saturados. O poder redutor e a concentração de TBARS não foram afetadas pelos tratamentos, enquanto os dienos conjugados aumentaram linearmente. A síntese de proteína microbiana não foi afetada pelos tratamentos e poucos efeitos foram observados nos parâmetros de fermentação ruminal. Devido ao impacto no consumo e qualidade do leite ao nível de 30 g/kg MS de CAN recomendou-se a suplementação de até 15 g/ kg MS de CAN para vacas leiteiras sem afetar a qualidade do leite e os parâmetros fermentativos.

Palavras-chave: aditivo alimentar, mitigação de metano, nitrato, nitrogênio não proteico, qualidade do leite

ABSTRACT

The objectives were to evaluate the effects of calcium nitrate (NO_3^-) and additional sources of NO_3^- on *in vitro* methane (CH_4) production and calcium nitrate supplementation for dairy goats and cows. In the first study, NO_3^- sources were associated with diets receiving either dry rolled corn (DRC) or high moisture corn (HMC) as the main source of starch. The experiment followed a randomized complete block design with a 5 × 2 factorial arrangement. Treatment were the sources of non-protein nitrogen (NPN) designed as: URE, urea; PON, potassium nitrate; CAN, calcium nitrate; DON, dolomite nitrate and AMN, ammonium nitrate) and starch sources (DRC, dry rolled corn and HMC, high moisture corn). The degradability of dry matter (DM) and neutral detergent fiber (NDF) were not affected by the sources of NO_3^-. The HMC increased propionate proportion, reduced the acetate: propionate ratio, and decreased CH_4 production. Methane production was reduced by NO_3^- compared to the control group regardless of the sources. Contrary to our main hypothesis, there was no interaction between NO_3^- and starch sources in all parameters. All NO_3^- sources were effective at mitigating CH_4 production regardless of the rate ruminal starch degradation. In the second study, 12 Saanen goats were enrolled in four 3 × 3 Latin squares. Each period lasted 21 days, with 14 days used as an adaptation phase and the last 7 days for sampling and data collection. Treatments were designed as: UREA, as a control group; CAN10, 10 g of calcium nitrate (7.65 g/kg of NO_3^- on DM basis) and CAN20, 20 g of calcium nitrate (15.3 g/kg of NO_3^- on DM basis). Dry matter intake and nutrient digestibility were not affected by CAN supplementation. There were no effects of CAN on milk production, composition, and milk fatty acids. Milk total antioxidant capacity was not affected by treatment, while the

concentration of conjugated dienes decreased and TBARS increased. Residual concentrations of NO_3^- and nitrite (NO_2^-) in milk were affected by the treatment, but it did not affect the proportions and total volatile fatty acids into the rumen, as well as the ammonia-nitrogen concentration. Calcium nitrate can be supplemented to lactating goats up to 20 g/kg DM without affecting ruminal fermentation and milk quality. Lastly, responses of CAN supplementation fed to dairy cows were evaluated. Six Holstein cows were enrolled in a replicated 3 × 3 Latin square design, with periods of 21 days, including 14 days for the adaptation of the animals and the remaining days for sampling and data collection. Treatments were designed as: UREA, as a control group; CAN15, 15 g of calcium nitrate (11.5 g/kg of NO_3^- on DM basis) and CAN30: 30 g of calcium nitrate (23 g/kg of NO_3^- on DM basis). Supplemental CAN reduced nutrient intake, but it did not affect nutrient digestibility. Treatment did not affect milk production; however, energy-corrected milk and fat-corrected milk decreased as the levels of CAN increased. Low levels of residual NO_3^- and NO_2^- were detected in milk, as well as the levels of methemoglobin in blood, although the latter has increased linearly. Milk fat content was negatively affected by CAN, and the proportion of saturated fatty acids decreased. Reducing power and TBARS concentration were not affected by the CAN, while conjugated dienes increased. Microbial protein synthesis was not affected by supplemental CAN and minor effects were observed on rumen fermentation parameters. Because of the negative effect of CAN on milk contents and quality at 30 g/kg DM, it was recommended the supplementation of 15 g/kg DM of CAN for dairy cows without affecting milk quality and fermentation parameters.

Keywords: feed additive, methane mitigation, milk quality, nitrate, non-protein nitrogen

I. INTRODUÇÃO

A demanda por alimentos em 2050 será intensivamente maior, com aumento estimado em 73 e 58% para carne e leite, respectivamente (Gerber et al., 2013). Simultaneamente, o uso da terra e a exploração de recursos naturais em decorrência do aumento da produção de alimentos de origem animal sem o devido planejamento e uso racional, podem gerar consequências irreparáveis ao meio ambiente.

O impacto ambiental causado pela expansão da atividade pecuária, principalmente pela produção de ruminantes ganharam destaque em pesquisas nas últimas décadas, desde que a emissão dos gases do efeito estufa e o aquecimento do planeta se tornaram preocupação mundial (Beauchemin et al., 2020). A produção de metano (CH_4) entérico pelos ruminantes contribui com aproximadamente 6% das emissões antropogênicas dos gases do efeito, e a maior parte foi atribuída aos animais voltados para a produção de leite, seguidos de animais destinados ao abate, com aproximadamente 46 e 43%, respectivamente (Gerber et al., 2013).

Nos últimos anos, muitos compostos químicos e biológicos têm sido investigados por grupos de pesquisa com o objetivo de mitigar a produção de CH_4 entérico a curto prazo, entre eles, aceptores de elétrons, ionóforos antibióticos e enzimas (Herrero et al., 2016). Além disso, melhor entendimento do metabolismo ruminal e das vias metabólicas relacionadas à metanogênese tem contribuído para o desenvolvimento de estratégias alimentares mais eficazes (Yang et al., 2016).

Dentre os aceptores de elétrons, os sais de nitrato (NO_3^-) destacam-se como viáveis aditivos alimentares sobretudo pela alta eficiência em reduzir a produção de CH_4 proveniente

da fermentação ruminal (Feng et al., 2020). Ademais, melhorar a digestibilidade das dietas e aperfeiçoar as estratégias alimentares, como a utilização de alimentos com maior degradabilidade podem modular o ambiente ruminal e impactar diretamente na metanogênese (Herrero et al., 2016).

Estudos *in vitro* têm comprovado a eficácia da utilização do NO_3^- na redução de metanógenos *Archaea*, e por conseguinte impactado na metanogênese (Patra & Yu, 2014; Zhou et al., 2012). Em estudos *in vivo*, dentre as fontes de NO_3^- avaliadas, o duplo sal de nitrato de cálcio e amônio decahidratado [$5Ca(NO_3^-)_2 \cdot NH_4\ NO_3^- \cdot 10H_2O$] tem sido o mais utilizado em pesquisas voltadas para a redução de CH_4 ao redor do mundo (Henry et al., 2020; Meller et al., 2019; Silveira et al., 2019). No entanto, fatores como dose utilizada, fonte de NO_3^-, manejo alimentar e tempo de adaptação dos animais, foi possivelmente o motivo da baixa consistência entre os estudos.

Além do impacto na produção de CH_4 entérico, os sais de NO_3^- são também fontes de nitrogênio não proteico (NNP), e de acordo com pesquisas recentes, podem ser um fator importante adicional de sua utilização pelo provimento de amônia a ser incorporada na síntese de proteína microbiana (Wang et al., 2018). Entretanto, a inconsistência de resultados em parâmetros como desempenho animal e composição do leite sugerem a necessidade de estudos adicionais.

Apesar de muitas pesquisas demonstrarem redução no consumo de nutrientes em resposta a suplementação de NO_3^- (Feng et al., 2020; Lee & Beauchemin, 2014), ainda faltam informações sobre os impactos na composição do leite de vacas, no perfil de ácidos graxos e na capacidade antioxidante. Ademais, até o momento, não existem trabalhos na literatura investigando os impactos da suplementação de nitrato de cálcio para cabras em lactação.

Diante do exposto, o objetivo do presente estudo foi avaliar a utilização do nitrato de cálcio em comparação com fontes adicionais de NO_3^- na produção *in vitro* de metano, assim como a suplementação de nitrato de cálcio em dietas para cabras e vacas em lactação sobre os parâmetros de qualidade do leite.

II. REVISÃO DE LITERATURA

1. Produção de metano em ruminantes

A produção de ruminantes tem como função principal produzir proteína animal, seja carne ou leite, através do fornecimento de dietas balanceadas com a quantidade de nutrientes necessária para a mantença e produção dos animais. A proporção e a variação do uso dos ingredientes dependem da espécie animal, da fase de produção e sobretudo do tipo de sistema. No entanto, a inclusão de pelo menos uma fonte de fibra fisicamente efetiva na dieta de ruminantes é altamente recomendada para garantir a fermentação ruminal adequada e, desta forma, prevenir distúrbios metabólicos.

A fermentação dos carboidratos no rúmen é um processo natural e essencial para a produção de ácidos graxos voláteis (AGV), entre eles o acetato, o propionato e o butirato, os quais são absorvidos na parede ruminal e posteriormente utilizados como fonte de energia e na síntese de gordura, lactose e proteína. Além disso, outros produtos são gerados pela fermentação ruminal da matéria orgânica, por exemplo, hidrogênio (H_2), dióxido de carbono (CO_2), formato, succinato, lactato, etanol, além de amônia (NH_3) que é fundamental para a produção de proteína microbiana (Janssen, 2010).

1.1 Metanogênese

O rúmen é um complexo ecossistema constituído de microrganismos específicos que atuam sinergicamente em processos químicos e ciclos de perda e geração de energia, por meio da degradação de substratos orgânicos. Entre os principais microrganismos, estão as

bactérias anaeróbicas, protozoários, fungos anaeróbicos, e os *Archaea,* estes últimos indispensáveis para a produção de CH_4 entérico, que é um essencial para manter o equilíbrio ruminal e, desta forma, garantir saúde ao hospedeiro (Huws et al., 2018).

Os microrganismos ruminais denominados *Archaea* são responsáveis pela metanogênese, processo de geração entérica de CH_4 que tem como objetivo primário reduzir o acúmulo de H_2 no rúmen, e consequentemente evitar distúrbios metabólicos nos animais. Os metanógenos *Archaea* são estritamente anaeróbicos e classificados como microrganismos distintos das bactérias e dos eucariotos (protozoários e fungos), possuem seu próprio domínio de classificação e são caracterizados por apresentar parede celular e membrana únicas, assim como vias metabólicas e enzimas específicas (Eme et al., 2017; Moissl-Eichinger et al., 2018).

A produção de CH_4 no rúmen desempenhada pelos metanógenos *Archaea* acontece através da utilização do H_2 livre como fonte de energia e do CO_2 como aceptor de elétrons, os quais são indispensáveis para a síntese de CH_4 entérico. Neste sentido, a produção de CH_4 é maior em ambientes com altas concentrações de CO_2 e menos favorável em meios ricos em outros aceptores de elétrons, entre eles o oxigênio (O_2), os NO_3^-, o ferro no estado férrico (Fe^{3+}) e os sulfatos (SO_4^{2-}) (Lyu et al., 2018).

Metanogênese é um processo essencial para o rúmen pois a redução das concentrações de H_2 livre neste ambiente permite o aumento das taxas de fermentação dos substratos presentes na dieta (Buddle et al., 2011). De acordo com Janssen (2010), as taxas de H_2 no rúmen aumentam imediatamente após a ingestão de alimentos pelos animais, fazendo deste processo uma importante via para dissolver o H_2 acumulado e manter o equilíbrio ruminal. Desta forma, o H_2 é considerado a principal fonte de energia para a formação de CH_4 no rúmen (Figura 1) através da reação química $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$, seguido de formato e metanol que também podem ser utilizados pelos metanógenos como fonte de energia (Buddle et al., 2011).

Diante do exposto, é notável a importância da produção de CH_4 para reduzir os impactos das altas concentrações de H_2 no rúmen. No entanto, os ruminantes necessitam liberar o gás produzido neste processo, o que se dá majoritariamente através da eructação (Janssen, 2010). Assim, apesar do benefício para a saúde dos ruminantes, segundo Hristov et al. (2013), a

liberação de CH_4 pode representar um risco potencial para a natureza a curto prazo, uma vez que é o segundo maior causador do efeito estufa proporcionalmente, por possuir poder de aquecimento de até 25 mais quando comparado ao CO_2.

De acordo com Lyu et al. (2018), as preocupações com o impacto do CH_4 ao meio ambiente vêm aumentando em decorrência da concentração atmosférica que dobrou desde a revolução industrial, ocasionando elevação a cerca de 20% no aquecimento da terra que deveria ocorrer de forma natural.

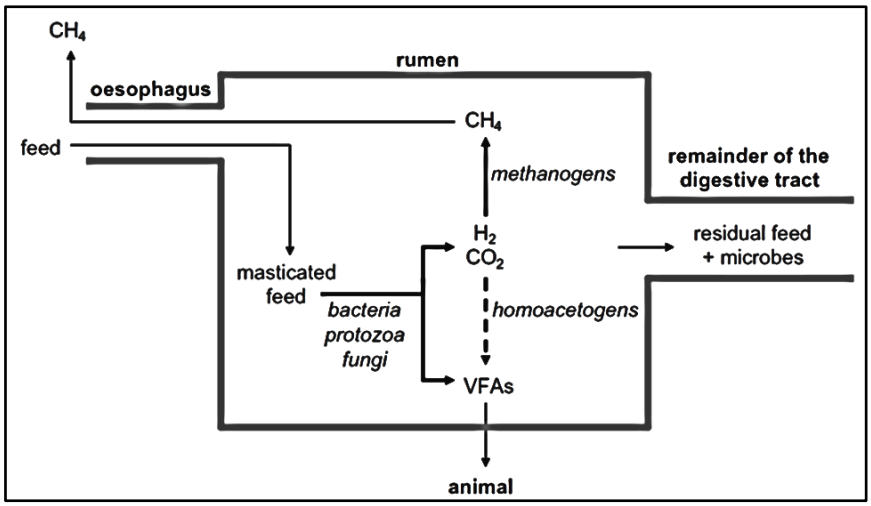

Figura 1. Esquema simplificado da produção de metano no rúmen (Buddle et al., 2011).

1.2 Impactos do metano ao meio ambiente

De acordo com Gerber et al. (2013), a fermentação entérica é a segunda maior fonte de produção de gases do efeito estufa relacionados à atividade pecuária, contribuindo a cerca de 40% do total de emissões. Neste cenário, os bovinos são os animais que mais produzem (77%), seguidos de búfalos (13%) e pequenos ruminantes (10%). Ademais, o CH_4 é o gás antropogênico mais emitido na pecuária (44%), com o restante distribuído em oxido nitroso (N_2O) com 29% e CO_2 estimado a cerca de 27% do total das emissões de gases, este último principalmente atribuído a atividades como transporte de alimentos e manejo de dejetos.

Ainda segundo Gerber et al. (2013), em escala global, a fermentação entérica em animais voltados para a produção de leite é responsável a cerca de 46% do total das emissões de

gases, seguidos de aproximadamente 43% para animais destinados para a produção de carne dentro destas cadeias produtivas. Devido a variação na qualidade das pastagens, os sistemas exclusivamente a pasto contribuem mais intensamente para o efeito estufa quando comparados a sistemas mistos (uso de suplementação) e confinados, de acordo com a classificação da *Food and Agriculture Organization of the United Nations (FAO)* (Robinson et al., 2011).

Somados as preocupações com o meio ambiente, estudos recentes têm objetivado mitigar a produção de CH_4 através do redirecinamento de H_2 para vias alternativas, seja pela utilização de aditivos alimentares ou pelo manejo alimentar, como por exemplo, a manipulação das proporções de volumoso e concentrado na dieta. Estas estratégias supostamente têm reduzido as perdas de energia no processo de metanogênse, e consequetemente aumentado o desempenho produtivo dos animais (Wang et al., 2018).

Neste contexto, mudanças no manejo alimentar e o uso de aditivos moduladores ruminais têm sido explorados para interferir em vias metabólicas da produção de CH_4, impactando positivamente no efeito estufa e ao mesmo tempo aumentando a efiência alimentar dos animais através do maior aproveitamento dos ingredientes da dieta. Além de tudo, o uso de ingredientes de melhor qualidade, ou seja, mais digestíveis, aumentam a taxa de passagem e consequentemente reduzem o tempo da fermentação entérica da fibra, que é considerado o fator principal para a produção de gases precursores do CH_4 no rúmen.

2. Mitigação de metano

A utilização de aditivos e a manipulação das dietas para ruminantes ganham destaque como estratégias alimentares eficazes para minimizar os impactos ambientais causados pela emissões de CH_4 entérico. Os aditivos mitigadores de gases do efeito estufa possuem propriedades singulares que interferem de forma específica no crescimento de metanógenos *Archaea* e, por sua vez, em fases da produção de CH_4 no rúmen, como por exemplo, no direcionamento de elétrons para vias metabólicas alternativas e na inibição de enzimas envolvidas no processo (Hristov et al., 2013). Além disso, a utilização de alimentos concentrados e a redução do teor de fibras em dietas para ruminantes têm sido alvo de estudos

objetivando minimizar a produção de CH_4 entérico através da modificação da produção de AGV no rúmen (Martin et al., 2010).

2.1 Manejo alimentar

A produção de CH_4 pode ser diretamente influenciada pela composição e proporção dos ingredientes da dieta, principalmente pela quantidade e composição dos carboidratos. Teoricamente, dietas com maior composição de carboidratos altamente degradáveis no rúmen, como o amido, podem modificar a microbiota ruminal, afetando consequentemente o pH e a proporção dos AGV (Johnson & Johnson, 1995).

Os principais produtos da fermentação dos carboidratos são os AGV, CH_4 e CO_2. De acordo com Van Soest (1994), a estequiometria geral para os produtos da fermentação de carboidratos foi classificada da seguinte forma:

$$C_6H_{12}O_6 \text{ (glicose)} + NH_3 \rightarrow \text{Microbiota ruminal} \rightarrow CH_4 + CO_2 + \text{AGV}$$

A alteração do ambiente ruminal pela inclusão de fontes de amido em substituição a forragens podem afetar a relação acetato:propionato. Essa modulação ruminal interfere na produção de CH_4, uma vez que a fermentação do amido favorece a produção de propionato que utiliza o H_2 livre no rúmen (Figura 2), e portanto, compete diretamente com a metanogênese (Janssen, 2010).

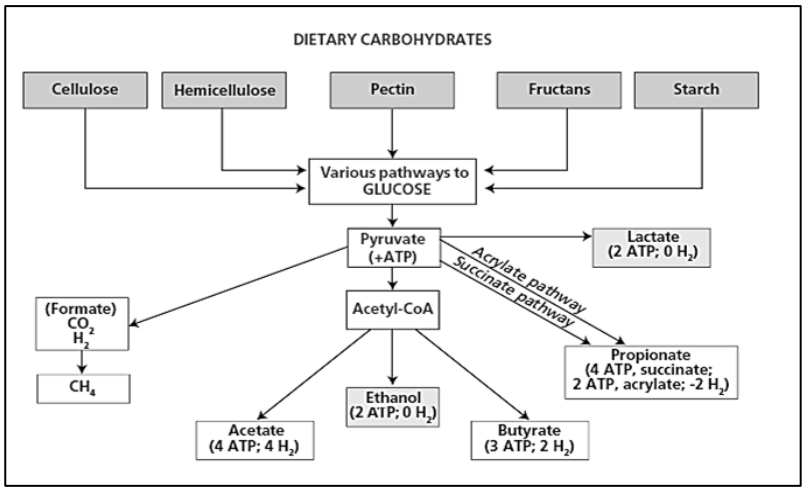

Figura 2. Fermentação dos carboidratos no rúmen (Van Soest, 1994). Adaptação: Hristov et al. (2013).

O aumento na proporção de propionato e redução do acetato podem acontecer pela substituição de carboidratos estruturais (forragens) por fontes de carboidratos facilmente degradáveis (concentrados). A redução da produção de CH_4 pode ser determinada por dois mecanismos principais, como anteriormente mencionado, o primeiro ocorre pela redução dos precursores de CH_4, como hidrogênio metabolizável ([H]) no rúmen, e o segundo pela redução na fermentação da fibra devido a efeitos associativos, neste caso, pelo aumento da taxa de passagem ruminal (Benchaar et al., 2001).

Um estudo *in vitro* comparando trigo e milho como fontes de amido associados a suplementação de NO_3^- (20 mg) por grama de matéria seca (MS) de substrato, gordura (50 mg/g de MS), ou 3-nitrooxopropanol (0,08 mg/g de MS), demonstraram que o efeito principal do uso de fontes de amido rapidamente degradáveis (trigo) aumentou a proporção de propionato, e desta forma, elevou a competição por [H], tendo como efeito secundário a redução de 22% na produção *in vitro* de CH_4. Os efeitos principais de nitrato, gordura e 3-nitrooxopropanol reduziram a produção *in vitro* de CH_4 em 21, 19 e 44%, respectivamente (Alvarez-Hess et al., 2019).

No estudo desenvolvido por Hatew et al. (2015), comparando dietas de rápida e lenta degradação ruminal associadas a dietas com alto e baixo nível de amido para vacas em lactação, foi observado redução na produção de CH_4 entérico expresso por kg de matéria orgânica fermentada no rúmen em dietas com maior taxa de degradação do amido, porém sem efeitos na produção de CH_4 quando relacionada com a ingestão de matéria seca e a produção de leite.

2.2 Aceptores de elétrons: Nitratos

O uso de aceptores de elétrons como mitigadores de CH_4 é uma estratégia alimentar bem estabelecida (Jones, 1972). Contudo, nos últimos anos, o uso destes aceptores como aditivo alimentar vem ganhando amplo destaque principalmente, pelo aumento nas preocupações com o impacto ambiental causado pela atividade humana, sobretudo pela produção de alimentos. Dentre os aceptores de elétrons, os sais de NO_3^- têm se destacado pela alta eficácia ao atuar como inibidor da metanogênese.

Nitratos são ânions inorgânicos que podem estar presentes naturalmente no solo, na água, nas pastagens e nas culturas. Dentre as variedades de plantas, os cultivares de azevém e sorgo são particularmente propensos a acumular NO_3^-, com mais intensidade no caule e menor acúmulo nas folhas (Leng, 2008). Em condições de estresse, como escassez de água, muitas plantas podem aumentar a deposição de NO_3^-, que uma vez consumida pelos animais, possivelmente causarão intoxicação aguda ou crônica (Wright & Davison, 1964).

O uso do ânion NO_3^- como aditivo inibidor de CH_4 em ruminantes tem recebido mais atenção em resposta aos diversos estudos desenvolvidos. Um dos motivos seria relacionado ao seu benefício adicional, uma vez que o NO_3^- também é considerado como fonte de NNP, essencial para a produção de proteína microbiana. No entanto, ainda existem preocupações com a possibilidade de intoxicação, mesmo ocorrendo em situações específicas, como é o caso da ingestão de plantas com altas concentrações de NO_3^- ou pelo fornecimento em dietas sem adaptação prévia e correta.

A suplementação dos sais de NO_3^- evidenciaram eficácia não apenas na mitigação do impacto ambiental, mas também na possível redução de perdas de energia ocasionadas pela produção de CH_4 entérico, que pode variar de 2 a 12% do total da energia bruta da dieta (Johnson & Johnson, 1995). Além disso, a possibilidade de usar fontes de NO_3^- em dietas para ruminantes como substituto da ureia e de fontes de proteína verdadeira, como farelo de soja, também foram investigados (Adejoro et al., 2020; Rebelo et al., 2019).

2.3 Metabolismo do nitrato no rúmen

O mecanismo de redução de NO_3^- pelos microrganismos ruminais foi reportado inicialmente por Sapiro (1949) há pelo menos 70 anos atrás. Na última década, as preocupações em encontrar estratégias eficazes para mitigar a produção de CH_4 entérico intensificaram os estudos utilizando NO_3^- na nutrição de ruminantes (Leng, 2008). Recentemente, estudos mais elaborados e dados adicionais relacionados ao metabolismo de NO_3^- no rúmen foram publicados, facilitando o entendimento das vias metabólicas que participam deste processo (Evans et al., 2019; Latham et al., 2016; Torres et al., 2016a; Yang et al., 2016).

Atualmente, considera-se estabelecido que o NO_2^-, intermediário da reação nitrato-amônia, pode ser absorvido na parede do rúmen, sobretudo em situações nas quais os animais não estejam adaptados aos sais de NO_3^- na dieta ou se a suplementação deste aditivo for feita em altas doses e de forma incorreta. O acúmulo de NO_2^- no rúmen pode causar prejuízos severos a saúde dos animais e impactar negativamente no desempenho produtivo (Nolan et al., 2010; Torres et al., 2016a).

Em geral, o metabolismo do NO_3^- no rúmen pode ocorrer através de duas vias metabólicas principais, denominadas via assimilatória e dissimilatória (Figura 3), ambas capazes de produzir NH_3 ao fim da reação (Leng, 2008; Yang et al., 2016). A redução de NO_3^- a NO_2^- é mais favorável em relação a energia termodinâmica, portanto, a segunda redução do NO_2^- a NH_3 ocorre mais lentamente no rúmen, podendo ocasionar o acúmulo de NO_2^- caso os microrganismos ruminais não estejam adaptados a introdução deste aditivo na dieta (Latham et al., 2016).

