Original Research Article

Dynamics of the ruminal microbial ecosystem, and inhibition of methanogenesis and propiogenesis in response to nitrate feeding to Holstein calves

Abimael Ortiz-Chura, José Gere, Gisela Marcoppido, Gustavo Depetris, Silvio Cravero, Claudia Faverín, Cesar Pinares-Patiño, Angel Cataldi, María E. Cerón-Cucchi

Institute of Pathobiology, CICVyA National Institute of Agricultural Technology, IPVet, UEDD INTA-CONICET, Hurlingham, C1686, Argentina
Engineering Research and Development Division, National Technological University (UTN), National Scientific and Technical Research Council (CONICET), Buenos Aires, C1179, Argentina
Agricultural Experimental Station of Balcarce, National Institute of Agricultural Technology (INTA), Balcarce, B7620, Argentina
Institute of Agrobiotechnology and Molecular Biology, IABIMO, National Institute of Agricultural Technology (INTA), National Scientific and Technical Research Council (CONICET), Hurlingham, C1686, Argentina
The Agribusiness Group, Lincoln University, PO Box 85016, Lincoln, 7674, New Zealand

Article info
Article history:
Received 18 November 2020
Received in revised form 25 June 2021
Accepted 8 July 2021
Available online 2 October 2021

Keywords:
Methane emission
Nitrate
Microbiota
Ruminal fermentation

Abstract
It is known that nitrate inhibits ruminal methanogenesis, mainly through competition with hydrogenotrophic methanogens for available hydrogen (H2) and also through toxic effects on the methanogens. However, there is limited knowledge about its effects on the others members of ruminal microbiota and their metabolites. In this study, we investigated the effects of dietary nitrate inclusion on enteric methane (CH4) emission, temporal changes in ruminal microbiota, and fermentation in Holstein calves. Eighteen animals were maintained in individual pens for 45 d. Animals were randomly allocated to either a control (CTR) or nitrate (NIT, containing 15 g of calcium nitrate/kg dry matter) diets. Methane emissions were estimated using the sulfur hexafluoride (SF6) tracer method. Ruminal microbiota changes and ruminal fermentation were evaluated at 0, 4, and 8 h post-feeding. In this study, feed dry matter intake (DMI) did not differ between dietary treatments (P > 0.05). Diets containing NIT reduced CH4 emissions by 27% (g/d) and yield by 21% (g/kg DMI) compared to the CTR (P < 0.05). The pH values and total volatile fatty acids (VFA) concentration did not differ between dietary treatments (P > 0.05) but differed with time, and post-feeding (P < 0.05). Increases in the concentrations of ruminal ammonia nitrogen (NH3-N) and acetate were observed, whereas propionate decreased at 4 h post-feeding with the NIT diet (P < 0.05). Feeding the NIT diet reduced the populations of total bacteria, total methanogens, Ruminococcus albus and Ruminococcus flaviiacs, and the abundance of Succinilactisum, Coprococcus, Treponema, Shuttlewortsia, Succinivibrio, Sharpea, Pseudobutyrivibrio, and Selenomonas (P < 0.05); whereas, the population of total fungi, protozoa, Fibrobacter succinogenes, Atopobium and Erysipelotrichaceae L7A_E11 increased (P < 0.05). In conclusion, feeding nitrate reduces enteric CH4 emissions and the methanogens population, whereas it decreases the propionate concentration and the abundance of bacteria involved in the succinate and acrylate pathways. Despite the altered fermentation profile and...
1. Introduction

The livestock sector contributes significantly to global food security, accounting for 17% and 33% of the world consumption of kilojoules and proteins (Rosegrant et al., 2009). An increase in global demands of meat (73%) and milk (58%) is projected for the year 2050, compared to 2010; due to projected increases in world population, urbanization, and income in developing countries (FAOSTAT, 2020; Gerber et al., 2013a). To meet this demand, the main challenge for the livestock sector is to increase production efficiency while reducing the impact on the environment.

Ruminal fermentation plays a crucial role in the digestion and transformation of structural and non-structural carbohydrates into useful products and metabolites beneficial to ruminant animals (Armstrong and Blaxter, 1957), a process that also generates methane (CH$_4$) which is then released into the atmosphere. This CH$_4$ source accounts for approximately 6% of global greenhouse gas (GHG) emissions (Gerber et al., 2013b). Methane is produced during ruminal methanogenesis by the methanogens that are closely associated with ciliated protozoa and hydrogen (H$_2$) producing bacteria (Morgavi et al., 2010).