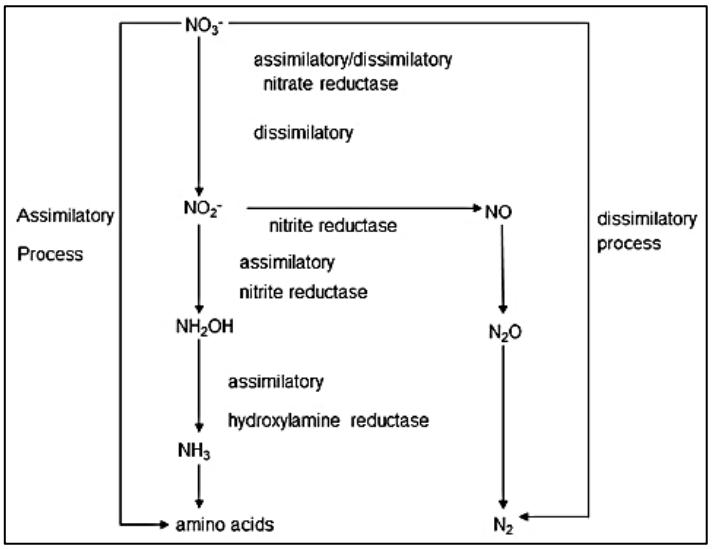

Figura 3. Redução do nitrato à amônia no rúmen através das vias assimilatória e dissimilatória (Yang et al., 2016).

Bactérias dos gêneros *Selenomonas ruminantium, Veillonella parvula,* e *Wolinella succinogenes* são consideradas como principais responsáveis pela redução do NO_3^- a NH_3 no rúmen (Simon, 2002). No entanto, de acordo Torres et al. (2016), as populações de bactérias *Escherichia coli* e *Salmonella typhimurium* também foram classificadas como nitrato-

redutoras. Entretanto, outros trabalhos demonstraram que tais gêneros de bactérias são dificilmente adaptados ao ambiente ruminal (Khafipour et al., 2011).

A redução nitrato-amônia é dependente das populações de bactérias redutoras e por sua vez, da concentração de enzimas presentes no rúmen, entre elas a nitrato e nitrito redutase (Figura 3). Assim, situações específicas, como a não adaptação dos animais a dietas experimentais, podem causar impactos negativos aos microrganismos ruminais e favorecer o acúmulo de intermediários da redução do NO_3^- a NH_3, entre eles o NO_2^-, óxido nítrico (NO) e óxido nitroso (N_2O), este último considerado como um gás importante para o efeito estufa (Lan & Yang, 2019; Torres et al., 2016a).

3. Efeitos da suplementação de nitrato para ruminantes leiteiros

Diversos estudos avaliaram a inclusão de sais de NO_3^- na dieta de ruminantes principalmente como substituto da ureia (Feng et al., 2020). No entanto, apesar do impacto positivo por meio da redução de metanógenos *Archaea* e subsequente mitigação de CH_4 entérico, efeitos adversos relacionados ao desempenho animal podem ocorrer. Assim, a investigação de parâmetros produtivos somados a preocupação com a saúde dos animais deve ser considerada na tomada de decisão do uso destes aditivos.

3.1 Metemoglobina

Um dos parâmetros mais utilizados para avaliar a presença de intoxicação decorrente da ingestão de NO_3^- advém da mensuração do nível sanguíneo de metemoglobina (MetHb). O aumento das taxas de MetHb pode ocorrer em animais não adaptados ao NO_3^-, pois o fato da não introdução gradual na dieta pode afetar os microrganismos ruminais, impactar na reação de redução nitrato-amônia, e desta forma, contribuir para o acúmulo de NO_2^- no rúmen (Lewis, 1951).

A acumulação do NO_2^- no rúmen pode favorecer a absorção através da parede ruminal, cair na corrente sanguínea e rapidamente converter a forma ferrosa (Fe^{2+}) da hemoglobina (Hb) para o estado férrico (Fe^{3+}), considerada como MetHb. Animais com níveis de MetHb superiores a 30% foram considerados em estado de metemoglobinemia, apresentando

hipoxia causado pela incapacidade da molécula heme de carrear oxigênio para os tecidos (Bruning-Fann & Kaneene, 1993).

Nos últimos anos, diversos estudos foram realizados utilizando a suplementação de NO_3^- para ruminantes. Em geral, o período de adaptação dos animais às dietas tem garantido baixos níveis de MetHb, evitando a intoxicação dos animais. Os níveis de MetHb em experimentos avaliando a suplementação de NO_3^- para vacas leiteiras foram em média 4,7% (Olijhoek et al., 2016; van Zijderveld et al., 2011). Silveira et al. (2019) avaliaram três doses de nitrato encapsulado (0, 9,4 e 18,3 g/kg de NO_3^- na MS da dieta total) em substituição ao farelo de soja para caprinos em crescimento e observaram que os índices de MetHb aumentaram linearmente, porém com o máximo índice observado de apenas 0,77%. Tal nível foi consideravelmente baixo em relação aos considerados como tóxicos para ruminantes, de acordo com os relatos mencionados anteriormente (Bruning-Fann & Kaneene, 1993).

3.2 Impactos no consumo, digestibilidade dos nutrientes e fermentação ruminal

Em geral, a ingestão de produtos compostos por NO_3^- tem gerado efeitos adversos na ingestão de nutrientes, que teoricamente podem ser relacionados como respostas a intoxicação dos animais. No entanto, de acordo com a revisão de Lee & Beauchemin (2014), muitos autores que avaliaram NO_3^- na dieta de ruminantes não observaram efeitos nos parâmetros produtivos e de composição do leite, como resultado de uma introdução gradual do NO_3^-, que também tem evitado o aumento dos níveis de MetHb sanguíneo.

Como já discutido, de acordo com Bruning-Fann & Kaneene (1993), os níveis de MetHb considerados tóxicos, e portanto capazes de impactar negativamente na produção dos animais variam de 20 a 30%, o que não tem sido observado até os dias de hoje em animais adaptados a suplementação com NO_3^-. Além de tudo, de acordo com Lee & Beauchemin (2014), a redução da ingestão de matéria seca e dos nutrientes pode não ser causado diretamente por distúrbios fisiológicos relacionados a intoxicação. Esta redução no desempenho animal pode ser causada apenas por erros de manejo alimentar, como pouca mistura da ração total levando a comportamentos de seleção, pois os suplementos que possuem NO_3^- apresentam baixa palatabilidade devido ao sabor amargo (Lee et al., 2015).

Olijhoek et al. (2016) avaliaram quatro níveis de nitrato de cálcio (0, 5,3, 13,6 e 21,1 g/kg de MS) na dieta de vacas leiteiras e não observaram efeitos na ingestão de água e da matéria seca, assim como na digestibilidade dos nutrientes das dietas. Adicionalmente, van Zijderveld et al. (2011) não observaram efeitos no consumo de matéria seca de vacas alimentadas com 21 g de NO_3^- por kg MS em substituição a ureia durante 89 dias de estudo. No mesmo estudo, não foram encontrados efeitos de tratamento nas digestibilidades do amido e da fibra em detergente neutro (FDN), assim como na produção de leite e no balanço de energia.

Conforme mencionado anteriormente, a não adaptação dos animais à suplementação de NO_3^- pode causar o acúmulo de NO_2^- no rúmen, podendo afetar negativamente a população de bactérias celulolíticas, e desta forma, reduzir a digestibilidade do FDN da dieta. De acordo com Zhou et al. (2012), a suplementação de NO_3^- na concentração de 48 μmol/mL^{-1} (aproximadamente 48 g/kg NO_3^- na MS da dieta) reduziu significativamente a quantidade de bactérias celulolíticas dos gêneros *Fibrobacter succinogenes, Ruminococcus albus,* e *R. flavefaciens,* porém sem efeitos no total de bactérias ruminais.

Em contrapartida, Patra & Yu (2015) afirmaram que a suplementação de nitrato de sódio na concentração de 5 $mmol^{-1}$ aumentou a população de bactérias celulolíticas e consequentemente a digestibilidade da fibra. Contrariamente, de acordo Wang et al. (2018) não foram observados efeito na digestibilidade do FDN em vacas alimentadas com 14,6 g de NO_3^- por kg de MS ingerida, justificado pela ausência de impacto na população de bactérias celulolíticas.

A inconsistência dos resultados supracitados pode ser justificada pela variação na dosagem de NO_3^-, fonte utilizada, método de avaliação (*in vitro* ou *in vivo*), eficiência na redução nitrato-amônia, espécie animal e efeitos associativos, quando há a combinação de NO_3^- com outros aditivos alimentares (Wang et al., 2018). Desta forma, é nítido que ainda faltam informações sobre o impacto de NO_3^- nas populações microbianas do rúmen. Além do que, experimentos *in vivo* adicionais são necessários para que esta inconsistência de resultados sobre a ingestão de MS seja amenizada.

Os efeitos no consumo e na digestibilidade dos nutrientes como resposta a suplementação de NO_3^- têm relação direta com a fermentação ruminal e as mudanças na

produção de AGV no rúmen. Teoricamente, as modificações no ambiente ruminal causadas pelo NO_3^- nos precursores da metanogênese podem causar efeitos secundários nas proporções de AGV.

De acordo com Olijhoek et al. (2016), o uso de NO_3^- suplementar até o nível de 21.1 g/kg MS na alimentação de vacas leiteiras não afetou o total de AGV ruminal, assim como as proporções individuais de AGV e a relação acetato:propionato. Em compensação, Meller et al. (2019) observaram redução na proporção de propionato, e consequentemente aumento na razão acetato:propionato quando o nitrato de cálcio foi introduzido na dieta de vacas em lactação ao nível de 20 g/kg de MS. Da mesma forma, Guyader et al. (2016) observaram redução no total de AGV, assim como na proporção de propionato e na relação de C_2:C_3 ao suplementar vacas em lactação com linhaça extrusada (100 g/kg MS) e nitrato de cálcio (18 g/kg MS).

Estudos *in vitro* (Guo et al., 2009; Lin et al., 2011) demonstraram que, em geral, a suplementação de NO_3^- tem aumentado a proporção de acetato e reduzido propionato, e consequentemente afetado a relação acetato:propionato no rúmen. Por outro lado, como detalhado no parágrafo anterior, estudos *in vivo* tem observado divergências nos efeitos de NO_3^- na produção de AGV no ambiente ruminal, provavelmente causados pela dose suplementada e pelo manejo alimentar adotado.

3.3 Implicações na síntese de proteína microbiana

Nos últimos anos, diversos estudos *in vitro* e *in vivo* tem avaliado a utilização de NO_3^- como substituto da ureia (Adejoro et al., 2020; De Raphélis-Soissan et al., 2016; Li et al., 2012). Os nitratos, assim como a ureia, têm a capacidade de atuar como fonte de NNP concomitantemente ao efeito na produção de CH_4 entérico. Desta forma, sua capacidade de fornecer nitrogênio disponível para a síntese de proteína microbiana pode ser também ponderada como foco de experimentos para animais ruminantes.

Lee & Beauchemin (2014) demonstraram por meio de uma revisão que os sais de nitrato têm em média 141% de equivalência a proteína bruta (PB), enquanto a ureia possui por volta de 291% de PB, na forma de NNP. De acordo com os mesmos autores, considerando que o NO_3^- pode ter o mesmo efeito da ureia como fonte de NNP, dietas que ultrapassem 25 g/kg

de NO_3^- com base na MS, equivalendo a aproximadamente 10 g/kg de ureia na MS total, podem ter efeitos negativos no consumo de MS, assim como na síntese de proteína microbiana e no desempenho animal.

De acordo com Wang et al. (2018), a suplementação de 14,6 g de nitrato de sódio por kg de MS, contribuiu positivamente para utilização de NH_3 na síntese de proteína microbiana em vacas leiteiras alimentadas com baixo nível de PB na dieta total. Esse efeito foi justificado como resposta da menor concentração de NH_3 no líquido ruminal de animais alimentados com NO_3^-. Como conclusão, os autores sugeriram que a suplementação de NO_3^- contribuiu positivamente para o incremento de nitrogênio na síntese de proteína microbiana de vacas leiteiras.

Silveira et al. (2019) avaliando caprinos em crescimento, observaram que a suplementação de até 25 g de nitrato encapsulado (GRASP Ind. & Com. LTDA, Curitiba, Paraná, Brazil), correspondendo a aproximadamente 18,3 g/kg de NO_3^- na MS em substituição ao farelo de soja, não afetou a síntese de proteína microbiana. No entanto, foram observados efeitos negativos no desempenho dos animais, levando os autores a concluir que o nível mais adequado de utilização de NO_3^- para caprinos aos 6 meses de idade foi de 12,5 g de nitrato encapsulado (9,4 g/kg de NO_3^- na MS) em substituição ao farelo de soja.

3.4 Efeitos na produção, composição e qualidade do leite

Estudos anteriores avaliando a utilização de NO_3^- na dieta de vacas leiteiras objetivaram principalmente reduzir a produção de CH_4 entérico e os danos ambientais causado pelos ruminantes (Meller et al., 2019; Olijhoek et al., 2016; van Zijderveld et al., 2011). Ademais, devido ao provimento de NNP no rúmen, outros autores tem avaliado a suplementação de NO_3^- como potencial estratégia para melhorar a síntese de proteína microbiana no rúmen e assim elevar índices produtivos dos animais (Wang et al., 2018).

No entanto, os sais de NO_3^- devem ser utilizados de forma correta, na dose certa e sempre com a adaptação prévia dos animais, assim como já é feito com outros aditivos específicos, como é o caso da ureia. Desta forma, a saúde dos animais seria assegurada pela utilização adequada do produto, além de garantir a segurança alimentar dos consumidores, pois animais

não adaptados podem favorecer a absorção ruminal de intermediários da redução nitrato-amônia e possivelmente elevar os resíduos no leite.

A produção de leite, em geral, não tem sido afetada pela inclusão de NO_3^- na dieta de vacas leiteiras (Klop et al., 2016; Olijhoek et al., 2016; van Zijderveld et al., 2011). No entanto, efeitos no consumo de nutrientes foram provavelmente a maior causa dos efeitos da suplementação de NO_3^- na composição do leite, o que por sua vez alteram as correções da produção de leite para gordura e energia.

Muitos estudos relataram que a suplementação de NO_3^- reduziu a quantidade de proteína verdadeira do leite (Guyader et al., 2016a; Meller et al., 2019). Contudo, o mecanismo de ação do NO_3^- na síntese de proteína ainda não está bem estabelecido. Klop et al. (2016) avaliaram a inclusão de 21 g de NO_3^- por kg de MS na dieta de vacas em lactação e observaram redução significativa na proteína verdadeira no leite. De acordo com estes mesmos autores, a menor ingestão de nutrientes, provavelmente interferiu a síntese de proteína, como resposta da redução dos precursores gliconeogênicos.

Os efeitos do NO_3^- no perfil de ácidos graxos do leite foram reportados pioneiramente na literatura através do experimento realizado por Klop et al. (2016). De acordo com estes autores, a suplementação de NO_3^-, em geral, não provocou efeitos negativos importantes no perfil de ácidos graxos do leite, aumentando significativamente as proporções de ácidos graxos poli-insaturados e não impactando as proporções de ácidos graxos saturados e monoinsaturados. Desta forma, estudos adicionais ainda são necessários para avaliar os efeitos da suplementação de NO_3^- na composição e na proporção dos ácidos graxos do leite.

Uma das limitações da suplementação de NO_3^- estão relacionadas ao possível aumento de resíduos de NO_2^- no leite como consequência da redução incompleta do NO_3^- a NH_3. Dentre os poucos estudos que avaliaram os resíduos de NO_3^- e NO_2^- no leite, Olijhoek et al. (2016) observaram maior nível de resíduo de NO_3^- (1,56 mg/L) como resultado do maior nível de suplementação de NO_3^- (21 g/kg MS), porém, de acordo com mesmo autores, tais níveis foram considerados seguros para o consumo humano, pois estão bem abaixo do nível máximo recomendado pela Organização Mundial de Saúde (WHO, 2011), que é de 50 mg/L para NO_3^- e 3 mg/L para NO_2^-. Outros estudos não observaram resíduos de NO_3^- e NO_2^- no

leite, assegurando que os níveis destes resíduos foram inferiores ao limite de detecção (Guyader, et al., 2016; Meller et al., 2019).

Até o momento, não existem trabalhos anteriores na literatura explorando os impactos do fornecimento de NO_3^- sobre a capacidade antioxidante do leite. Da mesma forma, ainda faltam informações sobre a possibilidade do aumento de resíduos de NO_3^- e NO_2^- no leite de animais alimentados com nitrato de cálcio.

Além de tudo, é importante ressaltar que não foram encontrados estudos avaliando os efeitos do nitrato de cálcio na alimentação de cabras em lactação sobre os parâmetros produtivos, a fermentação ruminal e a qualidade do leite, o que reforça a necessidade de pesquisas adicionais para avaliar a viabilidade deste aditivo como estratágia alimentar.

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III. OBJETIVO GERAL

O objetivo do presente estudo foi investigar a eficácia de fontes adicionais de nitrato na mitigação *in vitro* de metano e do nitrato de cálcio na digestibilidade dos nutrientes, na fermentação ruminal e na qualidade do leite de cabras e vacas em lactação.

OBJETIVOS ESPECÍFICOS

Estudo I

Objetivou-se avaliar fontes de nitrato associadas a dietas a base de milho seco ou silagem de grão úmido como fonte principal de amido em estudos *in vitro* sobre a degradabilidade dos nutrientes, a produção de metano, a emissão de óxido nitroso, e os parâmetros de fermentação ruminal.

Estudo II

Objetivou-se avaliar a suplementação de nitrato de cálcio na dieta para cabras em lactação sobre a composição do leite, o perfil de ácidos graxos, a capacidade antioxidante e os parâmetros de fermentação ruminal.

Estudo III

Objetivou-se avaliar a suplementação de nitrato de cálcio na dieta para vacas em lactação sobre a qualidade do leite, a síntese de proteína microbiana e os parâmetros de fermentação ruminal.

IV. Effects of nitrate sources on *in vitro* methane production, and ruminal fermentation parameters in diets differing in starch fermentability

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Abstract: We evaluated the effects of nitrate (NO_3^-) sources supplemented with feeds differing in starch degradability on *in vitro* DM and NDF degradability, methane (CH_4) production, nitrous oxide (N_2O) emissions, and ruminal fermentation parameters. The experiment followed a randomized complete block design with a 5 × 2 factorial arrangement. Treatments were five levels of non-protein nitrogen (NPN) including urea (URE), potassium NO_3^- (PON), calcium NO_3^- (CAN), dolomite-ammonium NO_3^- (DON), and ammonium NO_3^- (AMN), supplemented with total-mixed ration constituted with either high-moisture corn (HMC) or dry rolled corn (DRC). *In vitro* DM and NDF degradability were not affected by NO_3^- sources. High moisture corn improved *in vitro* DM and NDF degradability, increased propionate production, and consequently reduced acetate: propionate ratio. Methane production was reduced by 34% in HMC diets possibly due to the greater propionate proportion. Supplemental NO_3^- decreased CH_4 production (~24%) compared to urea regardless of the source without major effects on *in vitro* degradability and ruminal fermentation parameters. Concomitantly, N_2O emissions were increased by all NO_3^- sources. Contrary to our main hypothesis, no interactions were observed between NO_3^- and starch sources in all evaluated parameters, leading to conclude that NO_3^- sources acted independently at reducing CH_4 production regardless of the rate of ruminal starch degradation.

Keywords: energy loss, feed additives, greenhouse gases, methane inhibitor, starch degradability

1. Introduction

Dietary strategies to reduce methane (CH_4) emissions from ruminants have been extensively investigated in the past several years (Beauchemin et al., 2020). Enteric CH_4 emissions account for ~6 % of total greenhouse gas emissions and also represent 2-12 % of the gross energy intake of ruminants (Gerber et al., 2013; Johnson and Johnson, 1995). Hence, strategies aimed at reducing enteric CH_4 production have potential to mitigate environmental impact of livestock production and improve animal performance by conserving energy lost in CH_4 emissions (Beauchemin et al., 2020).

Feed additives have been widely explored to mitigate CH_4 emissions (Hristov et al., 2013). Recently, 3-nitrooxypropanol and nitrate (NO_3^-) have gained attention mainly because of their efficacy at reducing CH_4 emissions (Vyas et al., 2018; Wang et al., 2018). Supplemental NO_3^- is a competitive hydrogen ([H]) sink in the rumen at the expense of CH_4 synthesis while getting reduced to nitrite (NO_2^-) and subsequently ammonia (Lee and Beauchemin, 2014). Several *in vivo* and *in vitro* studies have investigated the efficacy of NO_3^- supplementation and observed significant reduction of CH_4 emissions without negative effects on animal performance and ruminal fermentation (Olijhoek et al., 2016; Zhou et al., 2012). Among sources of supplemental NO_3^-, both unencapsulated and encapsulated forms of the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3^-)_2 \cdot NH_4\ NO_3^- \cdot 10H_2O$] is most commonly studied (Henry et al., 2020; Lee et al., 2017; Olijhoek et al., 2016). However, we are still lacking studies on the efficacy of additional sources of NO_3^- in reducing CH_4 emissions as well as the effects of NO_3^- supplementation on nitrous oxide (N_2O) emissions (De Klein and Eckard, 2008; Petersen et al., 2015).

Starch is a major source of fermentable energy for ruminal microorganisms and glucogenic energy for lactating dairy cows (Hatew et al., 2015; Koenig et al., 2004). Starch fermentation results in greater proportion of ruminal propionate creating an alternative [H] sink to methanogenesis and subsequently reducing CH_4 production per unit of feed intake (g/kg DMI) (Janssen, 2010). The rate of starch fermentation also alters CH_4 emissions. Mills et al. (2001, 1999), observed lower CH_4 production with slowly fermentable starch sources such as corn compared to rapidly fermentable starch sources like barley and oats. The effects on lowering CH_4 production were attributed to shift in the site of starch digestion from rumen to small intestine. While the effects of varying starch fermentability on CH_4 has been studied, we are largely lacking studies evaluating CH_4 mitigation with supplemental NO_3^- when included in diets differing in starch fermentability.

Hence, this study was aimed at evaluating the effects of alternative NO_3^- sources; potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), and ammonium nitrate (NH_4NO_3) mixed with dolomite on *in vitro* CH_4 and N_2O production when supplemented with diets differing in starch fermentability. We hypothesized that supplemental NO_3^- will be more effective at mitigating CH_4 production in sources with higher solubility and efficacy will be greater when NO_3^- sources are included with rapidly rumen degradable starch source.

2. Material and Methods

All experimental procedures involving animals (rumen fluid collection and *in situ* incubation) were approved by the University of Florida Institutional Animal Care and Use Committee under protocol number 202009849.

2.1 Preparation of high moisture corn

Corn ears (Hybrid 26F87SX, Terral seeds®, Lake Providence, LA) were collected from four different plots in the same field at the University of Florida Plant Science Research and Education Unit (Citra, FL). Corn was shelled, homogenized, and coarsely ground using a Wiley mill (A. H. Thomas, Philadelphia, PA) without sieves to break the kernels and to simulate farm conditions. After grinding, corn samples were ensiled in vacuum-sealed in nylon-polyethylene standard barrier vacuum pouches (0.09 mm thickness, 25.4 × 35.6 cm; Doug Care Equipment Inc., Springville, CA) using a vacuum machine (Bestvac; distributed by Doug Care Equipment) in triplicates. All bags were filled and sealed within 3 h after harvesting and stored in a dark room for 45 days.

After the silo opening, 20 g of sample was dissolved in 200 mL of double-distilled water (DDH_2O). The extract was collected, and the pH was measured using a pH meter (Thermo-Orion Dual Star; Thermo Fisher Scientific Inc.) fitted with a glass pH electrode (Thermo-Orion 9172BNWP; Thermo Fisher Scientific Inc.). The remaining samples were acidified (1 mL of H_2SO_4 at 50%), centrifuged at 7,000 × *g* for 15 min at 4 °C and frozen at −20 °C for further ammonia-N (NH_3-N) and VFA analyses.

Particle size distribution was measured in dried samples (at 60 °C for 48 h) by a Tyler Ro-Tap Shaker (model RX-29; W.S. Tyler, Mentor, OH) using a set of 9 sieves (W.S. Tyler) with nominal square apertures of 4.75, 3.35, 2.36, 1.70, 1.18, 0.60, 0.30, and 0.15 mm and pan (ASABE, 2007). Geometric mean particle size (μm) was calculated using a log normal distribution (Baker and Herrman, 2002).

2.2 Chemical analyses

Feed ingredients (corn silage, dry rolled corn, and soybean meal) collected from University of Florida Dairy Research Unit (Alachua, FL) and high moisture corn samples were dried at 60 °C for 48 h in a forced-air oven (Heratherm OMS180; Thermo Fisher Scientific, Waltham, MA) to determine DM content. Samples were ground to pass through a 4-mm and 1-mm sieves in a Wiley mill (A. H. Thomas Scientific, Philadelphia, PA) for further *in situ* starch disappearance and chemical composition analyses, respectively.

Feedstuffs (Table 1) were analyzed according to AOAC (2012) for crude protein (CP) (method 990.03), acid detergent fiber (ADF) (method 973.18), neutral detergent fiber (aNDF) using sodium sulfite and heat-stable α-amylase (method: 2002.04) by Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, N.Y.), ash (method 942.05), and ether extract (EE) (method 920.39). Starch content was analyzed based on Hall et al. (2015).

2.3 Experimental design and diets

The experiment followed a randomized complete block design with a 5 × 2 factorial arrangement with 5 sources of non-protein N (NPN; urea and 4 sources of NO_3^-) and 2 sources of corn differing in the rate of starch degradability. The NPN sources used were: URE, urea (CH_4N_2O), 99.7% DM, 46% N (Thermo Fisher Scientific, Waltham, MA, USA) as a control; PON, potassium nitrate (KNO_3), 99.7% DM, 13.9% N, 61.6% NO_3^- (Thermo Fisher Scientific, Waltham, MA, USA); CAN, double salt of calcium ammonium nitrate decahydrate ($[5Ca(NO_3^-)_2 \cdot NH_4\ NO_3^- \cdot 10H_2O]$), 84% DM, 18.6% N, 76.5% NO_3^- (Yara North America, Inc. Tampa, FL, USA); DON, ammonium nitrate (NH_4NO_3) + dolomite, obtained by mixing ammonium nitrate solution with fine dolomite, 99.5% DM, 27% N, 59,8% NO_3^- (Yara North America, Inc. Tampa, FL, USA) and AMN, prilled ammonium nitrate (NH_4NO_3), 99.2% DM, 34.5% N, 76.6% NO_3^- (Yara North America, Inc. Tampa, FL, USA).

Starch sources used in the diets were either dry rolled corn (DRC) or HMC. Three independent runs were used as blocks.

Diets (Table 2) were formulated using Dairy NRC (NRC, 2001) to meet the requirements for a lactating cow of 690 kg of BW, 90 DIM, 40 kg/day of milk yield, 3.5% of milk fat, and 3% of milk true protein. All diets were isonitrogenous and isoenergetic, and the amount of NO_3^- was set at 20 g/kg DM for all diets, except 0 g/kg NO_3^- for urea as NPN source. Besides URE, other diets received urea to equilibrate the amount of NPN. Feed ingredients were previously dried, ground to pass through a 1 mm screen, and then mixed to obtain 300 g of total mixed ration (TMR), excluding urea and NO_3^- sources, which were weighted separately prior to the incubations in order to warrant the treatment effect.

2.4 In situ DM and starch disappearance

For ruminal *in situ* degradation (Table 1), 5 g samples (dried, 4 mm) were weighed in Dacron polyester bags of known weight (R1020, 10 × 20 cm, 50 ± 10 µm porosity; Ankom Technology, Macedon, NY, USA), and incubated in triplicates for 7 h in 2 ruminally cannulated lactating Holstein cows (3 bags per feedstuff per cow). Cows were fed a TMR (DM basis) consisting of corn silage (38.2%), alfalfa hay (4.0%), dry rolled corn (27.3%), soybean meal (14.5%), citrus pulp (9.1%), and a mix of minerals and supplements (6.8%).

After 7 h of incubation, bags were removed and placed immediately on ice to stop the fermentation, rinsed in a washing machine, and then dried at 60 °C for 48 h. *In situ* DM disappearance was calculated by the difference between the weight before and after incubation. For *in situ* starch disappearance, bags were opened, and the residues of replicates were pooled, resulting in one sample per feedstuff per cow for starch analysis. All ingredients

were analyzed for starch by the enzymatic method according to (Hall et al., 2015) with thermo-stable α-amylase (Ankom Technology, Macedon, NY) and amyloglucosidase (Megazyme E-AMGDF, Bray, Co. Wicklow, Ireland) enzymes, before and after the incubation period.

2.5 In vitro incubation, DM, and NDF degradability

Total mixed ration (dried, 1 mm) was used as substrate for *in vitro* incubations. Substrate were weighted (0.50 g) in Ankom F57 bags (25 µm porosity, Ankom Technology, Macedon, NY) in triplicate for each treatment combination with 5 sources of NPN and 2 sources of starch in TMR. Bags were sealed using an Uline Tabletop Poly Bag Sealer (Impulse® type AIE-200) and placed into 160-mL serum bottles. Rumen fluid was collected manually from the same cows used in the *in situ* trial, filtered through two-layer cheesecloth, and placed directly into prewarmed thermos flasks. Thermos were kept airtight until transported to the laboratory for final filtration with two more layers of cheesecloth, therefore a total of four layers of cheesecloth were used for rumen fluid filtration. The inoculum was added to a buffered pre-warmed (39 °C) media (McDougall, 1944) in a 1: 2 ratio (rumen fluid: artificial saliva). The media was continuously infused with CO_2 to maintain anaerobic environment for the rumen fluid inoculum.

Buffered rumen fluid (52 mL) was inoculated into 160 mL serum bottles with Ankom bags, and a continuous stream of CO_2 was flushed into the bottles during the whole inoculation process. The bottles were closed with rubber stoppers and sealed with aluminum seals. Serum bottles were incubated at 39 °C under a shaking system for 12 h and 24 h. Each run was repeated three times. Also, there were three blanks (bags without diet) per opening

time per run, which were used for further gas production calculations as well as for *in vitro* DM degradability (*IV*DMD) and *in vitro* NDF degradability (*IV*NDFD).

Incubations were terminated by placing bottles on ice after 12 and 24 h of incubation. Bags were taken out of serum vials, washed with tap water, and then dried in a forced-air oven set at 60 °C for 48 h. Dried residues were weighed, and the amount was used to estimate *IV*DMD. Bags were then analyzed for aNDF using sodium sulfite and heat-stable α-amylase in an Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, N.Y.). Bags were dried again at 60 °C for 48 h, weighted and the final values were used to estimate *IV*NDFD.

2.6 Gas production, CH_4, and N_2O analyses

Two sets of 30 bottles were evaluated after 12 and 24 h of incubation in each run. Headspace gas pressure was measured at 0, 3, 6, 9, 12, and 24 h using a pressure transducer in the 24 h bottles. Total gas production was estimated after 12 h and 24 h incubation. Net gas production was estimated by dividing total gas production per DM fermented. Gas pressure was calculated after correcting for gas pressure in blank bottles. Gas volume was calculated, based on our lab conditions, using the following equation.

$$GV\ (mL) = (GP * 4.8843) + 3.1296;\ r^2 = 0.97,$$

where, GV is the gas volume and GP is the gas pressure expressed by psi.

Gas samples were collected using 20 mL syringe from serum vials after 12 h and 24 h of incubation and immediately transferred to evacuated glass vials fitted with rubber stoppers. Glass vials with gas samples were crimped with aluminum seals.

Methane concentration was analyzed by gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA) with flame ionization detector and capillary column (Plot Fused Silica 25m by 0.32mm, Coating Molsieve 5A, Varian CP7536; Varian Inc. Lake Forest, CA) (Henry et al., 2020). Injector, column, and detector temperatures were maintained at 80, 160, and 200 °C, respectively. Injector pressure was 20 psi with a total flow of 191.58 mL/min and a split flow of 185.52 mL/min with a 100:1 split ratio. Column pressure was 20 psi with a flow of 1.8552 mL/min. Detector makeup flow was 21.1 mL/min. The carrier gas was N_2 and the run time was 3 min.

A second vial of gas sample was collected and analyzed for N_2O concentration by gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA). A micro-electron capture detector was used with a capillary column (Plot Fused Silica 25 m by 0.32 mm, Coating Molsieve 5A, Varian CP7536; Varian Inc. Lake Forest, CA). Injector, column, and detector temperatures were 110, 30, and 350 °C, respectively. Injector pressure was 30 psi with a total flow of 24.539 mL/min and a split flow of 18.427 mL/min with a 3:1 split ratio. Column pressure was 30 psi with a flow of 6.1422 mL/min. Detector makeup flow was 6.7834 mL/min. The carrier gas was N_2 and the run time was 4 min.

2.7 pH, volatile fatty acids, and NH_3 analyses

The pH of rumen inoculum was measured at 12 and 24 after incubation using a pH meter (Thermo-Orion Dual Star; Thermo Fisher Scientific Inc.) fitted with a glass pH electrode (Thermo-Orion 9172BNWP; Thermo Fisher Scientific Inc.). After pH measurements, 40 mL of the residual inoculum (after removing the bags) were placed into 50 mL centrifuge tubes, acidified with 0.4 mL of H_2SO_4 at 50% and centrifuged at 7,000 × g for 15 min at 4 °C. An aliquot of 2 mL was taken and frozen at −20 °C for further NH_3-N analysis.

For VFA analysis, another aliquot (2 mL) was collected and centrifuged for a second time at 10,000 × g for 15 min at 4 °C (Avanti J-E, Beckman Coulter Inc.). Subsequently, the supernatant was filtered with a 0.22 μm filter, placed into glass vials, and frozen at −20 °C for further analyses. The VFA concentration were measured using a High-Performance Liquid Chromatograph system, Hitachi, L2400, Tokyo, Japan) and an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) with 0.015*M* H_2SO_4 mobile phase and a flow rate of 0.7 mL/min at 47 °C. Ammonia-N concentration was measured via colorimetric quantification of N content using the phenol-hypochlorite reaction as described by Broderick and Kang (1980).

2.8 Statistical analyses

Data were analyzed as a randomized block design with a 5×2 factorial arrangement of treatments using GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Run was used as blocking factor. The UNIVARIATE procedure of SAS was used to test the residuals for normality prior to the final data analyses. Responses without normal distribution had data transformed using power transformation suggested by Box-Cox procedure using PROC TRANSREG in SAS (Cox and Box, 1964). Least square means were back transformed and the respective SEM was calculated (Jorgensen and Pedersen, 1998). Statistical model included NO_3^- source, starch source, and its interactions as fixed effects. Run was used as random effect.

Gas production over time was analyzed as a repeated measure. Means were determined using the LSMEANS statement. Statistical significance and trends were declared at $P \leq 0.05$ and $P > 0.05$ to $P \leq 0.10$, respectively, and Tukey-Kramer adjustments were used.

3. Results

3.1 Dry matter and NDF degradability

No interaction was observed between supplemental NPN and starch sources ($P > 0.05$) on *IV*DMD and *IV*NDFD after 12 and 24 h of incubation (Table 3). Supplemental NPN sources did not affect ($P > 0.05$) *IV*DMD after 12 or 24 h of incubation. Similarly, no differences were observed between NPN sources on *IV*NDFD after 12 h of incubation; however, *IV*NDFD tended to decrease ($P = 0.07$) with NO_3^- sources compared to URE diets after 24 h of incubation. The *IV*DMD was greater ($P < 0.01$) in diets receiving HMC compared to DRC diets regardless of the incubation duration (Fig. 1). Additionally, *IV*NDFD increased ($P < 0.01$) after 12 or 24 h of incubation in diets receiving HMC compared with DRC (Fig. 2).

3.2 Gas production, CH_4, and N_2O emissions

No interaction effects were observed between NPN and starch sources for total gas production, net gas, CH_4, and N_2O production ($P > 0.05$). Supplemental NO_3^- reduced total gas production ($P < 0.05$) compared to URE regardless of the incubation duration. However, starch sources had no effect on total gas production after 12 ($P = 0.24$) or 24 h ($P = 0.15$) of incubation (Table 4).

There was no interaction betweeen NPN and starch for gas production over time. Also, no effect was observed between starch vs. time; however, there was an effect of NPN sources and time on gas production as shown in Fig. 3. No effects of supplemental NPN sources were observed on gas production after 3 h of incubation ($P > 0.05$); however, gas production decreased ($P < 0.01$) with PON compared to other NPN sources after 6 h of incubation.

Supplemental NO_3^- sources decreased gas production compared to URE after 9, 12, and 24 h of incubation (Fig. 3).

Net gas production (mL/g DM fermented) was affected by NPN sources after 12 ($P < 0.01$) and 24 h ($P = 0.05$) of incubation. Net gas production was lower for PON diets after 12 and 24 h of incubation (Fig. 4). Nitrous oxide emissions were also affected by NPN sources ranging from the minimum production (0.96 µl/mL) observed for PON diets after 12 h of incubation and maximum production also observed for PON (1.24 µl/mL) after 24 h of incubation (Fig. 5). No starch effects were observed for net gas and N_2O production ($P > 0.05$), regardless of the incubation duration.

Methane production was independently affected by NPN and starch sources, regardless of the incubation duration. Methane production (mmol/g DM fermented) was lower for PON and DOM sources compared to URE ($P < 0.01$) after 12 h of incubation; however, NO_3^- salts decreased CH_4 production compared to URE regardless of the source after 24 h of incubation (Fig. 6). Likewise, HMC had lower ($P < 0.01$) CH_4 production (mmol/g DM fermented) compared to DRC diets regardless of the incubation duration (Fig. 7).

3.3 Ruminal fermentation parameters

No NPN or starch ($P > 0.05$) effect was observed for pH and total VFA. Similarly, no interaction was observed between NPN and starch sources for individual VFA parameters and NH_3-N after 12 and 24 h of incubation duration (Table 5).

Acetate proportion tended to increase ($P = 0.07$) by NO_3^- sources after 12 h of incubation, whereas it was greater ($P < 0.01$) for PON, DON and AMN compared to URE after 24 h of incubation (Fig. 8). Diets with HMC reduced acetate proportion after 12 h of incubation (*P*

< 0.01) compared with DRC diets (Table 5), while propionate proportion was greater (P < 0.01) with HMC compared to DRC diets after 12 (P < 0.01) and 24 h (P = 0.02) of incubation (Fig. 9).

Butyrate was affected (P < 0.05) by both NPN (P < 0.01) and starch sources (P = 0.04). Valerate was greater (P < 0.05) in HMC diets after 12 h of incubation, and the same effect was observed for isovalerate proportion after 24 h of incubation; however, no effects of NPN were observed (Table 5). Acetate:propionate (C_2:C_3) ratio was lower (P < 0.05) for HMC compared to DRC diets after 12 and 24 h of incubation; however, C_2:C_3 ratio increased by supplemental NO_3^- sources compared to URE after 12 and 24 h of incubation (Table 5). Finally, NH_3-N was lowered (P < 0.01) by PON and CAN salts when compared to URE regardless of the time, while DON and AMN did not differ from the control group at both incubation times (Fig. 10).

4. Discussion

The primary objective of this study was to evaluate different supplemental NO_3^- sources in combination with TMR composed of corn grain differing in ruminal starch disappearance on ruminal fermentation characteristics and nutrient degradability using *in vitro* batch culture experiments. We speculated greater efficacy of supplemental NO_3^- sources in mitigating CH_4 emissions when provided with highly degradable starch sources. However, contrary to our expectation, no interaction was observed between NO_3^- sources and starch degradability. Hence, our discussion will be focused on main effects of NO_3^- and starch sources observed in this study.

4.1 Nitrate supplementation

Supplemental NO_3^- has been proposed as useful NPN source for ruminants as substitute for URE. Most of the research studies have been conducted using CAN as NO_3^- source. To the best of our knowledge, the present study provides first documented evidence of efficacy of multiple supplemental NO_3^- sources on nutrient digestibility and ruminal fermentation characteristics.

Supplemental NO_3^- sources (PON, CAN, DON, and AMN) had no effects on *IV*DMD when compared to URE and effects are probably attributed to the optimal NO_3^- level (20 g/ kg DM) used in this study. These findings are in agreement with Lund et al. (2014) who evaluated double salt of calcium ammonium nitrate (up 20 g/ kg DM) and observed no effects on *IV*DMD after 48 h of incubation. Similarly, Liu et al. (2017) observed no differences on DM digestibility after 24 h of incubation evaluating sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl), with means of 535 g/kg DM and 542 g/kg DM, respectively.

The effects of supplemental NO_3^- on fiber degradability may be dose-dependent and probably attributed to NO_3^- effect on ruminal microbial population (Liu et al., 2017; Yang et al., 2016) specifically abundance of rumen cellulolytic bacteria (Villar et al., 2020; Zhou et al., 2012). Zhou et al. (2012) reported reduced cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*); however, no effects were observed on total rumen bacteria with $NaNO_3$ supplementation at highest dose (48 µmol mL^{-1}). In the present study, *IV*NDFD was not affected by NO_3^- sources after 12 h of incubation and only tended to decrease after 24 h. We can speculate that the lack of significant effects on DM and NDF degradability in this study suggests that dose (20 g/ kg DM) of NO_3^- sources had no adverse impacts on cellulolytic bacteria. Similarly, Wu et al. (2019) observed no effects on *IV*NDFD after 48 h incubation with $NaNO_3$ supplemented at

5 mmol L^{-1} under *in vitro* experimental conditions. Additionally, *in vivo* studies in dairy cows with fed calcium nitrate supplemented diet (up to 21 g/kg DM) showed no effect on ruminal, hindgut, and total-tract NDF digestibility (Olijhoek et al., 2016; van Zijderveld et al., 2011).

The effects of NO_3^- supplementation on lowering total gas production despite no effects on DM and NDF degradability may be attributed to lower CH_4 production. Capelari and Powers (2017), evaluated two distinct *in vitro* experiments (100% grass hay and 10% grass hay + 90% ground corn) and observed lower total gas production with the double salt of calcium ammonium decahydrate (up to 25 g/kg DM) and monensin sodium (up to 6 mg/L) compared to the control group even though no effects on *IV*DMD were reported.

Net gas production (mL/g DM fermented) was also decreased by NO_3^- supplementation; however, only PON differed from the control group after 24 h of incubation. The absence of effects on CAN, DON, and AMN compared to the control group (URE) may be explained by the presence of NH_3 in the sources. Supplementing NO_3^- salts with NH_3 seem to accelerate the reduction NO_3^- to NH_3, and consequently alleviate the toxic effect of nitrite (NO_2^-) as an intermediate of this reduction. This effect possibly reduced the negative effects of NO_2^- accumulation on rumen microbes probably resulting in absence of effects on net gas production and NDF degradability.

Nitrate supplementation is an effective way to decrease CH_4 production through the reduction of [H] availability as a consequence of its competition between methanogens and NO_3^- reducing bacteria. Thermodynamically, four [H] are consumed in this reaction, and consequently, one mole of CH_4 is inhibited (Van Zijderveld et al., 2010). Nitrate supplementation lowers CH_4 production by NO_3^- reduction to NH_3. This reaction of NO_3^- is energetically more favorable compared to methanogenesis. In addition, NO_3^-

supplementation can also decrease CH_4 production due to the toxic effects of NO_2^- on rumen methanogens. Nitrite is produced as intermediate during NO_3^- reduction to NH_3 (Lee et al., 2017; Leng, 2008; Van Zijderveld et al., 2010; Zhou et al., 2012). Contrary to our hypothesis, NO_3^- supplementation decreased CH_4 production with similar efficacy (~24%), regardless of the sources used. It was expected that sources of NO_3^- with greater solubility (DON and AMN) would increase CH_4 mitigation by accelerate conversion of NO_3^- into NH_3; however, differences in solubility and increasing the rate of NO_3^- reduction may not affect CH_4 production under *in vitro* conditions.

To date, few bacteria have been identified as NO_3^- reductors (*Selenomonas ruminantium, Veillonella parvula, Wolinella succinogenes*) (Simon, 2002). *Escherichia coli* and *Salmonella typhimurium* have been also considered NO_3^- reducing bacteria (Torres et al., 2016); however, both bacteria are poorly adapted to ruminal conditions (Khafipour et al., 2011; Rasmussen and Casey, 1993). The capacity of rumen microflora to reduce NO_3^- to NO_2^- may exceed the capacity for NO_2^- reduction resulting in ruminal accumulation of NO_2^-. Under *in vivo* conditions, NO_2^- can be readily absorbed across rumen wall and converts blood hemoglobin from ferrous to ferric state (methemoglobin), thereby losing capability of transporting oxygen to the tissues (Morgavi et al., 2010). Besides NO_2^- accumulation, N_2O production could be increased, whereas its metabolism still not well established (Torres et al., 2016).

Although N_2O represents only 0.03% of total greenhouse gas emissions, the global warming potential of N_2O is 300 times greater than carbon dioxide (Torres et al., 2016). In theory, N_2O is produced by denitrification, which is another pathway of the dissimilatory process, when NO_3^- is reduced to NH_3 (Latham et al., 2016; Torres et al., 2016). However,

as mentioned before, the main mechanism of N_2O production in the rumen is still unknown. Our results are in agreement with Petersen et al. (2015) who also evaluated N_2O emissions from feedstuffs and excreta, and observed no effects on those samples, leading to conclude that N_2O emissions came specifically from animals fed NO_3^-. Lee et al. (2017) observed lower N_2O with encapsulated NO_3^- compared with un-encapsulated NO_3^- suggesting that slow release of NO_3^- and slower NO_3^- availability to microbes is an effective strategy to reduce N_2O production from NO_3^- supplemented animals.

The reduction in total gas and CH_4 production with NO_3^- supplementation are highly associated with changes in rumen fermentation and effects on VFA profile. Nitrate supplementation increased acetate and reduced propionate proportion in both *in vitro* and *in vivo* studies (Li et al., 2012; Lin et al., 2011; Nolan et al., 2010). The effects on greater molar proportion of acetate may be attributed to NO_3^- reduction resulting in reduced availability of [H] for propionate production (Nolan et al., 2010). The NO_3^- reduction pathway is thermodynamically more favorable compared to propionate synthesis (Van Zijderveld et al., 2010). Although acetate was elevated with NO_3^- supplementation, regardless of the source used, no effects were observed on propionate proportion. The effects of NO_3^- supplementation of lowering butyrate proportion are in agreement with Lin et al. (2011) evaluating 12.6 g/kg DM of $NaNO_3$ after 12 and 24 h of *in vitro* incubations. The lack of effects on isobutyrate, isovalerate, valerate, and total VFA production are in agreement with Lund et al. (2014), who observed no effects on VFA production and associated it with a low impact on *in vitro* rumen fermentation when NO_3^- was added in a TMR up to 20 g/kg DM. The increase on acetate proportion also increased the C_2:C_3 ratio in both incubation times.

Based on previous studies it is well established that URE has greater conversion to NH_3 during initial hours of feeding (Guo et al., 2009; Lee et al., 2017; Wang et al., 2018)**.** This might have attributed greater NH_3-N concentration with URE supplemented group compared to NO_3^- sources. Although numerical, but greater NH_3-N concentration observed with DON and AMN compared with PON and CAN may be attributed to the presence of free NH_3 with DON and AMN resulting in greater NH_3 availability to rumen microbes. Leng (2008) suggested that supplemental NO_3^-, compared to URE, has generally lower NH_3-N concentration in the rumen because of two-step reduction of supplemental NO_3^- as it is first converted to NO_2^- and then to NH_3. The two-step reaction of supplemental NO_3^- is considered the key-factor to CH_4 mitigation since reduction at both steps are thermodynamically more favorable compared to methanogenesis under ruminal conditions (Latham et al., 2016).

4.2 Starch degradability

The considerable differences in the rates of *in situ* starch degradability explains greater DM observed with HMC compared to DRC diets. High-moisture corn has greater ruminal and total-tract starch degradability compared to DRC because the hydrophobic starch-protein matrix is broken down during the ensiling process (Ferraretto et al., 2013; Philippeau and Michalet-Doreau, 1998). The effects observed on increasing NDF degradability with HMC diets are not in agreement with earlier studies. Krause and Combs (2003), reported that NDF degradability may be reduced at lower pH as a consequence of greater ruminal starch degradability. Likewise, Ferraretto et al. (2013), reported in a meta-analytical study that diets with higher starch degradability generally decreased NDF digestibility in dairy cows mainly due to negative associate effects such as lower pH and lower abundance of cellulolytic

bacterial population (Wanapat et al., 2014). The pH of rumen inoculum was not affected, regardless of the starch source used, and it may have prevented negative effects on NDF degradability in the present study. Greater effects on NDF degradability with HMC diets are difficult to explain and may be attributed to greater energy availability with starch fermentation for microbial biomass synthesis. However, this speculation should be interpreted with caution since NH_3-N concentration tended to increase with HMC after 12 h of incubation suggesting inefficient capture of ruminally available N for microbial protein synthesis.

Diets containing HMC decreased CH_4 production by 34% in comparison with DRC diets, partially supporting our hypothesis. Diets with higher starch degradability reduce CH_4 synthesis by increasing propionate production, thereby providing an alternative [H] sink (Bannink et al., 2006; Moate et al., 2017). Alvarez-Hess et al. (2019) compared the effects of NO_3^- supplementation on wheat and corn-based diets and observed lower CH_4 production with wheat-based diets primarily due to rapid starch fermentation and higher propionate proportion. Diets with HMC reduced molar proportion of acetate proportion after 12 h of incubation; however, effects on acetate proportion disappeared after 24 h of incubation. High-moisture corn containing diets had lower NDF content; however, it may not affect lower acetate proportion after 12 h of incubation since NDF degradability was greater after 12 h and no changes were observed on total VFA concentration. Nevertheless, the changes in VFA proportions with HMC diets resulted in decreasing the C_2:C_3 ratio, leading to lower CH_4 production. The results are in agreement with Benchaar et al. (2001) who reported, through a modeling approach, lower CH_4 production due to the lower C_2:C_3 ratio.