Studies have shown that CH$_4$ emission is strongly related to feed intake and diet composition (Hellwing et al., 2018; Hristov et al., 2018), hence dietary interventions are among the most preferred GHG mitigation avenues leading to concurrent reduction of emission intensities by increasing animal performance (Beauchemin et al., 2020). One of these strategies is the supplementation of diets with nitrate (NO$_3$). The mitigation of CH$_4$ emissions in vivo through the use of NO$_3$ has proven to be effective, with an observed reduction potential ranging from 6.8% to 12.5% for each 1% of NO$_3$ added daily on a dry matter (DM) basis (Lund et al., 2014; van Zijderveld et al., 2011a; Olijhoek et al., 2016). It is known that NO$_3$ in the rumen acts as an electron acceptor, thereby competing for dissolved H$_2$ between nitrate-reducing microorganisms and methanogenic archaea (Latham et al., 2006). This mode of action is thermodynamically explained, given that reduction of NO$_3$ to NH$_3$ is more favorable (delta Gibbs [$\Delta$G] = $-$599.6 kJ/mol) than methanogenesis ($\Delta$G = $-$136 kJ/mol) (Thauer et al., 1977).

To date, there are no conclusive research results on the effects of NO$_3$ addition on ruminal fermentation and the ruminal microbiota, mainly due to the scarcity of research results. While some studies report a decrease in propionate production and an increase in acetate production (Olijhoek et al., 2016; Troy et al., 2015), another study reported an opposite effect (Wang et al., 2018), and others did not reveal significant changes (Popova et al., 2017; Zhang et al., 2019). It has been also reported that although methanogenesis was inhibited with the inclusion of NO$_3$, the concentration of H$_2$ (gaseous and dissolved) increased (Guyader et al., 2015; Olijhoek et al., 2016). All these reports suggest that competition for available H$_2$ cannot be regarded as the only mode of action of NO$_3$ to reduce CH$_4$ emissions. A possible explanation for the inhibition of methanogenesis could be the toxic effects of compounds derived from nitrate-reduction causing a reduction in the relative abundance and activity of methanogens (Granja-Salcedo et al., 2019; Iwamoto et al., 2002; Popova et al., 2019).

The production of volatile fatty acids (VFA), H$_2$, and CH$_4$ peak shortly following feeding (van Lingen et al., 2017). Also, the composition of ruminal microbiota varies widely during the day due to the different fermentation stages of feeds (Shaani et al., 2018). However, several studies that evaluated the effects of NO$_3$ on ruminal microbiota focused their analysis on a single follow-up time and did not report significant changes in the bacterial, protozoan, methanogenic and fungal communities (Granja-Salcedo et al., 2019; Popova et al., 2017). Therefore, it is important to consider the variation in the dynamics of the ruminal microbiota and its metabolites in response to the inclusion of NO$_3$ in the diet. Thus, the aim of this work was to simultaneously address the temporal changes in the population of ruminal microbiota (methanogens, bacteria, protozoa, and fungi), the concentration of dissolved metabolites (ruminal ammonia nitrogen [NH$_3$–N] and VFA), and the emissions of CH$_4$ in response to the inclusion of NO$_3$ in the diet of calves, with the purpose of understanding the effect in greater detail. In order to safeguard against unwanted effects on animal health and performance, an intermediate level of NO$_3$ inclusion (15 g/kg DM) was chosen based on the finding of previous studies (van Zijderveld et al., 2011a; Olijhoek et al., 2016), and a gradual acclimatization to increasing levels was implemented. We hypothesized that nitrate feeding would induce changes in ruminal microbiota composition, resulting in changes in ruminal fermentation profiles and lower CH$_4$ emission.

2. Materials and methods

2.1. Experimental design and animal procedures

The protocols, procedures, and the care of the animals were approved by the Institutional Committee for the Care and Use of Animals (CICUAE File No. 2017/124, approval date September 12, 2017) of the National Institute of Agricultural Technology (INTA), Argentina.

The experiment was conducted in the Experimental Dairy Centre of the Balcarce Experimental Station at INTA, Argentina. Eighteen Holstein calves (7 heifers and 11 steers) of 8.1 ± 0.5 months of age and 214 ± 13.5 kg of live weight (LW) were randomly allocated to either a control diet (CTR; including 5 steers and 4 heifers) or a nitrate diet (NIT; including 6 steers and 3 heifers). The CTR group received a total mix ration (on DM basis), containing ground corn (69.3%), soybean expeller (8.4%), urea (0.8%), vitamin-mineral premix (1.1%), and grass hay (20.4%); whereas the NIT group received a total mix ration (on DM basis), containing ground corn (68.4%), soybean expeller (8.4%), urea (0.2%), vitamin-mineral premix (1.1%), grass hay (20.4%), and 1.5% of nitrate (as calcium nitrate, Calcinit YARALIVA, Olso, Norway). (Appendix Table 1).

The trial included 30 d of acclimatization to diet, followed by a 15-d period of measurements (on d 31 to 45). To reduce the risks of toxicity from nitrates (ruminal microorganism convert NO$_3$ to nitrite [NO$_2$]), the amount of NO$_3$ in the diet was gradually increased as previously described (Ortiz-Chura et al., 2021). Throughout the experiment, the animals were kept in individual pens of 36 m$^2$ (9 m length × 4 m width), which were constructed outdoors using...
2.2. Diet chemical composition and feed intake

The experimental animals were fed at 100% of their ad libitum consumption observed during the last week of the acclimatization period. Feed dry matter intakes (DMI, in kilograms per day) by individual animals were measured daily as the difference between the amount offered and the amount refused. Refused feed was collected from the feeding bins immediately before feeding. Dry matter intake was also measured during the days of CH4 emission measurements (on d 36 to 40), when the feeding level was set to 95% of the ad libitum feed intake measurement period to ensure the complete intake of the diet. The experimental animals were weighed on d 1 and 45 (initial and final weight, respectively), following a fasting period of 24 h before initial and final weighing, when feed and water were removed.