Ammonia-N concentration tended to increase in diets receiving HMC compared to DRC after 12 h of incubation. The effects on NH_3-N concentration with HMC diets suggest lower incorporation of NH_3-N for microbial protein synthesis despite greater levels of starch fermentability. The greater levels of NH_3-N concentration may be attributed to higher protein fermentation from HMC. Hoffman et al. (2011) reported greater NH_3-N concentration during the fermentation process of HMC due to proteolytic degradation of hydrophobic proteins present in the starch-protein matrix.

5. Conclusions

Contrary to the main hypothesis, no interactions were observed between NPN and starch sources, leading to conclude that NO_3^- sources were individually efficient at reducing CH_4 production regardless of the rate of starch rumen degradation. Methane mitigation was observed by supplemental NO_3^-, regardless of the source used, without negatively affecting *in vitro* DM and NDF degradability and ruminal fermentation parameters. However, N_2O emissions were increased with supplemental NO_3^-, which should be studied further considering its global warming potential. Greater starch fermentability with HMC diets reduced CH_4 production because relative [H] sources decline due to lower acetate production and that of [H] sinks increase because of greater propionate synthesis. In addition, HMC diets improved both DM and NDF degradability

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Conflicts of Interest

The authors declare no potential conflicts of interest.

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Table 1

Chemical composition and *in situ* disappearance of feedstuffs used for the experimental diets.

Item	Ingredients[a]			
	HMC[b]	DRC[c]	CS[d]	SM[e]
Nutrient composition, g/kg DM				
DM, g/kg as fed	608 ± 0.83	939 ± 0.07	340 ± 1.20	954 ± 0.08
CP	98.8 ± 5.24	86.3 ± 4.03	83.1 ± 8.20	494 ± 3.06
aNDF	74.6 ± 4.57	100 ± 4.03	452 ± 6.60	118 ± 2.48
ADF	23.7 ± 1.91	20.6 ± 0.59	242.4 ± 4.02	69.3 ± 0.58
Ash	12.0 ± 1.13	18.3 ± 0.77	40.3 ± 1.38	85.9 ± 1.54
EE	41.6 ± 2.79	22.5 ± 2.20	30.0 ± 1.63	17.8 ± 1.35
Starch	575 ± 0.08	598 ± 0.12	353 ± 0.32	9.80 ± 0.20
In situ disappearance, g/kg DM[f]				
DM	679.3 ± 17.0	478.2 ± 17.2	553.1 ± 14.8	523.2 ± 34.3
Starch	718.9 ± 49.6	487.9 ± 18.5	864.1± 5.5	739.8 ± 18.9
Particle size				
Mean distribution, µm	2,817 ± 32			
Fermentation parameters, g/kg DM				
pH	4.04 ± 0.05	-	-	-
NH_3-N	0.35 ± 0.04	-	-	-
Total VFA	85.9 ± 2.67	-	-	-
Lactate	51.1 ± 2.34	-	-	-
Acetate	16.7 ± 0.52	-	-	-
Propionate	6.48 ± 0.31	-	-	-

[a]Mean ± standard deviation

[b]HMC: high moisture corn

[c]DRC: dry rolled corn

[d]CS: corn silage

[e]SM: soybean meal

[f]*In situ* disappearance after 7 h incubation

Table 2

Ingredient proportion and chemical composition of the experimental diets.

Item	Dry rolled corn					High moisture corn				
	URE	PON	CAN	DON	AMN	URE	PON	CAN	DON	AMN
Ingredient, g/kg DM										
CS[a]	550.1	550.0	550.4	550.0	550.1	550.2	550.0	549.9	550.2	550.0
DRC[b]	354.4	326.8	335.0	338.1	346.4	0.0	0.0	0.0	0.0	0.0
HMC[c]	0.0	0.0	0.0	0.0	0.0	359.5	331.8	340.9	343.1	351.6
SM[d]	75.9	80.8	79.4	78.4	77.4	70.9	76.0	74.0	73.2	72.3
Urea[e]	19.6	9.9	9.1	0.0	0.0	19.4	9.7	9.1	0.0	0.0
NO_3^-[f]	0.00	32.5	26.1	33.5	26.1	0.00	32.5	26.1	33.5	26.1
Chemical composition, g/kg DM										
DM	800.0	800.8	798.0	797.0	800.2	805.3	805.8	802.5	805.5	805.4
CP	170.0	170.0	170.0	170.0	170.0	172.2	172.0	172.0	172.1	172.1
NDF	249.0	245.0	246.0	247.0	248.0	226.0	224.0	225.0	225.0	226.0
ADF	135.0	134.0	134.0	134.0	135.0	133.0	132.0	132.0	132.0	136.0
EE	31.0	30.0	31.0	31.0	31.0	33.0	31.0	32.0	32.0	32.0
Starch	35.1	34.1	34.1	34.3	34.9	34.9	33.5	34.0	34.1	34.5
NO_3^-	0.0	20.0	20.0	20.0	20.0	0.0	20.0	20.0	20.0	20.0
NE_L[g]	1.55	1.55	1.55	1.55	1.55	1.56	1.57	1.56	1.56	1.56

[a]CS: corn silage

[b]DRC: dry rolled corn

[c]HMC: high moisture corn

[d]SM: soybean meal

[e]Urea (99.7% DM, 46% N)

[f]NO_3^- sources: PON = Potassium nitrate (99.7% DM, 13.9% N, 61.6% NO_3^-), CAN = Double salt of calcium ammonium nitrate decahydrate (84% DM, 18.6% N, 76.5% NO_3^-), DON = Ammonium nitrate + dolomite (99.5% DM, 27% N, 59.8% NO_3^-) and AMN- prilled ammonium nitrate (99.2% DM, 34.5% N, 76.6% NO_3^-)

[g]Net energy for lactation (mcal/kg DM)

Table 3

Effects of nitrate sources supplemented in diets with different starch fermentability on *in vitro* dry matter (*IV*DMD) and neutral detergent fiber degradability (*IV*NDFD).

Item[a]	Dry rolled corn					High moisture corn					SEM[b]	*P*-value		
	URE	PON	CAN	DON	AMN	URE	PON	CAN	DON	AMN		NPN[c]	Starch[d]	Int[e]
12 h incubation														
*IV*DMD	396.1	408.0	377.7	377.3	381.8	408.4	392.0	395.6	413.5	400.4	11.21	0.48	0.03	0.13
*IV*NDFD	530.6	529.4	547.9	552.2	551.3	612.7	602.9	608.3	606.5	603.3	24.03	0.79	<0.01	0.69
24 h incubation														
*IV*DMD	648.0	632.6	630.5	633.4	628.1	653.0	647.2	649.4	663.5	656.7	10.61	0.47	<0.01	0.43
*IV*NDFD	463.1	435.4	444.2	439.4	421.7	580.1	564.5	533.5	559.8	521.9	25.47	0.07	<0.01	0.71

[a]g/kg DM

[b]SEM: standard error of the mean

[c]NPN effect: URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate

[d]Starch effect: either DRC or HMC

[e]Int: Interaction NPN × starch sources

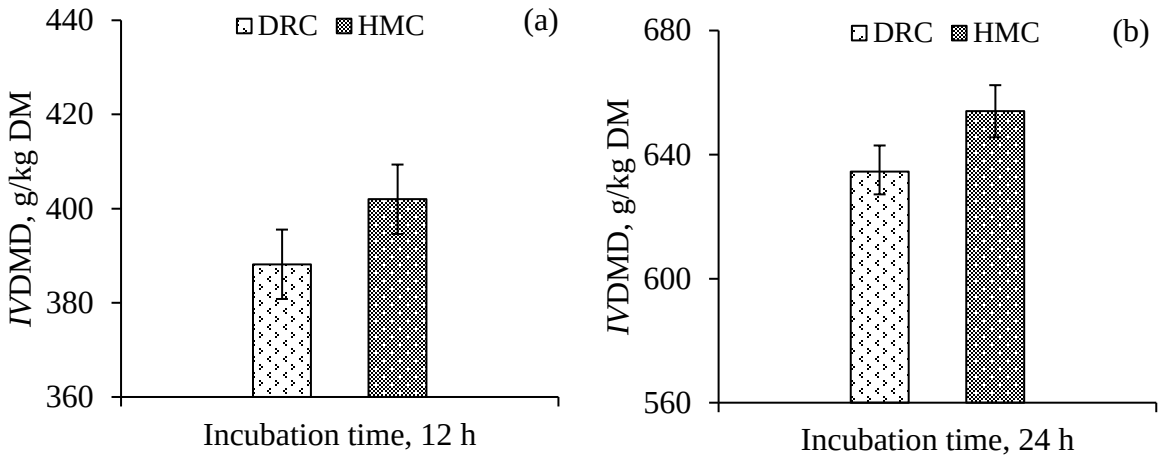

Fig. 1.

Effects of starch sources on *in vitro* dry matter degradability (a) after 12 h of incubation (P = 0.03; SEM = 7.37) and (b) after 24 h of incubation (P < 0.01; SEM = 8.39); DRC = dry rolled corn; HMC = high moisture corn.

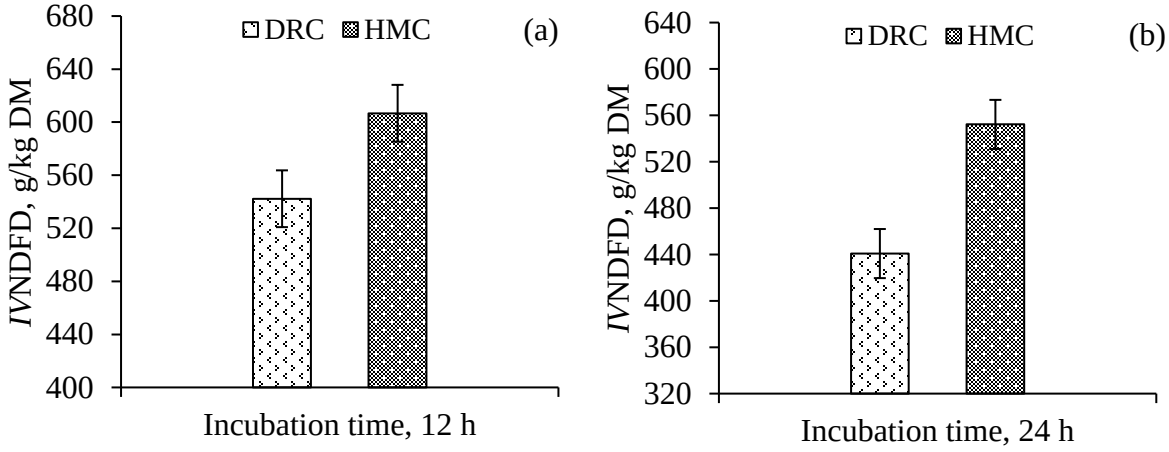

Fig. 2.

Effects of starch sources on *in vitro* neutral detergent fiber degradability (a) after 12 h of incubation (P < 0.01; SEM = 21.41) and (b) after 24 h of incubation (P < 0.01; SEM = 21.17); DRC = Dry rolled corn; HMC = High moisture corn.

Table 4

Effects of nitrate sources supplemented in diets with different starch fermentability on *in vitro* total gas, methane (CH_4), and nitrous oxide (N_2O) production.

Item	Dry rolled corn					High moisture corn					SEM[a]	*P*-value		
	URE	PON	CAN	DON	AMN	URE	PON	CAN	DON	AMN		NPN[b]	Starch[c]	Int[d]
12 h incubation														
Total gas, mL	28.9	26.1	25.6	25.9	26.6	31.2	24.5	25.5	26.9	27.7	1.68	<0.01	0.24	0.10
Net gas, mL/g DM[e]	148.5	130.8	135.6	138.2	140.6	156.0	127.4	130.1	133.2	141.4	7.20	<0.01	0.58	0.27
CH_4, mmol	0.210	0.158	0.185	0.169	0.176	0.104	0.056	0.070	0.066	0.075	0.01	<0.01	<0.01	0.92
CH_4, mmol/g DM[f]	0.718	0.556	0.650	0.579	0.611	0.523	0.289	0.355	0.328	0.388	0.05	0.01	<0.01	0.88
N_2O, µl/mL	0.94	0.95	1.00	1.11	1.21	1.02	0.98	1.19	1.12	1.14	0.14	0.03	0.24	0.50
24 h incubation														
Total gas, mL	51.1	47.9	48.7	47.2	48.2	52.7	46.5	49.6	50.4	50.2	2.82	0.03	0.15	0.52
Net gas, mL/g DM[e]	175.1	167.7	173.2	166.2	171.6	181.5	163.5	174.2	173.2	173.7	6.29	0.05	0.33	0.61
CH_4, mmol	0.203	0.156	0.161	0.151	0.128	0.089	0.064	0.069	0.068	0.065	0.02	<0.01	<0.01	0.30
CH_4, mmol/g DM[f]	0.692	0.540	0.567	0.527	0.455	0.458	0.321	0.363	0.364	0.340	0.07	<0.01	<0.01	0.43
N_2O, µl/mL	1.02	1.27	1.12	0.97	1.17	0.98	1.21	1.06	1.07	1.19	0.15	0.04	0.93	0.78

[a]SEM: standard error of the mean

[b]NPN effect: URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate

[c]Starch effect: either DRC or HMC

[d]Int: interaction NPN × starch sources

[e]Net gas production mL/g DM fermented

[f]CH_4 production mL/g DM fermented

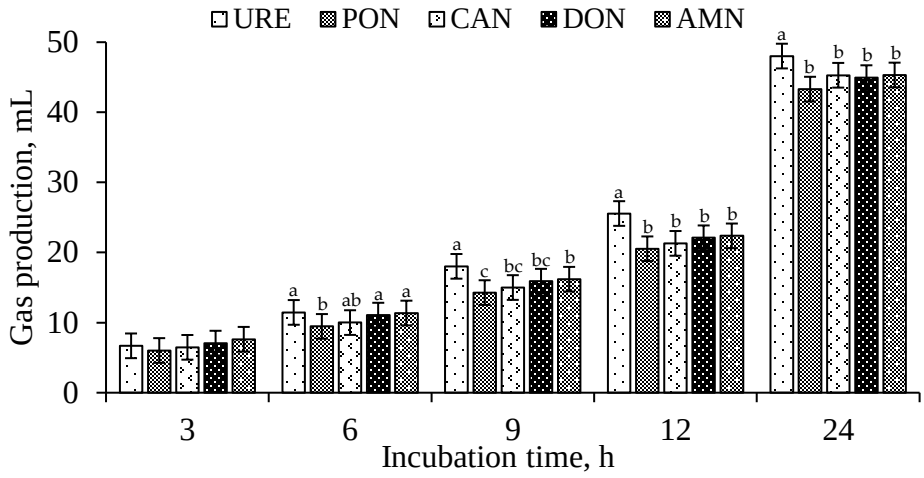

Fig. 3.

Effects of nitrate sources on *in vitro* gas production over time (NPN $P < 0.01$; Time $P < 0.01$; SEM = 1.75); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

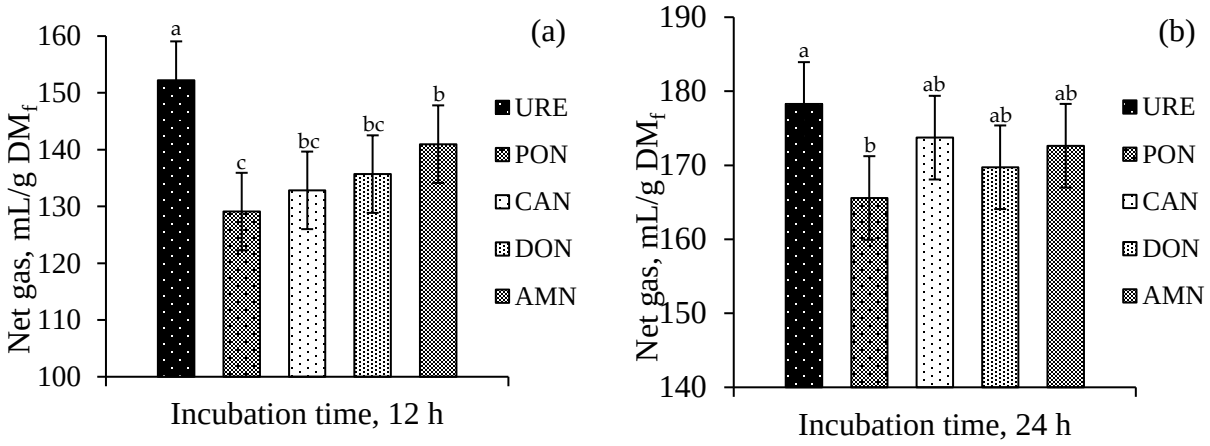

Fig. 4.

Effects of nitrate sources on *in vitro* net gas production (mL/g DM fermented) (a) after 12 h of incubation ($P < 0.01$; SEM = 6.83) and (b) after 24 of incubation ($P < 0.05$; SEM = 5.65); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

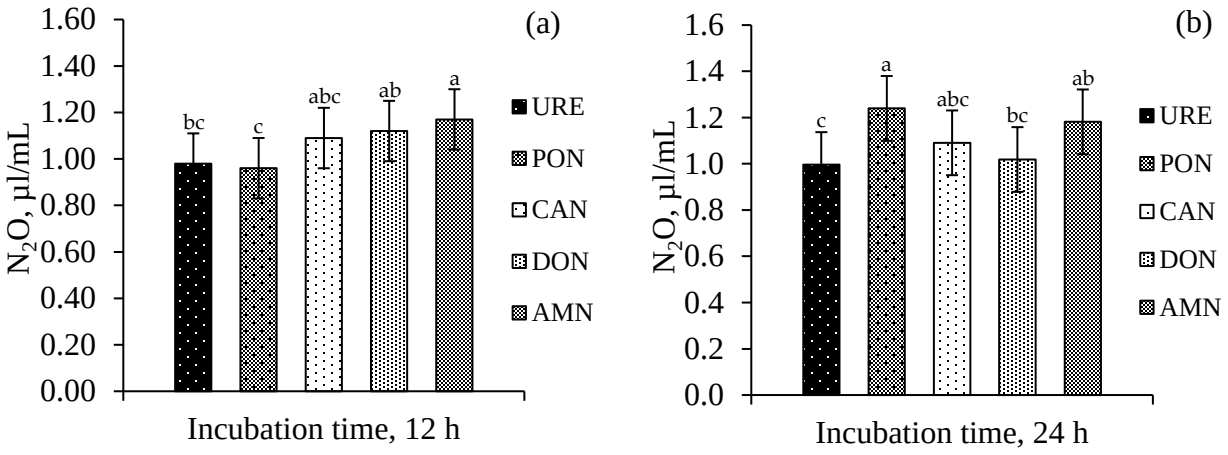

Fig. 5.

Effects of nitrate sources on *in vitro* N_2O emissions (µl/mL) (a) after 12 h of incubation (P = 0.03; SEM = 0.14) and (b) after 24 h of incubation (P = 0.04; SEM = 0.17); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

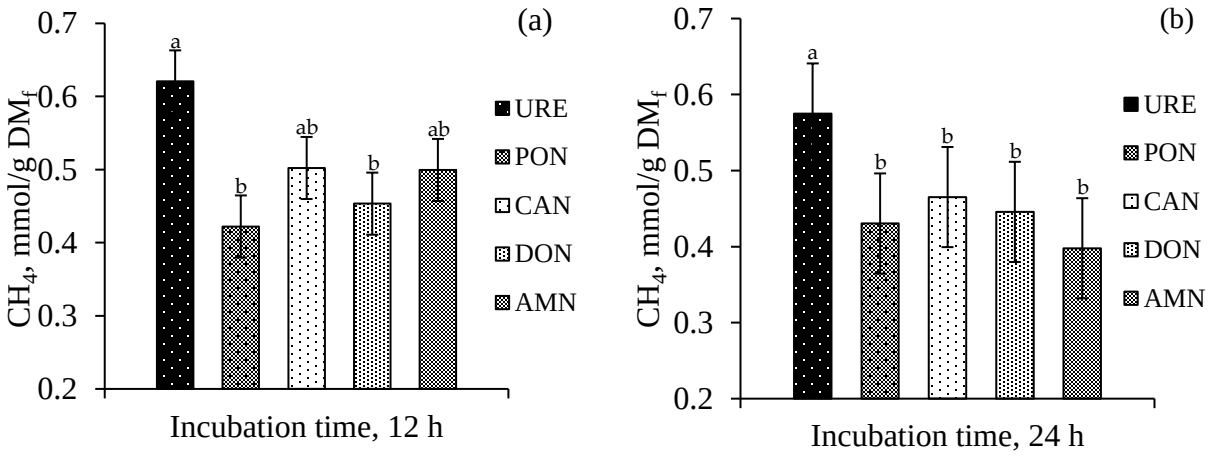

Fig. 6.

Effects of nitrate sources on *in vitro* CH_4 production (mmol/g DM fermented) (a) after 12 h of incubation (P < 0.01; SEM = 0.07) and (b) after 24 h of incubation (P < 0.01; SEM = 0.04); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

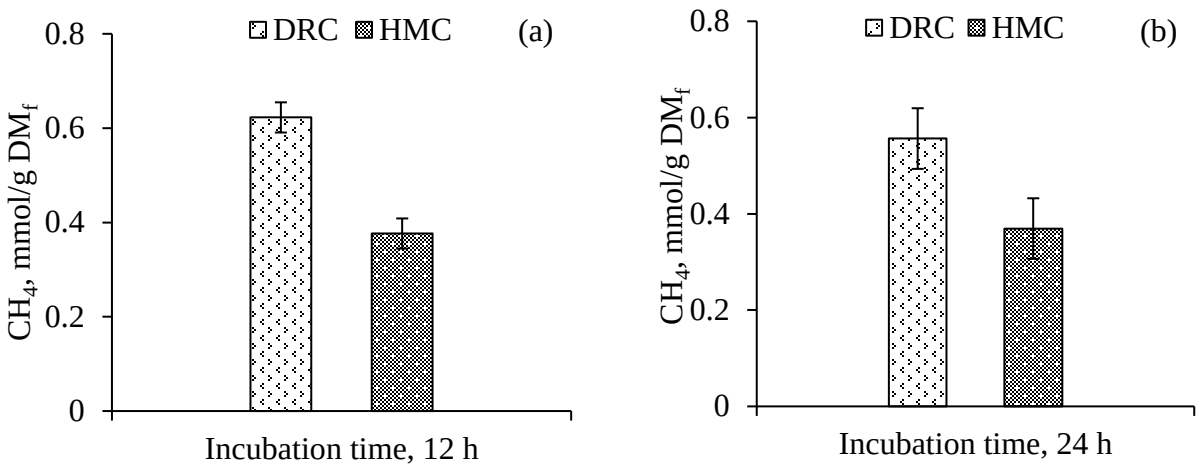

Fig. 7.

Effects of starch sources on *in vitro* CH_4 production (mmol/g DM fermented) (a) after 12 h of incubation ($P < 0.01$; SEM = 0.03) and (b) after 24 h of incubation ($P < 0.01$; SEM = 0.06); DRC = Dry rolled corn; HMC = High moisture corn.

Table 5

Effects of nitrate sources supplemented in diets with different starch fermentability on *in vitro* pH, volatile fatty acid, and NH_3-N.

Item	Dry rolled corn					High moisture corn					SEM[a]	*p*-value		
	URE	PON	CAN	DON	AMN	URE	PON	CAN	DON	AMN		NPN[b]	Starch[c]	Int[d]
12 h incubation														
pH	6.54	6.56	6.54	6.49	6.48	6.48	6.57	6.58	6.51	6.52	0.168	0.24	0.88	0.93
NH_3-N, m*M*/dL	31.8	24.5	25.2	27.3	28.6	34.7	28.8	25.5	33.4	31.1	3.259	0.04	0.06	0.82
Total VFA, m*M*	101	98	108	97	104	118	128	99	117	111	11.22	0.71	0.11	0.19
Individual VFA, mol/100 mol														
Acetate	51.9	54.8	54.2	55.4	54.6	50.1	50.6	52.0	50.3	53.2	0.922	0.07	<0.01	0.21
Propionate	21.6	19.9	19.6	20.0	19.8	21.0	21.1	21.3	21.0	21.1	0.802	0.28	<0.01	0.14
Isobutyrate	3.0	3.3	3.8	3.1	3.4	3.0	3.5	3.0	3.6	3.3	0.525	0.63	0.76	0.32
Butyrate	14.8	12.5	11.9	12.8	12.2	15.1	12.6	13.4	13.1	12.9	0.758	<0.01	0.06	0.41
Isovalerate	3.9	4.0	4.4	4.0	3.9	3.8	3.9	3.9	4.3	4.1	0.270	0.37	0.78	0.28
Valerate	4.5	5.4	6.1	4.7	6.1	7.5	5.9	6.8	5.8	6.3	0.789	0.33	0.01	0.19
C_2: C_3	2.41	2.76	2.78	2.78	2.76	2.38	2.40	2.45	2.39	2.54	0.105	0.02	<0.01	0.12
24 h incubation														
pH	6.12	6.24	6.51	6.20	6.25	6.27	6.22	6.36	6.30	6.20	0.168	0.24	0.88	0.93
NH_3-N, m*M*/dL	29.2	24.7	26.1	27.6	29.4	31.7	27.7	25.9	28.5	29.7	3.232	0.04	0.20	0.82
Total VFA, m*M*	133	136	137	132	142	125	128	127	122	114	15.07	0.99	0.17	0.93
Individual VFA, mol/100 mol														
Acetate	50.3	51.7	50.8	52.0	51.8	49.0	51.4	50.7	51.3	53.0	0.850	<0.01	0.53	0.28
Propionate	22.3	22.6	22.4	22.0	21.4	22.8	22.2	22.9	22.5	23.3	0.752	0.80	0.02	0.11
Isobutyrate	2.7	2.8	2.7	2.6	3.1	2.7	2.7	2.9	2.6	2.7	0.194	0.41	0.62	0.62
Butyrate	15.7	14.2	14.6	14.2	13.9	16.5	14.6	15.3	15.0	13.1	0.742	0.01	0.31	0.67
Isovalerate	4.0	4.0	4.1	4.0	4.2	4.2	4.3	4.4	4.3	4.3	0.146	0.89	0.01	0.90
Valerate	5.0	4.6	5.0	4.9	5.1	5.1	5.1	4.7	4.8	4.0	0.727	0.94	0.62	0.72
C_2: C_3	2.27	2.29	2.27	2.37	2.42	2.16	2.33	2.21	2.28	2.27	0.090	0.01	0.03	0.32

[a]SEM: standard error of the mean;

[b]NPN effect: URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate

[c]Starch effect: either DRC or HMC;

[d]Int: Interaction NPN × starch sources

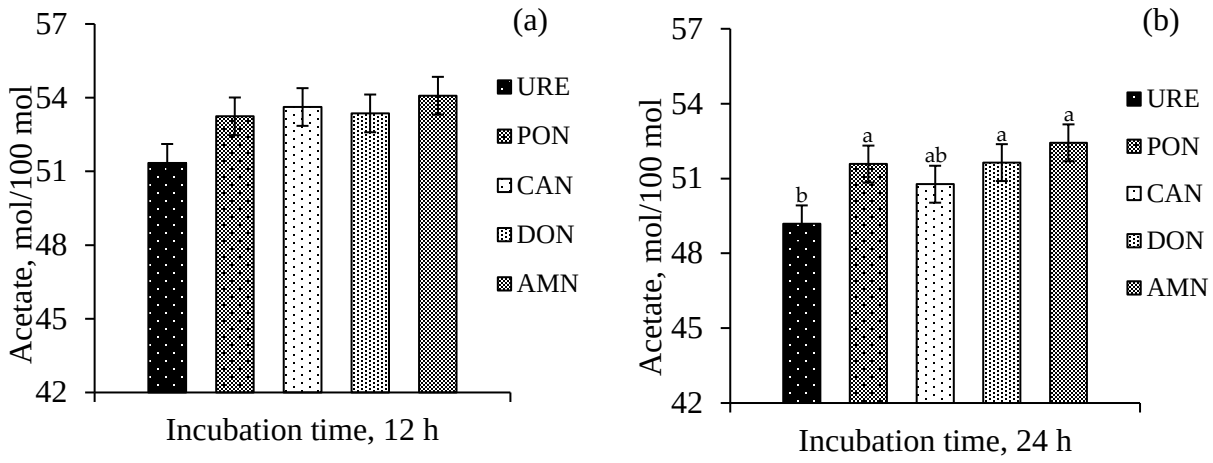

Fig. 8.

Effects of nitrate sources on *in vitro* acetate proportion (mol/100 mol) (a) after 12 h of incubation ($P = 0.07$; SEM = 0.65) and (b) after 24 h of incubation ($P < 0.01$; SEM = 0.74); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

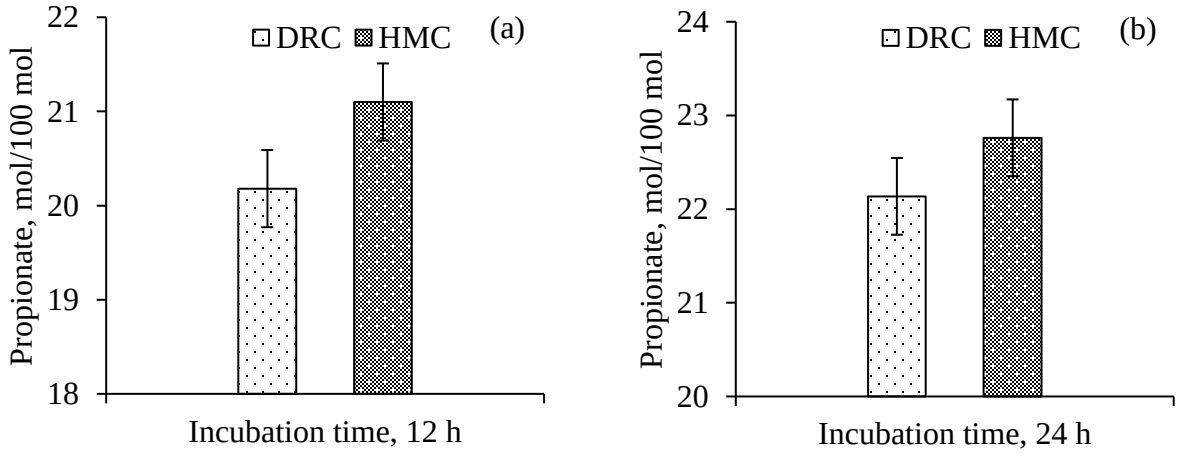

Fig. 9.

Effects of starch sources on *in vitro* propionate proportion (mol/100 mol) (a) after 12 h of incubation ($P < 0.01$; SEM = 0.03) and (b) after 24 h of incubation ($P = 0.02$; SEM = 0.06); DRC = Dry rolled corn; HMC = High moisture corn.

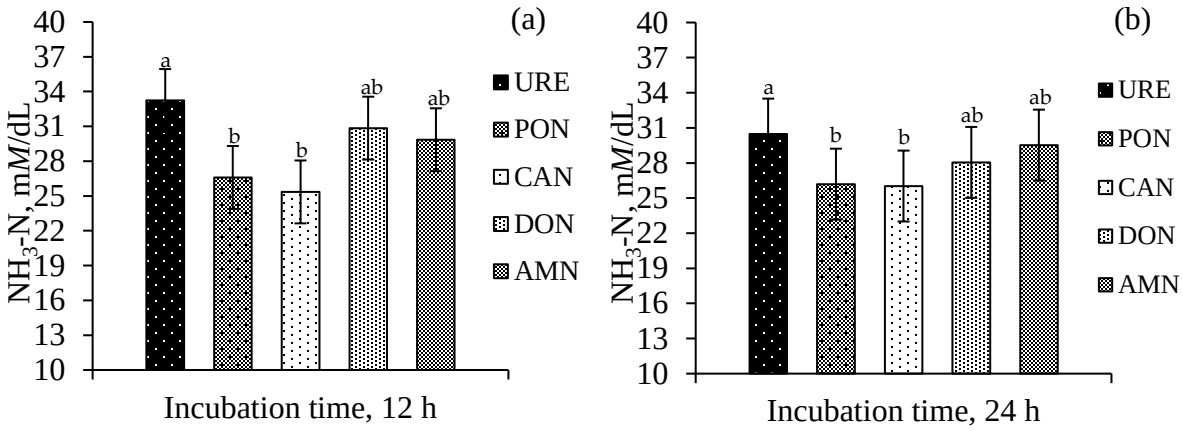

Fig. 10.

Effects of nitrate sources on *in vitro* NH_3-N concentration (m*M*/dL) (a) after 12 h of incubation ($P < 0.01$; SEM = 2.71) and (b) after 24 h of incubation ($P = 0.04$; SEM = 3.03); URE = Urea, PON = Potassium nitrate, CAN = Double salt of calcium ammonium nitrate decahydrate, DON = Ammonium nitrate + dolomite, and AMN = Prilled ammonium nitrate.

V- Effect of dietary calcium nitrate on milk composition, fatty acids profile, antioxidant capacity, and ruminal fermentation in dairy goats

Journal: Livestock Science

Abstract

We evaluated the effects of calcium nitrate (CAN) fed to dairy goats on milk composition, fatty acids profile, antioxidant capacity, and ruminal fermentation parameters. Twelve goats at 98.5 ± 13.1 DIM, with 53.5 ± 3.3 kg of BW were enrolled in four 3 × 3 Latin square design. Each period lasted in 21 days, with 14 days for the adaptation of diets and facilities and 7 days for sampling and data collection. Treatments were designed as UREA: control group (without nitrate), CAN10: 10 g of calcium nitrate (7.65 g/kg of NO_3^- on DM basis), and CAN20: 20 g of calcium nitrate (15.3 g/kg of NO_3^- on DM basis). Feed intake and nutrient digestibility were not affected by CAN. Milk yield, energy corrected milk, and fat corrected milk were similar between diets. Milk composition (fat, true protein, and lactose) and milk fatty acids had no influence of CAN. Total antioxidant capacity was unaffected by CAN, while conjugated dienes were elevated and TBARS was reduced. Nitrate and nitrite residuals in milk were altered by CAN. Supplemental CAN did not affect the concentration of urea in plasma. Calcium nitrate did not affect the total of volatile fatty acids and their proportions, as well as the ammonia-nitrogen concentration within the rumen. Calcium nitrate can be fed (up to 20 g/kg DM) to dairy goats as a urea replacer without affecting feed intake, nutrient digestibility, and milk quality.

Keywords: feed additive, hydrogen sink, nitrite, non-protein nitrogen

1. Introduction

Nitrate (NO_3^-) has been established as a feasible feed additive to reduce CH_4 production, as well as a potential substitute to urea because of its capacity to provide ammonia (NH_3) slowly to the microbes in the rumen, and, therefore, acting as an alternative non-protein nitrogen (NPN) source (Feng et al., 2020; Wang et al., 2018). However, supplementing NO_3^- to ruminants requires a gradual inclusion in diets in order to avoid nitrite (NO_2^-) accumulation into the rumen, as the intermediate of the reduction NO_3^- to NH_3 (Lee and Beauchemin, 2014).

Previous studies have shown that feeding NO_3^- to dairy cows with a prior adaptation avoided high concentrations of NO_3^- and NO_2^- residuals in milk (Guyader et al., 2016a; Meller et al., 2019; Olijhoek et al., 2016). Additionality, according to Klop et al. (2016) supplemental NO_3^- fed to dairy cows had minor impacts on milk fatty acids (FA), even though others have shown a negatively impact on milk composition by following NO_3^- supplementation (Guyader et al., 2016a; Meller et al., 2019; van Zijderveld et al., 2011).

Despite the well documented data of NO_3^- acting at the expense of enteric CH_4 production (Beauchemin et al., 2020; Feng et al., 2020), there is still a lack of information of its impacts on animal performance and milk quality. To date, according to the best of our knowledge, there are no previous studies evaluating the effects of NO_3^- fed to dairy goats on milk characteristics such as milk FA and antioxidant capacity.

We hypothesized that supplemental NO_3^- would replace urea as an alternative source of NPN for lactating goats without affecting animal performance, milk quality, and ruminal fermentation. Therefore, our objectives were to investigate the effects of two doses of calcium nitrate fed to dairy goats on dry matter intake, nutrient digestibility, milk production

and composition, fatty acids profile, antioxidant capacity, and ruminal fermentation parameters.

2. Materials and methods

Experimental procedures involving animals were approved and conducted under the surveillance of the State University of Maringa - Animal Care Ethics Committee to meet the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) under protocol number 9512221018. The experiment was conducted at the goat unit of the State University of Maringa, Maringa, Parana, Brazil.

2.1 Animals, experimental design, and diets

Twelve lactating Saanen goats at 98.5 ± 13.1 days in milk, with 53.5 ± 3.3 kg of body weight, and producing 2.53 ± 0.34 kg of milk were enrolled in four 3 × 3 Latin square design. The experiment lasted 63 days, distributed in three periods, with 14 days for acclimation to the facilities and adaptation to the experimental diets, and 7 days for collections of samples and data. Animals were housed in individual pens and fed in feeder and water fountain in order to estimate the voluntary feed intake. Experimental diets were isoenergetic and isonitrogenous, and formulated to meet the NRC (2007) requirements (Table 1).

Feed ingredients (corn silage, corn, and soybean meal) were analyzed prior to the diet formulation and the dry matter (DM) of corn silage was measured weekly during the entire experiment to readjust its inclusion in order to assure the same forage to concentrate ratio. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate

[5Ca(NO_3^-)2·$NH_4NO_3^-$·10H_2O], with 85.0% DM, 16.5% N, 19.6% Ca, and 76.5% NO_3^- on DM basis (Yara North America, Inc. Tampa, FL, USA).

Treatments were defined as UREA (without addition of calcium nitrate) as a control group, CAN10:10 g of calcium nitrate (7.65 g/kg of NO_3^- on DM basis), and CAN20: 20 g of calcium nitrate (13.5 g/kg of NO_3^- on DM basis). Animals were pre-adapted to the treatments during the first 4 days of each period whereby nitrate was added gradually (increasing 25% per day) until reaching the amount of each diet. Experimental diets were provided as total mixed ration (TMR) twice per day at 0800 and 1600 h in proportions of 70 and 30% of the total DM intake, respectively. Diets were readjusted daily in order to guarantee approximately 5% of refusals and to avoid sorting behavior. Body weight was recorded at the beginning and end of each period before the morning feeding. Voluntary DM intake was calculated daily by the difference between the DM offered and refused.

2.2 Sample collection and chemical analyses

Sampling and data collection were performed in the last 7 days of each experimental period. Fecal samples (~30 g) were collected directly in the rectum from day 14 to 21 at different time points (day 14 at 0600 h, day 15 at 0800 h, day 16 at 1000 h, day 17 at 1200 h, day 18 at 1400 h, day 20 at 1600 h, and day 21 at 1800 h), and frozen at −20°C for further analyses. Samples of concentrate, corn silage, and refusals were collected from day 15 to 20 and frozen at −20°C for further analyses. All feed, refusals, and fecal samples were dried at 60°C for 48 h in a forced-air oven (Heratherm OMS180; Thermo Fisher Scientific, Waltham, MA) to determine DM content. All samples were ground firstly to pass through a 4-mm sieve

and then to 1-mm in a Wiley mill (A. H. Thomas Scientific, Philadelphia, PA) for further chemical analyses.

Samples of concentrate and corn silage were pooled separately in order to obtain one sample per period, and fecal and refusals samples were proportionally composed based on their DM contents to achieve one sample per animal per period. All samples were analyzed according to AOAC (2012) for total DM content (method 934.01), crude protein (CP) (method 990.03), neutral detergent fiber (NDF) (method 2002.04), ash (method 942.05), and ether extract (EE) (method 920.39). Organic matter was calculated by the difference between DM and ash. Non-fibrous carbohydrates (NFC) were calculated based on Van Soest et al. (1991). Fecal excretion was estimated according to the methodology proposed by Cochran et al. (1986). In brief, indigestible neutral detergent fiber (iNDF) was used as an internal indicator by weighing ~500 mg of feed, feces, and refusals (dried and ground) into Ankom F57 bags (25 µm porosity, Ankom Technology, Fairport, N.Y.), and incubating in two rumen cannulated cows during 288 h, followed by NDF analyses in a Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, N.Y.).

2.3 Milk composition, fatty acids profile, and antioxidant capacity

Milk samples were collected on day 15 and 16 in each milking (morning and afternoon) and mixed proportionally according to the milk yield. Daily milk production was recorded during the last 7 days of each experimental period through meters coupled to the milking equipment. A 50 mL aliquot was collected into Bronopol® flask (2-bromo-2-nitropopano-1.3-diol) to analyze fat, protein, and lactose contents by infrared spectrophotometry (Bentley 2000; Bentley Instrument Inc., Chaska, MN), and milk urea nitrogen (MUN) by Berthelot

methodology (Chemspec 150, Bentley Instrument Inc., Chaska, MN). Fat corrected milk (FCM) was obtained by correcting milk yield per 3.5% of fat according to Sklan et al. (1992). Energy corrected milk (ECM) was calculated using the equations proposed by Sjaunja et al. (1990). Another five aliquots, including backup samples, were collected and frozen at −20°C for further analyses of NO_3^- and NO_2^- on milk, fatty acids profile, and antioxidant capacity.

Milk fatty acids were analyzed through fat extraction by centrifugation as proposed by Murphy et al. (1995) and esterification according to ISO 5509 method (ISO, 1978) using KOH/methanol and n-heptane and the methyl esters were quantified by gas chromatography (Trace GC 52 Ultra, Thermo Scientific, West Palm Beach, Florida, USA) self-sampling, equipped with a flame ionization detector at 240°C and a fused silica capillary column (100 m in length, 0.25 mm internal diameter and 0.20 μm, Restek 2560). Gas flow rate was 45 mL/min of H_2 (carrier gas), 45 mL/min for N_2 (auxiliary gas) and 45 a 400 mL/min of synthetic air (flame gas). Column temperature was initially set at 50°C (10 min) and raised gradually up to 200°C (15 min), and finally raised to reach 240°C (8 min) as final temperature. Fatty acids were quantified by comparing retention time of fatty acid methyl esters from standards (Sigma Aldrich, St. Louis, MO, USA) and milk samples.

Conjugated dienes (CD) was measured at 232 nm on a UV–vis spectrophotometer (Spectrum SP2000), calculated, and expressed as mmol/kg of fat (Kiokias et al., 2006). Thiobarbituric acid reactive substances (TBARS) was analyzed according to Vyncke (1970) at 532 nm in a UV–vis spectrophotometer (Spectrum SP2000). Results were expressed as mmol of malonaldehyde (MDA) per kg of fat. The total antioxidant capacity (TAC) was determined as described by Nenadis et al. (2004). Absorbance was measured at 734 nm on a UV–vis spectrophotometer (Spectrum SP2000). TAC was expressed in Trolox equivalent (μM Trolox/mL).

Concentration of NO_3^- was obtained by alkaline catalytic oxidation that converts nitrogenous compounds to NO_3^-. Subsequently, through the cadmium metal, NO_3^- was reduced to NO_2^- and determined by the diazotization with sulfanilamide and N-naphthyl (1-naphthyl-ethylenediaminodihydrochloride) as described by Cortas and Wakid (1990).

2.4 Blood and ruminal fluid collections

Blood was sampled by puncture of the jugular vein on day 19 of each experimental period before and 4 h after morning feeding into serum separator evacuated tubes, centrifuged at 3,200 × *g* for 15 min and stored at −20°C for subsequent analyses. Plasma urea nitrogen (PUN) was analyzed colorimetrically by commercial kits (Gold Analisa®, Belo Horizonte, BH, Brazil) using a spectrophotometer (Bioplus 2000®, São Paulo, SP, Brazil).

Ruminal fluid was collected on day 20 of each period using an esophageal tube accoupled to a vacuum pump 2 and 8 h after feeding. An aliquot of 50 mL was collected, and pH was measured immediately using a pH meter (Tecnal®, Piracicaba, SP, Brazil). Another aliquot of 50 mL was filtered through four layers of cheesecloth, acidified with 1 mL of sulfuric acid (1:1), and stored at −20°C for further analyses. Volatile fatty acids (VFA) concentration was determined by gas chromatography using a chromatograph (Shimadzu GC-2010 Plus, Shimadzu®, Kyoto, Japan) equipped with an AOC-20i automatic injector, Stabilwax-DA ™ capillary column (30 m, 0.25 mm ID, 0.25 μm df, Restek®, Bellefonte, PA, USA) and a flame ionization detector (FID), after acidifying with 1 M phosphoric acid and fortifying with a WSFA-2 standard. A 1 μL aliquot of each sample was injected with a 40:1 split rate using He as the carrier gas. Injector and detector temperatures were 250 and 300°C, respectively. Column temperature ramp started at 40°C, was raised to 120°C at a rate of 40°C/min,

followed by a gradient of 120 to 180°C at the rate of 10°C/min and a rate of 120°C/min for 180 to 240°C, maintaining the temperature at 240°C for an additional 3 min. Ammonia-N (NH_3-N) concentration was measured via colorimetric quantification of N content using the phenol-hypochlorite reaction as described by Broderick and Kang (1980).

2.5 Statistical analyses

Data were previously checked for the normality of residuals using the Shapiro-Wilk test. Responses that violated the assumptions of normality were subjected to power transformation as described by Box and Cox (1964). The least square means (LSM) and standard error of the mean (SEM) were back transformed prior to the presentation of results (Jorgensen and Pedersen, 1998).

Data were analyzed by the MIXED procedure of SAS (SAS ver. 9.4, SAS Institute Inc., Cary, NC). The statistical model was:

$$Yijkl: \mu + LSi + Perj + Ani(LS)ki + Treatl + \epsilon ijkl,$$

where Yijkl = is the dependent variable, μ is the overall mean, LSi = *i-th* Latin square as a random effect (i = 1 to 4), Perj = *j-th* as a random effect of period (j = 1 to 3), Ani(LS) ki = random effect of the *k-th* animal nested within the *i-th* Latin square, Treatl = *l-th* is the fixed effect of treatment (1= control group, 2= CAN10, and 3= CAN20), and ϵijkl = the residual error associated with each observation as a random effect.

Orthogonal polynomial contrasts were used to determine linear and quadratic effects of treatments on the responses analyzed. Differences of LSM were adjusted by the Tukey-Kramer test. Treatment significances and trends were declared at $P \leq 0.05$, and $0.05 < P \leq 0.10$, respectively.

3. Results

3.1 Dry matter intake and nutrient digestibility

Supplemental CAN did not affect ($P > 0.05$) DM intake with an average of 1.77 kg per day (Table 2). Similarly, no effects ($P > 0.05$) of CAN10 and CAN20 were observed on the intake of OM, CP, EE, NDF, and NFC (Table 2).

There was a quadratic trend on digestibility of DM ($P > 0.08$), OM ($P > 0.09$), and ($P > 0.06$) by supplementing CAN to lactating goats. However, treatment did not affect ($P > 0.05$) EE, NDF, and NFC digestibility (Table 3).

3.2 Milk composition, fatty acids profile, and antioxidant capacity

Feeding CAN to lactating goats did not affect ($P > 0.05$) milk yield as well as its corrections for fat (FCM) and energy (ECM), with means 2.10, 2.02, and 1.97 kg per day, respectively (Table 4). Likewise, feed efficiency (ECM/DMI) was similar ($P > 0.05$) between treatments (URE, 1.11; CAN10, 1.14; CAN20, 1.14). Furthermore, there were no effects ($P > 0.05$) of supplemental CAN on milk contents of fat, true protein, and lactose, as well as the concentration of MUN (Table 4).

Dietary CAN did not affect ($P > 0.05$) the proportions of saturated fatty acids (C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0) in milk of lactating goats with an average of 77 g/100 g of total lipids (Table 5, 6). No effects ($P > 0.05$) of treatment were observed on grouped short, medium, and long chain fatty acids (Table 6).

There were no effects ($P > 0.05$) of supplemental CAN on proportions of monounsaturated fatty acids and polyunsaturated fatty acids (Table 6).

Treatment did not affect ($P > 0.05$) the total antioxidant capacity of the milk with a total mean of 203.1 μM of Trolox equivalent/mL (Table 7). However, the TBARS concentration in milk reduced ($P < 0.01$) as the levels of CAN increased. Contrarily, conjugated dienes concentration in milk increased (P = 0.02) linearly as the levels of CAN increased (Table 7).

Residual NO_3^- concentration in milk increased ($P < 0.01$) linearly (URE, 0.33 mg/L; CAN10, 0.31 mg/L; CAN20, 0.44 mg/L; Figure 1a). In contrast, supplemental CAN caused a quadratic effect ($P = 0.03$) on the residual NO_2^- in milk, with the maximum concentration for CAN10 (0.065 mg/L), followed by CAN20 (0.056 mg/L), and URE (0.042 mg/L) as shown in Figure 1b.

3.3 Plasma urea nitrogen, volatile fatty acids profile, and NH_3-N concentrations

Concentration of PUN presented a quadratic effect ($P = 0.02$) by supplemental CAN and increased ($P < 0.01$) over time, with the greatest values after 4 h of feeding; however, no interactions were observed between CAN vs. time (Figure 2).

The ruminal pH was not affected ($P > 0.05$) by supplemental CAN nor time. Ruminal NH_3-N concentration was not affected ($P > 0.05$) by supplemental CAN; however, it was decreased over time varying from 20.63 to 9.95 mg/dL between 2 and 8 h after feeding, respectively. No interaction was observed ($P > 0.05$) between CAN and time on ruminal NH_3-N concentration (Table 8).

Dietary CAN did not affect acetate proportion ($P > 0.05$), although a time effect was verified for this parameter. There was no effect ($P > 0.05$) of CAN on propionate and butyrate

proportion; however, propionate was reduced (P = 0.02) over time while isobutyrate increased (P = 0.03). Butyrate and valerate proportions were not affected by CAN (P > 0.05), whereas isovalerate reduced (P = 0.02) linearly as the levels of CAN increased (Table 8).

Total VFA concentration was unaffected by CAN (P > 0.05). Similarly, supplemental CAN had no influence (P > 0.05) on acetate: propionate ratio, however it was reduced (P < 0.01) according to sampling time from 3.31 (2 h) to 2.88 (8 h) (Table 8).

4. Discussion

4.1 Feed intake and nutrient digestibility

Feeding CAN to lactating goats up to 20 g/kg on a DM basis did not affect DM and nutrient intake. Treatment was gradually included in the diet during the adaptation period, and diet was provided as a TMR to prevent sorting behavior, which could partially explain the absence of effects. According to Lee and Beauchemin (2014), nitrate has a bitter-taste, which might reduce feed intake in ruminants. Such effects were previously observed by De Raphélis-Soissan et al. (2014) in sheep by supplementing 31 g of calcium nitrate (~20 g/kg of NO_3^- on DM basis) compared to urea. However, corroborating with our findings, others have not observed effects in dairy cows by supplementing calcium nitrate up to 21.1 g/kg on DM basis or feeding sodium nitrate (14.6 g on DM basis) as a urea replacer in low protein diets (Olijhoek et al., 2016; Wang et al., 2018).

Nutrient digestibility was also unaffected by supplementing CAN to dairy goats. We can assume with such response that, providing CAN gradually until amounts of 20 g/kg (on DM basis) avoided negative effects on rumen microorganisms. Previous *in vitro* studies have

shown that supplemental NO_3^- (~48 g/kg NO_3^- on DM basis reduced cellulolytic bacteria population, which consequently might reduce NDF digestibility (Zhou et al., 2012). Nevertheless, according to Wang et al. (2018) supplementing sodium nitrate (14.6 g of NO_3^-) to dairy cows did not affect fiber digestibility, supported by the absence of changes in cellulolytic bacteria (*Ruminococcus albus, R. flavefaciens,* and *Fibrobacter succinogenes*). Corroborating with our findings, Olijhoek et al. (2016) observed no effects on DM, OM, CP, and NDF digestibility in the rumen, small intestine, and hindgut by feeding calcium nitrate (up to 21.1 g/kg on DM basis) to lactating cows.

4.2 Milk production, composition, and quality

Dietary CAN did not affect ECM, FCM, and feed efficiency (ECM/DMI). Such effects are directly related to the milk composition, which also was unaffected by treatment. The lack of response on feed intake, nutrient digestibility, and VFA proportions such as acetate and propionate, also contributed to the unchanged response on milk components. In line with our findings, Olijhoek et al. (2016) demonstrated that supplemental CAN (up to 21.1 g/kg on DM basis) fed to dairy cows did not change milk yield and ECM. The current study did not find effects on milk protein, fat, and lactose. Contrarily, previous studies investigating dietary nitrate to lactating cows have observed a reduction in milk true protein, which was supported by the lower ingestion of nutrient that possibly affected the synthesis of milk components as a consequence of the lack of gluconeogenic precursors (Guyader et al., 2016a; Meller et al., 2019; Van Zijderveld et al., 2010).

To our knowledge, there is no previous data evaluating the effects of CAN on milk FA profile of dairy goats. Milk FA are generally derived from two main sources, diet and ruminal

microbial activity (Parodi, 2004). According to Månsson (2008), changes on ruminal fermentation might affect milk FA proportions mainly because acetate and butyrate, where the latter is converted to β-hydroxybutyrate, absorbed through the rumen wall, and contribute to the *de novo* synthesis in the mammary gland. The absence of response on ruminal fermentation parameters in our study suits as the best explanation for the lack of effects on milk FA composition. Similar to our results, Klop et al. (2016) investigated for the first time the impacts of dietary nitrate fed to dairy cows and observed minor effects on milk FA composition. Indeed, it is still needed information in regard to the rumen microbial role on milk FA synthesis, especially in goats (Giger-Reverdin et al., 2020).

According to our knowledge, the effect of CAN on milk antioxidant capacity of dairy goats has also not been reported yet in the literature. Total antioxidant capacity was not affected by treatment, whereas the concentration of TBARS on milk was reduced by dietary CAN. Conjugated dienes were elevated by following treatment, which can be related as an indicator of lipid oxidation (Guillén and Cabo, 2002). Nevertheless, the absence of effects on TAC could be related as a positive effect because such parameter provides, in general, the milk status as antioxidant potential, which has been found in higher concentration in milk goats compared to milk cows (Beghelli et al., 2016). Additionally, the reduction in TBARS concentration may also be defined as a positive effect meaning that dietary CAN might avoid milk oxidation, which according to Kiokias et al., (2006) enhances resistance of the product from sensory deterioration, and therefore, increased the shelf-life.

Nitrate residual concentration in milk increased by treatment. However, the maximum value observed (0.44 mg/L) by feeding 20 g of CAN is still under the recommendations of WHO (2011) for human consumption, which is up to 50 mg/L. Corroborating with our

results, others have observed low or undetectable NO_3^- concentration in milk (Meller et al., 2019; Olijhoek et al., 2016).

Regarding NO_2^- in milk, it was observed an unexpected effect whereby CAN10 presented the greatest concentration. Such effect was not well understood by the authors in the current study, although, this concentration (0.07 mg/L) is still not considered risky for human safety, since is under the guidelines of the WHO (2011) that require amounts below 3 mg/L to be considered safe for human consumption. Also, because of the concern of fatal methemoglobinemia in infants, it is important to investigate the NO_3^- and NO_2^- concentrations in food supply and drinking water (Hord et al., 2011).

4.3 Plasma urea nitrogen and ruminal fermentation parameters

The PUN was affected by treatment, with CAN at 10 g/kg MS presenting the highest concentration regardless of the collection time. One plausible explanation for such effect is that providing a low dose of calcium nitrate in the diet (7.65 g/kg NO_3^- on DM basis) favored a faster conversion from NO_3^- to NH_3 within the rumen, meaning that CAN20 had a slower reduction to NH_3 by rumen bacteria, and therefore, a more effective NH_3 utilization.

Overall, supplementing CAN to lactating goats did not affect ruminal fermentation parameters. As mentioned before, the lack of these effects was likely the main reason that kept nutrient digestibility and milk composition unaltered. According to Giger-Reverdin et al. (2020), despite the possible changes in the rumen environment by diets, microbiota of goats has usually high stability and resilience, which can sustain the absence of impacts on rumen fermentation in the current study. Similar effects on total VFA and their proportions were observed by Olijhoek et al. (2016), who also declared no effects on feed intake and milk

production in cows fed calcium nitrate (up to 21.1 g/kg of NO_3^- on a DM basis). In contrast, Asanuma et al. (2015) observed a reduction on acetate, propionate proportion, and total VFA concentration by supplementing potassium nitrate (up to 9 g/day) to male goats.

Finally, dietary CAN did not affect ruminal NH_3-N concentration. Such response could be considered as a positive effect, meaning that rumen microbiota acted similarly regardless of the CAN level. Thus, we can assume that the adaptation period and doses adopted in our study were effective. Theoretically, the nitrate-ammonia reduction within the rumen occurs in two steps, whereby NO_3^- is converted to NO_2^- rapidly because of higher thermodynamic energy, and subsequently NO_2^- is converted to NH_3-N (Latham et al., 2016). Corroborating with our findings, Van Zijderveld et al. (2010) did not find effect on NH_3-N concentration by supplementing 34 g/kg of calcium nitrate(~25.5 g/kg of NO_3^- on a DM basis) to growing male lambs.

5. Conclusions

Our findings indicated that CAN can be supplemented up to 20 g (15.5 g/kg NO_3^- on a DM basis) as a urea replacer for dairy goats without affecting feed intake, nutrient digestibility, ruminal fermentation, as well as milk quality.

Author statement

The authors declare that they have approved the submission.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Table 1. Ingredient proportion and nutritional composition of the experimental diets

Item	Treatment		
	UREA	CAN10	CAN20
Ingredient proportion, g/kg DM			
Corn silage	450.0	450.0	450.0
Ground corn	388.2	387.5	386.8
Soybean meal	137.9	138.1	138.2
Urea[1]	7.32	3.66	0.00
Calcium nitrate[2]	0.0	10.0	20.0
Limestone[3]	11.54	5.77	0.00
Mineral supplement[4]	5.00	5.00	5.00
Nutritional composition, g/kg DM[5]			
DM, as-fed basis	504.3	503.9	503.5
OM	949.1	946.3	943.6
CP	160.0	160.0	160.0
RDP	106.7	106.7	106.7
EE	32.6	32.6	32.6
NDF	299.8	299.8	299.7
NO_3^-	0.00	7.65	15.30
Ca	6.6	6.6	6.6
P	3.6	3.6	3.6
Ca:P	1.83	1.83	1.83

[1]Prote-N (GRASP Ind. & Com. LTDA, Curitiba, Brazil). Composition: 99.5% DM and 41.7% N on a DM basis.
[2]Double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$] manufactured by Yara North America, Inc. Tampa, FL, USA. Composition: 85.0% DM; 16.5% N, 19.6% Ca, and 76.5% NO_3^- on a DM basis.
[3]Granisul, Ind. & Com. LTDA, Rio Branco do Sul-Paraná, Brazil. Composition (per kg of product): 340 g of Ca and 40 g of Mg.
[4]Composition (per kg of product):150 g Ca, 60 g P, 50 g S, 5 g Mg, 136 g Na, 90 mg Co, 150 mg Cu, 180 mg I, 400 mg Mn, 13 mg Se, and 3000 mg Zn.
[5]Unless otherwise stated.

Table 2. Effects of calcium nitrate fed to dairy goats on dry matter and nutrient intake

Item[1]	Treatments[2]			SEM[3]	*P*-value[4]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
DMI	1.73	1.78	1.76	0.05	0.29	0.33	0.21
OMI	1.65	1.70	1.68	0.05	0.32	0.38	0.22
CPI	0.25	0.26	0.25	0.00	0.20	0.15	0.27
EEI	0.03	0.03	0.03	0.00	0.24	0.29	0.18
NDFI	0.66	0.66	0.67	0.02	0.36	0.52	0.20
NFCI	0.71	0.72	0.72	0.02	0.44	0.37	0.36

[1]kg per day
[2]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[3]Standart error of the mean.
[4]Treat = effect of treatment (UREA vs. CAN10 vs. CAN10); Linear = linear effect of calcium nitrate and Quadratic = quadratic effect of calcium nitrate.

Table 3. Effects of calcium nitrate fed to dairy goats on apparent dry matter and nutrient digestibility

Item[1]	Treatment[2]			SEM[3]	*P*-value[4]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
DMD	589.5	618.2	553.3	20.9	0.10	0.23	0.08
OMD	608.9	635.2	574.0	20.2	0.11	0.23	0.09
CPD	590.8	648.8	612.8	19.9	0.13	0.44	0.06
EED	710.5	727.7	685.5	16.2	0.17	0.26	0.12
NDFD	444.6	459.9	416.8	15.6	0.12	0.19	0.11
NFCD	839.2	849.5	815.2	22.7	0.40	0.36	0.32

[1]g per kg of DM
[2]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[3]Standart error of the mean.
[4]Treat = effect of treatment (UREA vs. CAN10 vs. CAN20); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 4. Effect of calcium nitrate fed to dairy goats on milk yield and milk composition

Item	Treatment[4]			SEM[5]	*P*-value[6]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
Milk yield, kg/d	2.05	2.13	2.13	0.12	0.27	0.16	0.41
FCM[1]	1.97	2.06	2.04	0.14	0.38	0.29	0.36
ECM[2]	1.92	2.00	1.99	0.13	0.37	0.26	0.40
ECM/DMI	1.11	1.14	1.14	0.07	0.73	0.52	0.65
Fat, %	3.25	3.25	3.19	0.17	0.71	0.48	0.68
Fat, kg/d	0.067	0.070	0.068	0.00	0.53	0.49	0.38
True protein, %	2.76	2.73	2.75	0.07	0.87	0.92	0.61
True protein, kg/d	0.056	0.058	0.059	0.00	0.39	0.20	0.66
Lactose, %	4.09	4.08	4.07	0.06	0.92	0.70	0.98
Lactose, kg/d	0.083	0.87	0.086	0.00	0.32	0.23	0.36
MUN[3], mg/dL	22.8	22.5	23.4	2.06	0.75	0.66	0.54

[1]Fat-corrected milk
[2]Energy-corrected milk
[3]Milk urea nitrogen
[4]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[5]Standart error of the mean.
[6]Treat = effect of treatment (UREA vs. CAN10 vs. CAN20); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 5. Effect of calcium nitrate fed to dairy goats on milk fatty acids proportions

Item[1]	Treatment[2]			SEM[3]	*P*-value[4]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
C6:0	0.47	0.43	0.51	0.11	0.88	0.80	0.68
C8:0	1.74	1.91	2.05	0.29	0.66	0.37	0.96
C10:0	13.36	13.58	14.17	1.27	0.80	0.52	0.86
C11:0	0.22	0.23	0.23	0.02	0.96	0.79	0.94
C12:0	7.73	7.59	8.15	0.58	0.60	0.47	0.48
C13:0	0.25	0.25	0.25	0.02	0.98	0.86	0.99
C14:0	18.01	17.97	17.90	0.43	0.93	0.70	0.98
C14:1	0.88	0.94	0.87	0.05	0.40	0.82	0.19
C15:0	1.29	1.19	1.19	0.10	0.66	0.43	0.64
C15:1	0.20	0.24	0.19	0.01	0.15	0.46	0.18
C16:0	32.55	33.03	32.25	1.52	0.78	0.79	0.52
C16:1	0.57	0.55	0.56	0.03	0.81	0.84	0.55
C17:0	0.58	0.55	0.53	0.03	0.53	0.29	0.75
C17:1	0.11	0.11	0.10	0.01	0.86	0.60	0.91
C18:0	6.49	6.14	5.71	0.45	0.31	0.13	0.92
C18:1 n9t	2.87	2.99	2.96	0.16	0.86	0.70	0.71
C18:1 n9c	8.05	7.78	7.75	0.39	0.77	0.52	0.75
C18:2 n6t	0.41	0.45	0.45	0.02	0.48	0.29	0.45
C18:2 n6c	0.68	0.65	0.68	0.05	0.82	0.96	0.54
C18:2 c9t11-CLA	0.05	0.07	0.12	0.04	0.33	0.14	0.80
C20:0	0.11	0.11	0.11	0.00	0.81	0.77	0.57
C20:2	0.04	0.04	0.04	0.00	0.91	0.80	0.74
C21:0	0.15	0.14	0.17	0.03	0.74	0.61	0.57

[1]g/100g of total lipids
[2]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[3]Standard error of the mean.
[4]Treat = effect of treatment (UREA vs. CAN10 vs. CAN20); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 6. Effect of calcium nitrate fed to dairy goats on milk fatty acids grouped

Item[1]	Treatment[8]			SEM[9]	*P*-value[10]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
SCFA[2]	2.22	2.35	2.56	0.37	0.77	0.48	0.92
MCFA[3]	61.19	61.45	61.03	1.24	0.92	0.89	0.72
LCFA[4]	20.16	19.60	19.23	0.82	0.63	0.35	0.91
MUFA[5]	12.75	12.66	12.50	0.51	0.91	0.67	0.94
PUFA[6]	1.16	1.19	1.26	0.09	0.65	0.36	0.86
SFA[7]	76.52	76.98	77.52	0.82	0.59	0.31	0.96

[1]g/100g of total lipids
[2]SCFA - Short chain fatty acids;
[3]MCFA - Medium chain fatty acids;
[4]LCFA - Long chain fatty acids;
[5]MUFA - Monounsaturated fatty acids;
[6]PUFA - Polyunsaturated fatty acids;
[7]SFA - Saturated fatty acids
[8]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[9]Standard error of the mean.
[10]Treat = effect of treatment (UREA vs. CAN10 vs. CAN20); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 7. Effect of calcium nitrate fed to dairy goats on milk antioxidant capacity

Item	Treatment[4]			SEM[5]	*P*-value[6]		
	UREA	CAN10	CAN20		Treat	Linear	Quadratic
CD[1]	47.11	55.67	66.02	4.89	<0.01	<0.01	0.78
TBARS[2]	9.74	7.00	7.34	0.79	0.00	0.01	0.06
TAC[3]	202.0	207.4	199.9	8.21	0.29	0.67	0.13

[1]Conjugated dienes (mmol/kg of fat).
[2]TBARS - Thiobarbituric acid reactive substances (mmol of malondialdehyde/kg of fat).
[3]TAC - Total antioxidant capacity (μM of Trolox equivalent/mL).
[4]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[5]Standart error of the mean.
[6]Treat = effect of treatment (UREA vs. CAN10 vs. CAN20); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

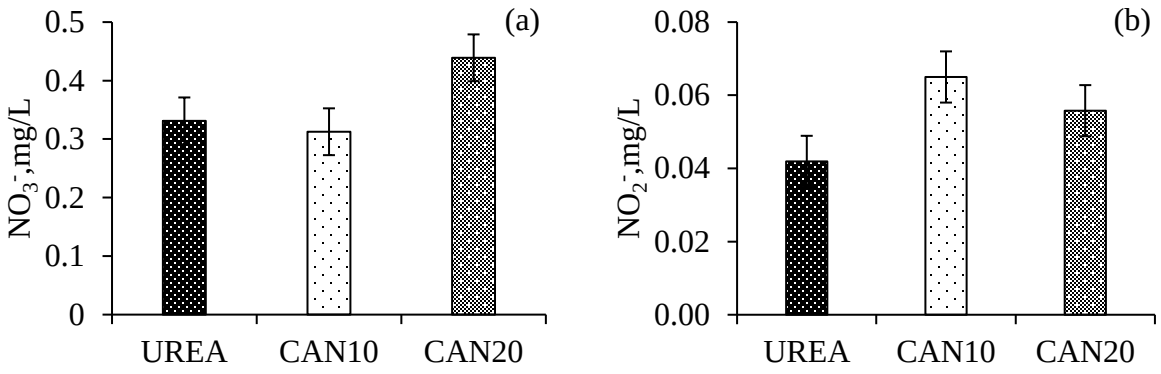

Figure 1. Effect of calcium nitrate fed to dairy goats on (a) nitrate (NO_3^-) *P*-values: Treat = 0.00; Linear = 0.01; Quadratic = 0.06; SEM= 0.04 and on (b) nitrite (NO_2^-) concentration in milk *P*-values: Treat = 0.03; Linear= 0.11; Quadratic = 0.03; SEM= 0.007.

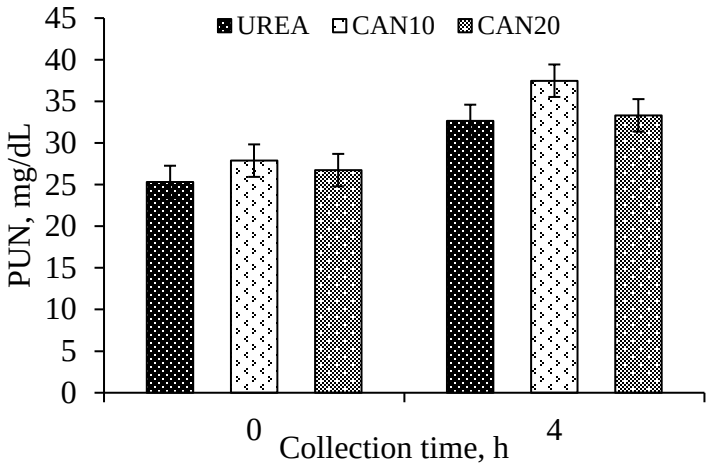

Figure 2. Effect of calcium nitrate fed to dairy goats on plasma urea nitrogen (PUN) before (0 h) and after (4 h) feeding. *P*-values: Treat = 0.07; Time <0.01; Treat × time = 0.37 Linear = 0.52; Quadratic = 0.02; SEM = 1.43.

Table 8. Effect of calcium nitrate fed to dairy goats on ruminal pH, volatile fatty acids profile, and NH_3-N concentration

Item[1]	Treatment[2]			Time[3]		SEM[4]	*P*-value[5]				
	UREA	CAN10	CAN20	2 h	8 h		Trt	Time	Int	Lin	Quad
pH	6.89	7.17	6.99	6.92	7.12	0.26	0.20	0.11	0.18	0.41	0.12
Acetate	62.37	63.09	65.54	65.51	61.82	1.91	0.18	0.03	0.59	0.10	0.47
Propionate	22.69	21.84	19.23	20.36	22.15	1.86	0.22	0.02	0.19	0.12	0.53
Isobutyrate	0.81	0.85	0.70	0.87	0.77	0.14	0.52	0.03	0.23	0.43	0.42
Butyrate	12.19	12.56	12.94	11.54	13.59	0.77	0.78	0.16	0.67	0.49	0.99
Isovalerate	0.85	0.71	0.71	0.75	0.77	0.11	0.02	0.80	0.45	0.01	0.04
Valerate	1.07	0.93	0.85	0.96	0.94	0.10	0.20	0.84	0.90	0.10	0.70
Total VFA, mM	49.54	50.55	56.96	46.14	58.55	8.40	0.98	0.83	0.20	0.85	0.97
C_2: C_3	2.76	2.91	3.60	3.31	2.88	0.37	0.18	0.00	0.07	0.10	0.40
NH_3-N, mg/dL	15.99	15.25	14.63	20.63	9.95	3.79	0.60	<0.01	0.16	0.37	0.95

[1]Molar proportion (unless otherwise stated).
[2]UREA: control group (without nitrate); CAN10: 10 g of calcium nitrate per kg of DM; CAN20: 20 g of calcium nitrate per kg of DM.
[3]Time of collection
[4]Standart error of the mean.
[5]Trt = effect of treatment (UREA vs. CAN10 vs. CAN20); Time = effect of time (2 and 8 h); Interaction: treatment x time. Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

VI. Effect of calcium nitrate fed to dairy cows on feed intake, nutrient digestibility, milk quality, microbial protein synthesis, and ruminal fermentation parameters

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INTERPRETIVE SUMMARY

By Almeida et al. Objectives were to determine the effects of calcium nitrate fed to dairy cows on milk quality, microbial protein synthesis, and fermentative parameters. Cows received diets containing urea, as a control, 15, and 30 g/ kg DM of calcium nitrate. Calcium nitrate reduced dry matter and nutrient intake; however, did not affect digestibility and fermentation parameters. Milk yield was unaffected, whereas ECM, FCM, and milk components were reduced. Minor effects of CAN on milk FA and antioxidant capacity were observed. Treatment did not affect purine derivates and microbial protein synthesis. Dietary CAN can be fed to dairy cows up to 15 g/kg of DM without affect milk quality and ruminal fermentation parameters.

ABSTRACT

The objectives were to evaluate the effects of calcium nitrate (CAN) fed to dairy cows on dry matter intake, nutrient digestibility, milk yield and composition, fatty acids profile, antioxidant capacity, microbial protein synthesis, and rumen fermentation. Six multiparous Holstein cows were enrolled (106.3 ± 14.8 DIM; 550.7 ± 21.8 kg of BW; mean ± SD) in a

replicated 3 × 3 Latin square design. Experimental period lasted 21 d, with 14 d as an adaptation phase and the remaining days for sampling and data collection. Treatments were designed as UREA, as a control group (only urea), CAN15: 15 g of CAN (11.5 g/kg of NO_3^- on a DM basis), and CAN30: 30 g of CAN (23 g/kg of NO_3^- on a DM basis). Diets were isonitrogenous and cows were fed ad libitum after 5 d of gradual introduction to CAN. Sampling of feed, orts, feces, urine, blood, and ruminal fluid were carried out in the last 7 d of each experimental period. Supplemental CAN reduced dry matter and nutrient intake, whereas nutrient digestibility was not affected. Treatment did not affect milk yield; however, ECM and FCM decreased as the levels of CAN increased. Nitrate and nitrite residuals were detected in low quantities in milk, as well as the level of methemoglobin in blood. Milk fat content was negatively affected, and the proportion of saturated fatty acids was decreased by CAN. Reducing power and TBARS had no effect of treatment, whereas conjugated dienes increased. Microbial protein synthesis was not impacted by treatment and minor effects on ruminal fermentation were observed. Supplemental CAN at 30 g/kg increased conjugated dienes in milk and reduced its contents, leading to recommend that CAN can be fed up to 15 g/kg of DM to dairy cows without affect milk quality and ruminal fermentation.

Key words: antioxidant capacity**,** non-protein nitrogen source, methemoglobin, milk fatty acids

INTRODUCTION

Nitrate (NO_3^-) is an inorganic anion that has been explored in ruminant production as a feed additive by supplementing low doses on diets mainly because of its potential to decrease methane (CH_4) production. In a recent meta-analysis, Feng et al. (2020) have shown through

an average of 24 studies that NO_3^- was effective at reducing CH_4 emissions in both dairy cows (17%) and beef cattle (12%) production. Therefore, NO_3^- has been considered an important feed strategy to reduce the environmental impact caused by enteric CH_4 generation, which according to Gerber et al. (2013) represents up to 6% of the total anthropogenic greenhouse gas emissions.

Providing an additional electron acceptor such as NO_3^- salts would also reduce the energy losses by enteric CH_4 production that according to Johnson and Johnson (1995) ranges from 2 to 12% in ruminants. Besides acting as a hydrogen ([H]) sink, and therefore, impacting at the expense of CH_4 production, supplemental NO_3^- could replace urea and act concomitantly as non-protein nitrogen (NPN) source, that after being converted to ammonia (NH_3) has been incorporated by rumen microorganisms to synthesize essential AA (Nolan et al., 2010). Indeed, Wang et al. (2018) have reported that sodium nitrate increased the NH_3 utilization for microbial protein synthesis in dairy cows fed to low protein diets.

The reduction from NO_3^- to NH_3 is more thermodynamically favorable than carbon dioxide (CO_2) to the CH_4 synthesis and occurs by two steps into the rumen, whereby NO_3^- is first converted to nitrite (NO_2^-) and subsequently to NH_3 by a dissimilatory pathway (Latham et al., 2016; Leng, 2008). Nitrite as an intermediate of NO_3^- reduction can be accumulated within the rumen, absorbed into the bloodstream through the rumen wall, and cause methemoglobinemia in ruminants. Methemoglobin (MetHb) is caused as a result of the transformation from ferrous iron (Fe^{2+}) form of hemoglobin to the ferric iron (Fe^{3+}) producing MetHb (Takahashi and Young, 1991). Under certain circumstances (e.g. animals not adapted to the diets) the percentage of MetHb in red blood cells might increase, and consequently reducing its capacity to carry oxygen (Leng, 2008). Therefore, a previous

adaptation is extremely required to avoid MetHb and to prevent NO_3^- or NO_2^- accumulation in body tissues and products.

Beauchemin et al. (2020) have reported in a recent review that supplemental NO_3^- might produce residues in tissues and products such as milk even though it has not affected food security due to its low concentration. Indeed, a previous study has shown that supplementing NO_3^- up to 21 g/kg DM in dairy cow diets produced negligible concentrations of NO_3^- in milk, and therefore considered safe for human consumption (Olijhoek et al., 2016). According to Klop et al. (2016), dietary NO_3^- had no major impacts on milk fatty (FA) acids composition.

It is well documented that NO_3^- is a feasible CH_4 inhibitor (Feng et al., 2020; Lee and Beauchemin, 2014). However, it is important to keep investigating NO_3^- effects in ruminant nutrition since there is still a lack of information on how its supplementation would change milk components and milk properties such as FA profile and antioxidant capacity. We hypothesized that including calcium nitrate (CAN) as a source of NPN in dairy cow diets would improve microbial protein synthesis without affecting milk components, fatty acids profile, antioxidant capacity, as well as the parameters of digestibility and ruminal fermentation. Therefore, objectives were to determine the effects of a low and high dose of CAN fed to dairy cows on dry matter intake, nutrients digestibility, milk yield, milk composition, milk fatty acids, antioxidant capacity, microbial protein synthesis, and ruminal fermentation parameters.

MATERIAL AND METHODS

All procedures involving animals in the experiment were approved by the State University of Maringa - Animal Care Ethics Committee under protocol number 9512221018.

The experiment and laboratorial analyses were conducted at the dairy unit and ruminant nutrition lab of the State University of Maringa, Brazil.

Cows, Experimental design, and Diets

Six multiparous Holstein cows (106.3 ± 14.8 DIM; 550.7 ± 21.8 kg of BW; mean ± SD) were enrolled in a replicated 3 × 3 Latin square design. The experiment lasted 63 d, distributed in three periods of 21 d each, whereby 14 d were destined for acclimation of animals to the facilities and adaptation to the experimental diets, and 7 d for sampling and data collection. Animals were housed in tie-stalls and fed by a feeder and water fountain in order to evaluate the individual feed intake. Experimental diets were formulated to meet the NRC (2001) requirements for a Holstein cow at mid-lactation with 600 kg of BW, 110 DIM, and producing 25 kg of milk per day (Table 1). Diets were isocaloric and isonitrogenous.

Feedstuffs (corn silage, corn, and soybean meal) were analyzed prior to the diet formulation and the corn silage DM was measured weekly during the entire experiment for readjusting the diets and keeping the same forage to concentrate ratio. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3^-)_2 \cdot NH_4NO_3^- \cdot 10H_2O$], with 85.0% DM, 16.5% N, 19.6% Ca, and 76.5% NO_3^- on DM basis (Yara North America, Inc. Tampa, FL, USA). To ensure that all treatments had the same amount of nitrogen and calcium, urea (Prote-N, GRASP Ind. & Com. LTDA, Curitiba, Brazil. Composition: 99.5% DM, and 41.7% N), and limestone (Granisul, Ind. & Com. LTDA, Rio Branco do Sul-Paraná, Brazil, composition per kg of product: 340 g of Ca and 40 g of Mg) were used to balance the diets.

Treatments were control group, defined as UREA (urea without adding nitrate), CAN15: 15 g of calcium nitrate (11.5 g/kg of NO_3^- on a DM basis), and CAN30: 30 g of calcium nitrate (23 g/kg g/kg of NO_3^- on a DM basis). Animals were pre-adapted to the treatments

during the first 4 d of each experimental period, whereby nitrate was added gradually (increasing 25% per day) until reaching the amount of each diet. Experimental diets were provided as total mixed ration (TMR) twice a day at 0630 and 1530 h in proportions of 70 and 30% of the total DM intake, respectively. Diets were readjusted daily in order to guarantee 5% of refusals and to avoid sorting behavior. Animals were weighed at the end of each period before the morning feeding and the voluntary DM intake was calculated daily during the week of data collection by the difference between the total DM offered and refused.

Sampling and Proximate Analyses

Sampling and data collection were performed in the last 7 d of each experimental period. Fecal samples (~100 g) were collected through the rectum from d 14 to 21 at different times throughout the week (d 14 at 0600, d 15 at 0800, d 16 at 1000, d 17 at 1200, d 18 at 1400, d 20 at 1600, and d 21 at 1800 h) and frozen at −20°C for further analyses. Grain mix, corn silage, and refusals were collected from d 15 to 20 and frozen at −20°C for further analyses. All feed and fecal samples were dried at 60°C for 48 h in a forced-air oven (Heratherm OMS180; Thermo Fisher Scientific, Waltham, MA) to determine DM content and then ground to pass through a 4-mm and 1-mm sieves in a Wiley mill (A. H. Thomas Scientific, Philadelphia, PA) for further chemical analyses.

Feedstuffs were pooled in order to obtain one sample per period, and fecal and refusals samples were proportionally composed based on its DM to achieve one sample per animal per period. All samples were analyzed according to AOAC (2012) for total DM content (method 934.01), crude protein (CP) (method 990.03), neutral detergent fiber (NDF) (method 2002.04), ash (method 942.05), and ether extract (EE) (method 920.39). Organic matter was calculated by the difference between DM and ash. Non-fibrous carbohydrates (NFC) were calculated based on Van Soest et al. (1991). Fecal excretion was estimated according to

Cochran et al. (1986). Briefly, indigestible neutral detergent fiber (iNDF) was used as an internal indicator by weighing ~500 mg (dried and ground) of feed, feces, and refusals into Ankom F57 bags (25 µm porosity, Ankom Technology, Fairport, N.Y.), and incubating in two rumen cannulated cows during 288 h, followed by NDF analysis in a Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, N.Y.).

Milk Collections and laboratorial Assays

Milking was performed mechanically twice daily at 0600 and 1500 h and milk production was recorded during the last 7 d of each experimental period. Milk samples were collected on d 15 and d 16 proportionally to the production of each milking (morning and afternoon). A 50 mL aliquot was collected and placed into Bronopol® flask (2-bromo-2-nitropopano-1.3-diol) to analyze fat, protein, and lactose contents by infrared spectrophotometry (Bentley 2000; Bentley Instrument Inc., Chaska, MN), and milk urea nitrogen (MUN) by Berthelot methodology (Chemspec 150, Bentley Instrument Inc., Chaska, MN). Fat corrected milk (FCM) was obtained by correcting milk yield per 3.5% of fat according to Sklan et al. (1992). Energy corrected milk (ECM) was calculated using the equations proposed by Sjaunja et al. (1990). Another five aliquots, including backup samples, were collected and frozen at −20°C for analyses of NO_3^- and NO_2^- on milk, fatty acids profile, and antioxidant capacity.

Concentration of NO_3^- was obtained by alkaline catalytic oxidation that converts nitrogenous compounds to NO_3^-. Subsequently, through the cadmium metal, NO_3^- was reduced to NO_2^- and determined by the diazotization with sulfanilamide and N-naphthyl (1-naphthyl-ethylenediaminodihydrochloride) as described by Cortas and Wakid (1990).

Milk fatty acids were analyzed through fat extraction by centrifugation as proposed by Murphy et al. (1995) and esterification according to ISO 5509 method (ISO, 1978) using KOH/methanol and n-heptane, and the methyl esters were quantified by gas chromatography

(Trace GC 52 Ultra, Thermo Scientific, West Palm Beach, Florida, USA) self-sampling, equipped with a flame ionization detector at 240°C and a fused silica capillary column (100 m in length, 0.25 mm internal diameter and 0.20 μm, Restek 2560). Gas flow rate was 45 mL/min of H_2 (carrier gas), 45 mL/min for N_2 (auxiliary gas) and 45 a 400 mL/min of synthetic air (flame gas). Column temperature was initially set at 50°C (10 min) and raised gradually up to 200°C (15 min), and finally raised to reach 240°C (8 min) as final temperature. Fatty acids were quantified by comparing retention time of fatty acid methyl esters from standards (Sigma Aldrich, St. Louis, MO, USA) and milk samples.

Milk reducing power was analyzed according to Zhu et al. (2002), whereby the absorbance was measured at 700 nm on a UV–vis spectrophotometer (Spectrum SP2000, Castelnuovo, DB, Italy). Reducing power was expressed as gallic acid equivalent (GAE) per kg of milk. Conjugated dienes (CD) was measured at 232 nm on a UV–vis spectrophotometer (Spectrum SP2000, Castelnuovo, DB, Italy), calculated, and expressed as mmol/kg of fat (Kiokias et al., 2006). Thiobarbituric acid reactive substances (TBARS) in milk was analyzed as described by Vyncke (1970) at 532 nm on a UV–vis spectrophotometer (Spectrum SP2000, Castelnuovo, DB, Italy). Results were expressed as mmol of malonaldehyde (MDA) per kg of fat.

Urine, Blood, and Ruminal Fluid Analyses

Urine samples were collected on d 17 and d 18 within 4 h after morning feeding, filtered through layers of cheesecloth, and then placed into 50 mL plastic bottles. Uric acid and creatinine were analyzed subsequently by enzymatic and colorimetric methods (Gold Analisa®, Belo Horizonte, MG, Brazil), respectively. An aliquot of 10 mL of urine was acidified with 40 mL of sulfuric acid (0.036 N) for further allantoin analyses according to the methodology of Chen and Gomes (1992). Creatinine was used as a marker to estimate daily

urinary volume, assuming a factor for urinary creatinine excretion of 29 mg/kg of BW per day (Valadares et al., 1999). Microbial synthesis was calculated considering 1 mmol of derivate of purines per kg of milk (Chen and Gomes, 1992).

Blood was sampled by puncture of the jugular vein on d 20 of each experimental period before and 4 h after morning feeding and transported to a commercial laboratory within 1 h after collecting for MetHb analysis. Other samples were collected into serum separator evacuated tubes, centrifuged at 3,200 × g for 15 min and stored at −20°C for subsequent analyses. Plasma urea nitrogen (PUN) was analyzed colorimetrically by commercial kits (Gold Analisa®, Belo Horizonte, MG, Brazil) using a spectrophotometer (Bioplus 2000®, São Paulo, SP, Brazil).

Ruminal fluid was collected on d 21 of each period using an esophageal tube accoupled to a vacuum pump 2 and 8 h after feeding. An aliquot of 50 mL was collected, and pH was measured immediately using a pH meter (Tecnal®, Piracicaba, SP, Brazil). Another aliquot of 50 mL was filtered through four layers of cheesecloth, acidified with 1 mL of sulfuric acid (1:1), and stored at −20°C for further analyses. Volatile fatty acids (VFA) concentration was determined by a gas chromatograph (Shimadzu GC-2010 Plus, Shimadzu®, Kyoto, Japão) equipped with an AOC-20i automatic injector, Stabilwax-DA ™ capillary column (30 m, 0.25 mm ID, 0.25 μm df, Restek®, Bellefonte, PA, USA) and a flame ionization detector (FID), after acidifying with 1 M phosphoric acid and fortifying with a WSFA-2 standard. A 1 μL aliquot of each sample was injected with a 40:1 split rate using He as the carrier gas. Injector and detector temperatures were 250°C and 300°C, respectively. Column temperature ramp started at 40°C, was raised to 120°C at a rate of 40°C/min, followed by a gradient of 120°C to 180°C at the rate of 10°C/min and a rate of 120°C/min for 180°C to 240°C, maintaining the temperature at 240°C for an additional 3 min. Ammonia-N (NH_3-N) concentration was

measured via colorimetric quantification of N content using the phenol-hypochlorite reaction as described by Broderick and Kang (1980).

Statistical Analyses

Normality of residuals and homogeneity of variance were examined for each variable analyzed using the Shapiro-Wilk test. Responses that violated the assumptions of normality were subjected to power transformation as described by Box and Cox (1964). The LSM and SEM were back transformed for the presentation of results (Jorgensen and Pedersen, 1998)

Data were analyzed by the MIXED procedure of SAS (SAS ver. 9.4, SAS Institute Inc., Cary, NC) using treatment (UREA, CAN-L, and CAN-H) as a fixed effect, cow within Latin square, and period as random effects. Responses with repeated measures were analyzed with statistical models that included the fixed effects of treatment, time, and their interactions, and the random effects of cow within Latin square, and period. Hour was used as the term in the REPEATED statement.

Orthogonal polynomial contrasts were used to determine linear and quadratic effects of treatments on the responses analyzed. Differences of LSM were adjusted by the Tukey-Kramer test. Treatment significances and trends were declared at $P \leq 0.05$, and $0.05 < P \leq 0.10$, respectively.

RESULTS

Intake and Digestibility of DM and Nutrients

Treatment did not affect ($P > 0.05$) the body weight of the animals with an average of 555 kg (Table 2). Nevertheless, feeding calcium nitrate reduced linearly ($P < 0.001$) the

intake of DM, OM, CP, EE, NDF, and NFC. Similarly, CP intake had also a quadratic effect ($P < 0.001$) in cows fed CAN (Table 2).

Supplemental CAN did not affect ($P > 0.05$) parameters of DM, OM, CP, EE, and NDF digestibility. However, NFC digestibility was increased ($P < 0.01$) linearly as treatment levels increased (Table 3).

Milk Composition, Nitrate, and Nitrate Concentration

Cows fed supplemental NO_3^- did not differ ($P > 0.05$) on milk yield (Table 4). On the other hand, FCM and ECM were affected ($P = 0.05$) negatively by treatments. Furthermore, treatment increased ($P = 0.03$) linearly the ECM:DMI ratio, and therefore, changed feed efficiency. Additionally, supplemental CAN reduced ($P = 0.03$) linearly fat content, whereas true protein had a quadratic effect ($P = 0.05$). Lactose content was unaffected ($P > 0.05$) by NO_3^- levels. Similarly, treatment did not affect ($P > 0.05$) MUN concentration (Table 4).

Nitrate concentration in milk had a positive linear response ($P = 0.02$) with the increase of NO_3^- levels in the diet (Figure 1a). Nevertheless, supplemental CAN did not affect ($P < 0.05$) concentration of NO_2^- residual in milk (Figure 1b).

Milk Fatty Acids and Antioxidant Capacity

Dietary treatment did not affect ($P > 0.05$) the proportions of C6:0, C8:0, C10:0, and C11:0 (Table 5). However, feeding NO_3^- decreased linearly ($P = 0.03$) the proportions of C12:0 and C13:0. The proportion of C14:0 was unaffected ($P > 0.05$) by treatment, whereas C14:0 tended ($P = 0.07$) to increase linearly. Treatment did not affect ($P > 0.05$) the proportion of C15:0, but a quadratic response ($P = 0.05$) was observed on C15:1 proportion, and a linear effect ($P = 0.02$) was observed on the proportion of C16:0. Nevertheless, increasing supplemental NO_3^- in dairy cow diets did not affect ($P > 0.05$) the proportions of

C16:1, C17:0, C17:1, C18:1 n9t, C18:1 n9c, C18:2 n6c, C18:2 n6t, C18:2 n6c, C18:3 n6, C18:3 n3. Similarly, no effects ($P > 0.05$) of NO_3^- supplementation were observed ($P > 0.05$) on C18:2 c9 t11-CLA and C18:2 t10 c12-CLA. Finally, supplemental CAN increased linearly ($P < 0.05$) the proportions of C20:0 and C21:0, whereas C20:1 and C20:2 were not affected by treatments (Table 5).

Total CLA was not ($P > 0.05$) affected by supplemental CAN fed to dairy cows (Table 6). Likewise, short chain FA in total was not influenced ($P > 0.05$) by treatments, whereas medium chain FA decreased linearly ($P < 0.05$), and a positive linear tendency was observed on the total of long chain FA ($P = 0.06$) and monounsaturated FA ($P = 0.08$). The total of saturated FA was reduced ($P < 0.05$) linearly; however, treatment did not affect ($P > 0.05$) the total of polyunsaturated FA. Similarly, there were no treatment effects ($P > 0.05$) on n-3, n-6, as well as on n-6/n-3 ratio (Table 6).

Treatment did not affect ($P > 0.05$) the reducing power of the milk (Table 7). Similarly, no effect ($P > 0.05$) of CAN supplementation was observed on TBARS concentration; however, conjugated dienes concentration in milk increased ($P = 0.02$) linearly as the levels of CAN increased (Table 7).

Blood, Microbial Protein Synthesis, and Ruminal Fermentation Parameters

Plasma urea nitrogen was not affected by supplemental CAN; however, its concentration was higher 4 h after feeding when compared to the collections before feeding (0 h) (Figure 2). Additionally, there was no interaction ($P > 0.05$) between PUN and time in cows fed supplemental CAN. Methemoglobin proportion increased ($P < 0.01$) linearly as the levels of CAN increased (Figure 3). Also, there was an increase ($P < 0.01$) on MetHb by supplemental CAN over time, although no interactions ($P > 0.05$) between treatment and time was observed.

Supplemental CAN did not affect ($P > 0.05$) allantoin excretion, whereas uric acid tended to increase ($P = 0.06$) linearly according to the levels of CAN (Table 8). Similarly, no effect ($P > 0.05$) of CAN supplementation was observed on allantoin:creatinine ratio as well as on uric acid:creatinine, and allantoin + uric acid:creatinine. Additionally, treatment did not affect ($P > 0.05$) microbial protein synthesis (Table 8).

The pH was not affected ($P > 0.05$) by treatment either 2 or 8 h after feeding. Ammonia-N concentration was not affected ($P > 0.05$) by supplemental CAN and similarly had no effect of collection time. Also, no interaction was observed ($P > 0.05$) between treatment and time on NH_3-N concentration (Table 9).

The proportion of acetate increased linearly ($P < 0.01$) as the levels of CAN increased. Acetate proportion decreased linearly ($P < 0.01$) throughout time; however, there was no interaction ($P > 0.05$) between treatment and time. Propionate proportion was unaffected ($P > 0.05$) by supplemental CAN, but an increase was observed ($P < 0.01$) over time, even though there was no interaction ($P > 0.05$) between treatment and time. Contrarily, the proportion of isobutyrate reduced ($P < 0.01$) throughout time and tended ($P = 0.08$) to decrease linearly. Treatment had no influence ($P > 0.05$) on butyrate proportion but reduced linearly ($P < 0.01$) the proportions of isovalerate and valerate. Acetate: propionate ratio tended to increase linearly ($P = 0.07$) by supplemental CAN and decreased ($P < 0.01$) over time (Table 9).

DISCUSSION

Supplementing CAN has been established as a feasible nutritional strategy acting at the expense of CH_4 synthesis in ruminants (Beauchemin et al., 2020; Feng et al., 2020; Lee and Beauchemin, 2014). However, there still a lack of information regarding the effects of NO_3^-

on milk quality, microbial protein synthesis, and ruminal fermentation in dairy cows. Despite the current experiment did not show the impact of NO_3^- on methane production, once was not its objective, proving additional data on dairy production are necessary to support it as a feasible nutritional strategy and would contribute for the development of novel commercial products.

Feed Intake and Nutrient Digestibility

Dry matter intake was lowered up to 13% by supplemental CAN. Similar to urea, it is believed that nitrate has a bitter taste, which could decrease feed palatability and be a limitation for animal feeding (Lee and Beauchemin, 2014). In line with our findings, Lund et al. (2014) reported a significant decrease on DMI by supplementing 20 g/kg DM of calcium nitrate in a metabolic study. Similarly, supplemental CAN reduced intake of both pasture and concentrate (up to 23 g/kg NO_3^- on DM basis) in grazing cows, impacting negatively on milk production. Furthermore, in a recent study, Meller et al. (2019) also observed a reduction on DMI caused by the main effect of CAN in a factorial study (Live yeast culture vs. 20 g/kg NO_3^- on DM basis) in Jersey cows.

Nutrient intake was also reduced by supplemental NO_3^- fed to dairy cows. Such effects are related directly as a response to the reduction on total DMI and to the negative effects on milk performance in this study. Poor palatability was possibly the main effect that caused negative effects on nutrient intake. The encapsulation of nitrate salts would be a possible solution to prevent such effects; however, previous studies using an encapsulated nitrate source with vegetable oil (GRASP Ind. & Com. LTDA, Paraná, Brazil; EW| Nutrition GmbH, Visbek, Germany) has not alleviated negative effects on feed intake (Lee et al., 2015a; Rebelo et al., 2019). New studies are necessary to explore whether feed additives capable to increase feed palatability, such as molasses (DeVries and Gill, 2012), would

minimize negative effects on dry matter intake by supplementing NO_3^- and possibly enhancing milk yield.

Supplemental CAN did not affect DM, OM, CP, and NDF digestibility in dairy cows. Such effects indicated that the adaptation of the animals to diets was properly applied. The lack of response on nutrient digestibility has been observed across many trials evaluating NO_3^- in the diet of dairy cows (Olijhoek et al., 2016; van Zijderveld et al., 2011; Wang et al., 2018). Zhou et al. (2012) observed in an *in vitro* approach that supplementing NO_3^- at the dose of 12 μmol ml^{-1} (~12 g/kg DM) reduced methanogens population properly (~97% less) but also reduced drastically cellulolytic bacteria (*Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens*) caused possibly by a raising in the redox potential and nitrite accumulation, which might affect negatively NDF digestibility. An unexpected increase on NFC digestibility was observed as the levels of supplemental CAN increased; however, no effects on NDF digestibility was observed, which possibly would impact in such parameters.

Milk contents, Nitrate and Nitrite residuals

Supplemental NO_3^- did not impact affect milk yield. However, FCM and ECM were negatively impacted by treatment. Fat corrected milk decreased as a result of the lower fat content observed in our study. Similarly, ECM, that besides fat considers protein content in its calculation was reduced due to their linear reduction and tendency to decrease, respectively. Similar to our findings, Olijhoek et al. (2016) did not find differences on milk yield by supplementing calcium ammonium nitrate up to 21 g/kg on DM basis, and related such effect to the absence of responses on DM digestibility and VFA proportions. Altogether, van Wyngaard et al. (2018) have shown negative effects on FCM and ECM also due to the reduction in fat and protein content, respectively, supporting the explanation for our findings.

As mentioned sooner, milk fat content decreased as the CAN levels increased. This effect could be directly related to the reduction on NDF intake. Another plausible explanation would be a reduction on NDF digestibility, which, although did not show statistical significance was decreased numerically. Supplemental CAN did not change true protein yield (kg/ per day); however, the percentage of protein had a quadratic effect, meaning that CAN15 improved its content, and CAN30, on the contrary, negatively affected protein content compared to the control group. Corroborating with our results, van Zijderveld et al. (2011) observed a reduction in milk protein content by supplementing 21 g/kg NO_3^- on DM basis for 89 days and attributed such impact to a dilution effect, whereas Klop et al., (2016) in a factorial study (nitrate vs. docosahexaenoic) also observed low protein content by also supplementing 21 g/kg NO_3^- to dairy cows. Lactose content and yield was not affected by treatment with an average of 4.61%, 1.06 kg/d, respectively. Milk urea nitrogen was also unaffected by treatment, assuming that CAN15 and CAN30 were properly converted to NH_3, and similar to URE, provided enough nitrogen for rumen microbiota utilization. In line with our findings, others have found no effects on MUN by supplemental NO_3^- (van Zijderveld et al., 2011; Klop et al., 2016; van Wyngaard et al., 2018).

It is important to emphasize that supplemental NO_3^- still not approved as a feed additive in many countries worldwide due to a concern of likely toxicity for the animals and possible residuals of NO_3^- or NO_2^- in milk, which in high concentrations may cause health problems in humans (Bryan and van Grinsven, 2013). Our study observed an increase of NO_3^- in milk by supplementing CAN for dairy cows, varying from 0.30 to 0.38 mg/L between URE and CAN30, respectively. However, such effects were much lower than those reported by Olijhoek et al. (2016) who found 1.56 mg/L of NO_3^- residual in milk by supplementing levels (21.1 g/kg on DM basis) of calcium nitrate for dairy cows, although, as well as our findings,

it was considered safe for human consumption for being under the guidelines of WHO (2011) which is at maximum 50 mg/L. In the same study, NO_2^- in milk was not found because its concentration was considered below the detection limit (<30 μg/L) in their evaluation method (Olijhoek et al., 2016). Despite the absence of effect, our results demonstrated an average between treatments of 43 μg/L of NO_2^- in milk. Altogether, other studies have shown low and undetectable NO_3^- and NO_2^- residuals in milk, respectively, supporting that supplementing NO_3^- at adequate levels for dairy cows would not be a concern for human food safety (Guyader et al., 2016a; Meller et al., 2019).

Milk Fatty Acids and Antioxidant Capacity

It is well documented that supplemental NO_3^- may reduce DMI and NDF digestibility due to its impact on palatability and toxicity for cellulolytic bacteria, respectively, which in our understanding may affect milk FA composition. According to our knowledge, Klop et al. (2016) was the pioneer to investigate the effects of NO_3^- and its interactions with DHA fed to dairy cows on milk FA composition. Hence, there is still a lack of information on whether supplementing NO_3^- in diets for dairy cows would impact milk FA composition. Corroborating with Klop et al. (2016), our findings demonstrated minor effects on individual milk FA profile, supported by the absence of effects on nutrient digestibility. For a better understanding, besides presented the effects of NO_3^- on milk FA individually, we have shown such parameters grouped.

The absence of effects on CLA indicates that fat depression was not caused by its synthesis. In theory, C18:2 *trans*10-*cis*12-CLA, produced via biohydrogenation, has been considered a potent inhibitor of milk fat synthesis and secretion (Baumgard et al., 2000). Supplemental CAN reduced the proportion of medium chain FA, and individually, treatment reduced C12:0, C13:0, and C16:0 proportions. Saturated FA were reduced by increasing

NO_3^- levels in the diet; however, separately, treatment effect was only observed on C12:0, C13:0, C16:0, and C20:0 proportions, the latter in agreement with the findings by Klop et al. (2016). Such effect may be considered positive for milk quality, as according to Lock and Bauman (2004), saturated FA in bovine milk has led to a negative consumer perception ever since intaking saturated fats became a health concern, representing up to 70% of total fat weight (Lindmark Månsson, 2008). One plausible cause for the reduction on saturated FA can be the reduction on NDF intake by treatment, even though no effect on its digestibility was observed.

To date, according to our knowledge, there is no previous data with regard to supplemental NO_3^- fed to dairy cows on milk antioxidant capacity. Besides FA, vitamin, and minerals, other properties of milk have been also linked to the benefits of its antioxidant capacity for improving health in humans by removing free radicals from the body (Khan et al., 2019). In our study, reducing power and TBARS were unaffected by treatment, meaning that supplemental CAN did not change milk oxidative properties. Conjugated dienes increased with increasing levels of CAN, which has been considered an indicator of lipid peroxidation caused by lower stability on milk FA (Guillén and Cabo, 2002). The reduction on SFA proportion possibly was the main reason for increasing conjugated dienes in milk of cows receiving supplemental CAN.

Blood Methemoglobin and Plasma Urea Nitrogen

As mentioned sooner, there is still a concern of supplemental CAN regarding animal toxicity. However, a prior and proper adaptation to the animals has been guaranteed low levels of MetHb. The incidence of MetHb in blood in our study was augmented as the levels of NO_3^- in the diet of dairy cows increased over time, however, the highest level observed 4 h after feeding (3.26%) for CAN30 was much lower than the considered sufficient to cause

methemoglobinemia, which according to Bruning-Fann and Kaneene (1993) ranges from 30% to 40% of the total hemoglobin. Our data also are lower than the observed in previous studies evaluating dietary nitrate salts fed to dairy cows either in short (Olijhoek et al., 2016) and long-term studies (van Zijderveld et al., 2011), with 4.8% and 4.7%, respectively. Thus, supplementing CAN up to 23 g/kg on DM basis with a prior and adequate adaptation, increasing the addition of NO_3^- in the diet gradually, has guaranteed low levels of MetHb in the blood, and therefore, without risk of intoxication to the animals.

Treatment did not affect the concentration of PUN; however, there was an increase on its amount over time. We assume, with such effect, that URE and CAN provided similar amounts of N to be absorbed through the rumen wall, and posteriorly dropping into the bloodstream. Differently to our findings, (Wang et al., 2018) have observed higher amounts of NH_3 in the plasma of cows receiving urea compared to supplemental NO_3^- (14.6 g/kg DM).

Microbial Protein Synthesis, Ammonia-N, and VFA proportions

Besides acting as a [H] sink, reducing CH_4 production, nitrate salts are sources of non-protein nitrogen, essential for the rumen microbiota to synthesize microbial protein. Indeed, Wang et al., (2018) affirmed that sodium nitrate increased NH_3 incorporation into microbial protein in the rumen compared to urea in cows fed a low-protein diet. However, in our study, treatment did not affect microbial protein synthesis, meaning that compared to URE, supplemental CAN seemed to have the same efficacy at providing NH_3 for bacteria growth, which is in line with Olijhoek et al., (2016) who evaluated NO_3^- fed to dairy cows up to 21 g/kg DM. The absence of response may be caused by the lack of effects observed in the excretion of purine derivates, demonstrated by their concentrations and ratios (e.g., allantoin, uric acid). Additionally, estimation of microbial protein synthesis through purine derivates must be interpreted with caution, since Hristov et al. (2019), in a recent review, affirmed that

there is an inaccurate relationship between microbial protein synthesis and urinary excretion of purine derivatives.

Treatment did not affect the concentration of NH_3-N within the rumen, corroborating with previous studies evaluating supplemental NO_3^- fed to dairy cows (Guyader et al., 2016a; Meller et al., 2019). Despite the absence of effects in our study, it is well established that supplemental NO_3^- has a slower conversion to NH_3-N within the rumen compared to urea mainly because nitrate reduction occurs by two steps, whereby first NO_3^- is converted into NO_2^-, and posteriorly NO_2^- into NH_3 (Lee et al., 2017; Leng, 2008).

Total VFA was unaffected by treatment. Collectively, the present results corroborated findings from others showing that supplementing CAN for dairy cows does not seem to have implications on VFA concentrations (Meller et al., 2019; Olijhoek et al., 2016). However, VFA concentrations in our study were lower than the latter studies probably due to the rumen fluid collection method, since it has been shown that using a stomach tube to collect rumen fluid in ruminants generally affect such parameters (Shen et al., 2012)

Supplemental CAN increased the proportion of acetate, resulting in a tendency to increase C_2:C_3 as the levels of NO_3^- increased. Our findings corroborate with Latham et al. (2016), who observed that dietary NO_3^- salts generally increased acetate production, and different from other feed additives aiming at methane mitigation, could reduce propionate production, although, such effect was not observed in the current study. Indeed, the current findings are also supported by other studies exploring NO_3^- supplementation that indicated changes on VFA proportions caused by NO_3^- action as a [H] sink during the nitrate-ammonia reduction (Guyader et al., 2016b; Patra and Yu, 2013; Wang et al., 2018). As mentioned sooner, the collection method applied in our study might have implicated fermentation

parameters, although a common inconsistency was observed between studies that accessed rumen fluid via cannula (Meller et al., 2019; Olijhoek et al., 2016; Wang et al., 2018).

CONCLUSIONS

Supplementing CAN at 30 g/kg DM to dairy cows reduced nutrient intake and negatively affected milk fat, and milk protein content. Minor effects were observed on milk FA and its antioxidant capacity. A proper and gradual adaptation to dietetic NO_3^- guaranteed low NO_3^- and NO_2^- residuals in milk, as well as the low proportion of MetHb in blood. Providing NO_3^- to dairy cows seemed not to affect microbial protein synthesis and ruminal fermentation parameters, although such effects must be carefully interpreted when estimated by purine derivates and rumen collection via stomach tube, respectively. Altogether, dietary CAN at 15 g/kg DM, according to the results in this study, can be considered proper for dairy cow diets as a feed additive replacing urea without affecting milk quality.

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Table 1. Ingredient proportion and nutritional composition of the experimental diets

Item	Treatment		
	UREA	CAN15	CAN30
Ingredient proportion, g/kg DM			
Corn silage	530.00	530.00	530.00
Ground corn	304.99	304.49	303.98
Soybean meal	120.84	120.92	121.02
Urea[1]	11.87	5.94	0.00
Calcium nitrate[2]	0.00	15.00	30.00
Limestone[3]	17.30	8.65	0.00
Mineral and vitamin supplement[4]	15.00	15.00	15.00
Nutritional composition, g/kg DM[5]			
DM, as-fed basis	469.06	468.50	467.94
OM	932.29	928.20	924.10
CP	164.22	164.22	164.23
RDP	114.25	114.25	114.26
EE	29.84	29.82	29.80
NDF	341.76	341.71	341.67
Forage NDF	288.85	288.85	288.85
Nonfibrous carbohydrates	420.63	420.27	419.91
NO_3^-	0.00	11.47	22.94
Ca	9.71	9.71	9.71
P	3.67	3.66	3.66
Ca:P	2.65	2.65	2.65

[1]Prote-N (GRASP Ind. & Com. LTDA, Curitiba, Brazil). Composition: 99.5% DM and 41.7% N on a DM basis. [2]Double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2{\cdot}NH_4NO_3{\cdot}10H_2O$] (Yara North America, Inc. Tampa, FL, USA): 85.0% DM; 16.5% N, 19.6% Ca, and 76.5% NO_3^- on a DM basis). [3]Granisul, Rio Branco do Sul-Parana, Brazil. Composition per kg: 340g of Ca and 40g of Mg. [4]Bovigold® CRINA® (DSM Tortuga, Sao Paulo, Brazil) Composition per kg: 120g Ca, 35g P, 15g S, 38g Mg, 50g K, 106g Na, 15mg Co, 417mg Cu, 16.5mg Cr, 875mg Fe, 17.5mg I, 1,165mg Mn, 15mg Se, 2,300mg Zn, 350mg F, 170,000 IU of vit A, 60,000 IU of vit D, 1,000 IU of vit E. [5]Unless otherwise stated.

Table 2. Effect of calcium nitrate fed to dairy cows on body weight, dry matter intake, and nutrients intake

Item[1]	Treatment[3]			SEM[4]	*P*-value[5]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
BW[2]	555.17	558.00	551.00	10.27	0.45	0.46	0.32
DMI	19.06	18.88	16.51	0.68	<0.001	<0.001	0.09
OMI	18.16	17.87	15.67	0.65	<0.001	<0.001	0.11
CPI	2.74	2.78	2.28	0.09	<0.001	<0.001	<0.001
EEI	0.42	0.44	0.37	0.01	<0.001	<0.001	0.01
NDFI	7.07	7.13	6.21	0.25	<0.001	<0.001	0.05
NFCI	7.60	7.48	6.57	0.26	<0.001	<0.001	0.13

[1]kilograms per day (unless otherwise stated).
[2]kilograms
[3]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[4]Standart error of the mean.
[5]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 3. Effect of calcium nitrate fed to dairy cows on apparent dry matter, and nutrient digestibility

Item[1]	Treatment[2]			SEM[3]	*P*-value[4]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
DMD	632.70	627.00	638.99	10.09	0.53	0.55	0.34
OMD	649.42	645.23	657.59	10.10	0.49	0.44	0.37
CPD	593.79	607.27	587.95	20.32	0.61	0.77	0.35
EED	660.66	671.15	670.30	10.91	0.41	0.27	0.45
NDFD	474.33	472.83	456.05	21.87	0.69	0.45	0.71
NFCD	818.83	824.54	869.37	17.01	0.03	0.01	0.22

[1]grams per kilograms of DM
[2]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[3]Standart error of the mean.
[4]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 4. Effect of calcium nitrate fed to dairy cows on milk yield, and milk composition

Item	Treatment[4]			SEM[5]	*P*-value[6]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
Milk yield, kg/d	23.32	23.17	22.94	1.31	0.83	0.55	0.93
FCM[1]	22.83	22.50	20.73	1.03	0.09	0.05	0.38
ECM[2]	22.63	22.43	20.69	0.99	0.08	0.05	0.32
ECM/DMI	1.19	1.19	1.26	0.05	0.06	0.03	0.27
Fat, %	3.39	3.35	2.94	0.23	0.06	0.03	0.25
Fat, kg/d	0.78	0.77	0.66	0.04	0.06	0.03	0.30
True protein, %	3.03	3.10	2.91	0.07	0.05	0.09	0.05
True protein, kg/d	0.71	0.72	0.67	0.04	0.25	0.19	0.29
Lactose, %	4.64	4.61	4.58	0.07	0.63	0.35	1.00
Lactose, kg/d	1.08	1.07	1.05	0.06	0.63	0.35	0.90
MUN[3], mg/dL	13.36	14.04	13.83	0.96	0.64	0.53	0.50

[1]Fat-corrected milk
[2]Energy-corrected milk
[3]Milk urea nitrogen
[4]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[5]Standart error of the mean.
[6]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

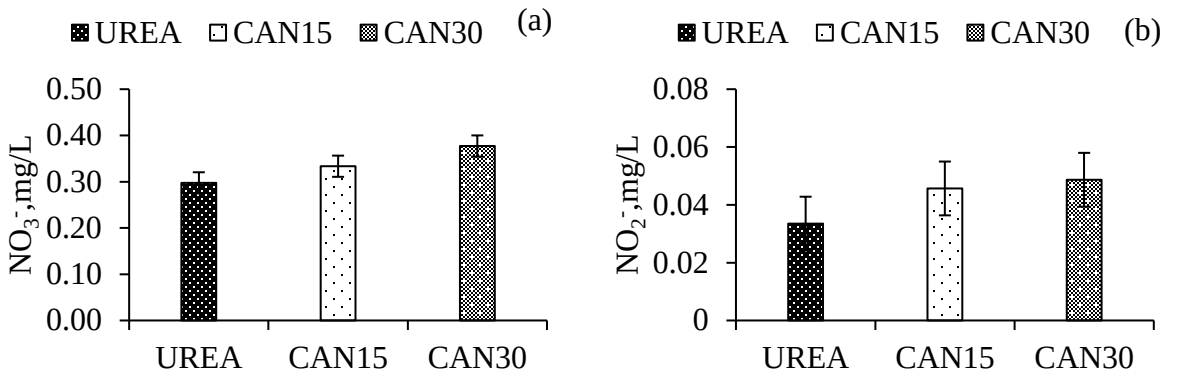

Figure 1. Effect of calcium nitrate fed to dairy cows on (a) nitrate (NO_3^-) (*P*-values: Treat = 0.05; Linear = 0.02; Quadratic = 0.87; SEM= 0.02) and (b) nitrite (NO_2^-) concentration in milk (*P*-values: Treat = 0.49; Linear = 0.26; Quadratic = 0.69; SEM= 0.009).

Table 5. Effect of calcium nitrate fed to dairy cows on milk fatty acids proportions

Item[1]	Treatment[2]			SEM[3]	*P*-value[4]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
C6:0	0.40	0.39	0.46	0.05	0.61	0.42	0.58
C8:0	1.00	0.97	1.02	0.07	0.80	0.82	0.54
C10:0	4.64	4.49	4.46	0.32	0.76	0.50	0.79
C11:0	0.29	0.28	0.26	0.03	0.41	0.20	0.81
C12:0	7.26	6.82	6.53	0.43	0.06	0.02	0.74
C13:0	0.39	0.36	0.31	0.03	<0.01	<0.01	0.57
C14:0	21.2	21.5	21.3	0.51	0.91	0.96	0.68
C14:1	1.65	1.66	1.81	0.08	0.13	0.07	0.34
C15:0	2.40	2.36	2.22	0.18	0.23	0.10	0.62
C15:1	0.28	0.26	0.33	0.03	0.05	0.11	0.05
C16:0	37.5	36.9	35.0	1.18	0.06	0.02	0.42
C16:1	1.30	1.32	1.38	0.13	0.86	0.61	0.90
C17:0	0.73	0.73	0.79	0.05	0.39	0.24	0.48
C17:1	0.14	0.14	0.17	0.03	0.37	0.21	0.56
C18:0	6.51	6.56	7.08	0.45	0.54	0.33	0.63
C18:1 n9t	3.29	2.27	3.73	0.36	0.36	0.23	0.44
C18:1 n9c	9.08	9.79	10.66	0.92	0.38	0.18	0.93
C18:2 n6t	0.63	0.65	0.73	0.05	0.19	0.08	0.55
C18:2 n6c	0.75	0.86	0.94	0.09	0.30	0.13	0.94
C18:3 n6	0.03	0.04	0.03	0.00	0.54	0.86	0.28
C18:3 n3	0.04	0.05	0.06	0.01	0.41	0.19	0.96
C18:2 c9 t11-CLA	0.00	0.14	0.09	0.07	0.42	0.38	0.33
C18:2 t10 c12-CLA	0.01	0.01	0.01	0.01	0.99	0.94	0.99
C20:0	0.10	0.11	0.12	0.00	0.12	0.04	0.86
C20:1	0.02	0.02	0.03	0.01	0.55	0.28	0.96
C20:2	0.05	0.05	0.06	0.01	0.60	0.37	0.66
C21:0	0.16	0.20	0.31	0.04	0.08	0.03	0.46

[1]g/100g of total lipids
[2]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[4]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 6. Effect of calcium nitrate fed to dairy cows on milk fatty acids grouped

Item[1]	Treatment[9]			SEM[10]	*P*-value[11]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
Total CLA[2]	0.02	0.15	0.10	0.08	0.51	0.45	0.39
SCFA[3]	1.41	1.37	1.49	0.12	0.75	0.63	0.57
MCFA[4]	71.1	70.1	67.8	1.43	0.08	0.03	0.55
LCFA[5]	22.8	24.0	26.2	1.57	0.13	0.06	0.69
MUFA[6]	15.8	16.5	18.1	1.25	0.19	0.08	0.66
PUFA[7]	1.46	1.79	1.86	0.19	0.28	0.14	0.57
SFA[8]	76.2	75.2	72.9	1.50	0.12	0.05	0.64
n-3	0.04	0.05	0.06	0.01	0.41	0.19	0.96
n-6	1.42	1.74	1.80	0.18	0.28	0.14	0.55
n-6/n-3	28.2	29.5	27.7	1.75	0.72	0.83	0.45

[1]g/100g of total lipids
[2]Total CLA - Conjugated linoleic acid;
[3]SCFA - Short chain fatty acids;
[4]MCFA - Medium chain fatty acids;
[5]LCFA - Long chain fatty acids;
[6]MUFA - Monounsaturated fatty acids;
[7]PUFA - Polyunsaturated fatty acids;
[8]SFA - Saturated fatty acids
[9]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[10]Standard error of the mean.
[11]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 7. Effect of calcium nitrate fed to dairy cows on antioxidant capacity

Item	Treatment[4]			SEM[5]	*P*-value[6]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
RP[1]	12.66	13.66	12.31	1.91	0.86	0.89	0.61
CD[2]	47.56	52.66	63.39	6.56	0.06	0.02	0.59
TBARS[3]	6.42	6.04	7.03	1.23	0.68	0.60	0.49

[1]Reducing power, mg of gallic acid equivalent/L
[2]Conjugated dienes, mmol/kg of fat
[3]TBARS - Thiobarbituric acid reactive substances, mmol of malondialdehyde/kg of fat
[4]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[5]Standart error of the mean.
[6]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate

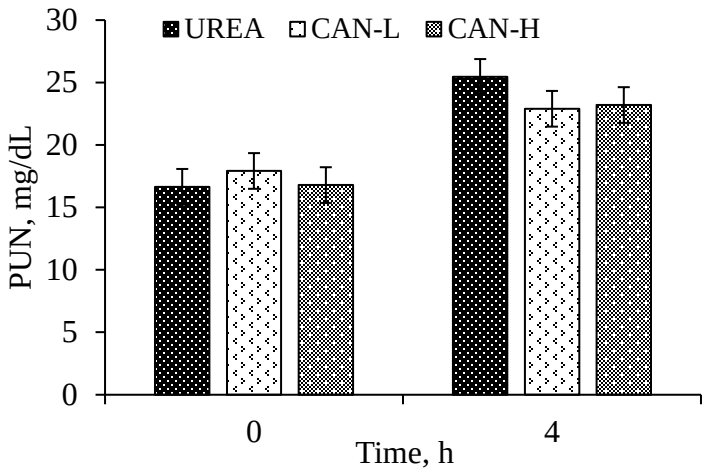

Figure 2. Effect of calcium nitrate fed to dairy cows on plasma urea nitrogen (PUN) before (0 h) and after (4 h) feeding. *P*-values: Treat = 0.64; Time <0.01; Treat x time = 0.28 Linear = 0.37; Quadratic = 0.91; SEM = 1.43

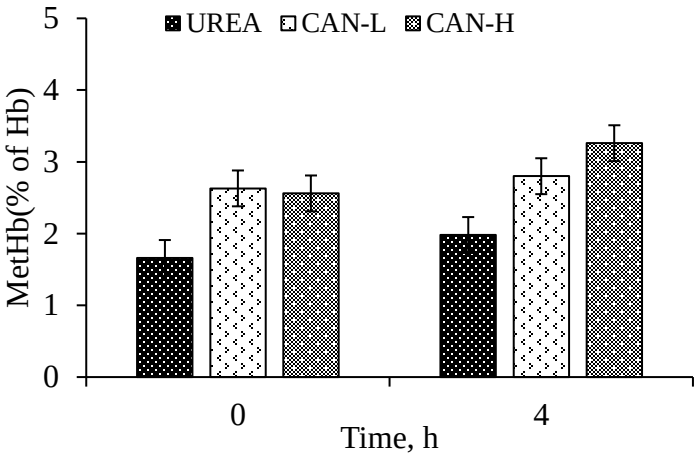

Figure 3. Effect of calcium nitrate fed to dairy cows on methemoglobin (% of total hemoglobin) before (0 h) and after (4 h) feeding. *P*-values: Treat <0.01.; Time <0.01; Treat x time = 0.17 Linear <0.01; Quadratic = 0.05; SEM = 0.25

Table 8. Effect of calcium nitrate fed to dairy cows on allantoin, uric acid excretion, and microbial protein synthesis

Item	Treatment[1]			SEM[2]	*P*-value[3]		
	UREA	CAN15	CAN30		Treat	Linear	Quadratic
Allantoin, mmol/d	343.25	354.12	345.19	25.1	0.78	0.90	0.50
Uric Acid, mmol/d	50.89	55.83	67.25	8.56	0.14	0.06	0.63
Allant:Creat	3.03	2.97	2.96	0.38	0.94	0.76	0.90
Uric Acid:Creat	0.43	0.43	0.57	0.07	0.18	0.11	0.33
Allant+Uric Acid:Creat	3.46	3.40	3.54	0.42	0.84	0.74	0.64
Microbial Protein, g/d	1996.30	2079.10	2093.37	27.5	0.63	0.39	0.72

[1] UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[2]Standart error of the mean.
[3]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

Table 9. Effect of calcium nitrate fed to dairy cows on pH, volatile fatty acids, and NH_3-N concentration

Item[1]	Treatment[2]			Time[3]		SEM[4]	*P*-value[5]				
	UREA	CAN15	CAN30	2 h	8 h		Treat	Time	Int	Lin	Quad
pH	6.80	6.75	6.78	6.74	6.81	0.12	0.92	0.35	0.95	0.88	0.71
NH_3-N, mM	13.30	10.86	10.43	12.22	10.84	2.44	0.48	0.25	0.61	0.27	0.64
Total VFA, mM	73.00	72.51	71.69	72.88	71.92	7.25	0.98	0.83	0.20	0.85	0.97
Acetate	59.20	60.66	62.98	62.15	59.74	1.33	<0.01	<0.01	0.10	<0.01	0.54
Propionate	23.61	23.88	21.67	22.10	24.01	1.74	0.29	0.01	0.37	0.20	0.34
Isobutyrate	0.83	0.75	0.77	0.88	0.69	0.05	0.07	<0.01	0.17	0.08	0.10
Butyrate	12.75	11.37	11.79	11.61	12.33	0.82	0.28	0.18	0.68	0.27	0.24
Isovalerate	1.81	1.61	1.33	1.61	1.56	0.19	0.04	0.57	0.37	0.01	0.81
Valerate	1.77	1.71	1.44	1.63	1.64	0.16	0.03	0.84	0.70	0.01	0.33
C_2:C_3	2.59	2.65	2.95	2.86	2.59	0.21	0.13	<0.01	0.16	0.07	0.42

[1]Molar proportion (%) (unless otherwise stated).
[2]UREA: control group (without nitrate); CAN15: 15 g of calcium nitrate per kg of DM; CAN30: 30 g of calcium nitrate per kg of DM.
[6]Standart error of the mean.
[4]Treat = effect of treatment (UREA vs. CAN15 vs. CAN30); Interaction: treatment x time. Linear = linear effect of calcium nitrate; Quadratic = quadratic effect of calcium nitrate.

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CONSIDERAÇÕES FINAIS

O nitrato de cálcio e as fontes adicionais de NO_3^- (nitrato de amônio, nitrato de amônio + calcário dolomítico e nitrato de potássio) foram eficazes em reduzir a produção *in vitro* de metano. Ademais, o uso de silagem de grão úmido como fonte de amido rapidamente degradável no rúmen também reduziu a emissão *in vitro* de metano, embora tais estratégias alimentares não interagiram entre si.

A suplementação de nitrato de cálcio para cabras em lactação pode ser realizada em até 20 g/kg de MS, o que corresponde a 15.3 g de NO_3^-, sem afetar a digestibilidade dos nutrientes e os parâmetros de qualidade do leite.

O fornecimento de nitrato de cálcio na dose de 30 g/kg de MS (23 g de NO_3^- com base na MS) para vacas em lactação reduziu o consumo e os componentes do leite. Portanto, recomenda-se a suplementação deste aditivo em até 15 g/kg de MS na dieta de vacas em lactação sem alterar a digestibilidade dos nutrientes e a qualidade do leite.