The dry matter concentration of feed offered was determined by oven drying at 105 °C for 24 h. Ash concentrations were determined by incineration at 550 °C for 4 h (AOAC, 1990; method 942.05). The total nitrogen (TN) concentration was determined using a combustion type auto-analyzer (Leco FP-2000, Leco Corp., St. Joseph, MI) according to AOAC Official Methods (1990; method 990.03) and the crude protein (CP) was calculated as TN × 6.25. The ether extract (EE) concentration was determined after extraction with petroleum ether using a Soxhlet System Apparatus (Electromantle ME1000, UK) according to AOAC (1990; method 920.39). The concentration of the neutral detergent fiber (NDF) was determined using the Ankom 220 fiber analyzer (ANKOM Technology, Macedonia NY–USA) according to Van Soest et al. (1991). The starch concentration was analyzed using the enzymatic method (MacRae and Armstrong, 1968).

2.3. Enteric methane emission

Methane emissions from individual animals were estimated using the principles of the sulfur hexafluoride (SF6) tracer technique (Zimmerman, 1993; Johnson et al., 1994), with a slight modification as described by Pinares-Patiño et al. (2012). This modification involved extending the duration of breathed air sample collection from every 24 h (traditional technique) to 5 consecutive days (adapted technique). Briefly, at the beginning of the acclimatization period, the calves were orally dosed with permeation tubes containing the tracer. The tubes were chosen from a larger number based on their high linearity of mass loss ($R^2 > 0.99$) and narrow range of pre-calibrated rates of SF6 permeation (4.57 ± 0.64 mg/d; range 3.85 to 5.99 mg/d). The sample collection system consisted of polyvinyl chloride yoke-shaped devices (2.5 L volume) and the sample flow regulator was a metal capillary (10 cm length), with a small section (5 mm) pressed until the desired flow was achieved (initial flow rate 0.25 mL/min). Approximately, 500 mbar of internal pressure in the collection device at the end of sample collection was aimed (Pinares-Patiño and Clark, 2008). The sampling period was 5 consecutive days as proposed by Gere and Gratton (2010) and validated by Pinares-Patiño et al. (2012). Parallel to the animal breath sample collections, background air samples were also collected at a surrounding site, away from the animal pens to determine the baseline atmospheric concentrations of CH4 and SF6. These background samples were collected in duplicate, facing the wind direction. Given the disposition of the animal pens, it was assumed that concentrations of CH4 and SF6 in the inspired air by animals would be similar to those at the background samplers.

Sampling for CH4 emission started at 08:30 on d 36 and ended at 08:00 on d 40. The concentrations of CH4 and SF6 were analyzed using a gas chromatograph (PerkinElmer 6000, USA) as described by Gere et al. (2019).

Methane emissions were calculated using the permeation rate of each SF6 permeation tube and the concentration of CH4 and SF6 measured in each sample, according to the equation described by Williams et al. (2011):

$$\text{CH}_4(\text{g/d}) = \frac{\text{PR}_{\text{SF}_6}(\text{g/d}) \times (\frac{[\text{CH}_4] - [\text{BG}_\text{CH}_4]}{[\text{SF}_6] - [\text{BG}_{\text{SF}_6}]}) \times \text{MW}_{\text{CH}_4}}{\text{MW}_{\text{SF}_6}} \times 1.000$$

where PR SF6 is the permeation rate of the SF6 tubes; [CH4] and [SF6] are the concentrations of these gases in the samplers; [BG] is the baseline atmospheric concentrations (baseline values were 2.8 ± 0.3 parts per million and 6.3 ± 0.7 parts per trillion for CH4 and SF6, respectively); MWCH4 and MWSF6 are the molecular mass of CH4 (16.04 g/mol) and SF6 (146.06 g/mol), respectively.

2.4. Sampling and processing of ruminal fluid

The ruminal fluid of the 18 animals in study was sampled at 3 evaluation times: 0 h pre-feeding (08:00), 4 h post-feeding (12:00), and 8 h post-feeding (16:00; immediately before the afternoon feeding) on d 41, 42 and 43, respectively. Samples of ruminal fluid (approximately 200 mL per animal) were obtained by esophageal tubing using a flexible PVC tube (2 mm of wall thickness and 8 mm of internal diameter; Tecnocom, Buenos Aires, Argentina) with about 25 holes of 3 mm diameter in the 15 cm-probe head, and an electric vacuum pump (down to 7 mbar; PXC-100, BOMBAS PASCAL S.A., Argentina). Then, the samples were filtered with a 3-cloth gauze, and immediately afterward the pH of each sample was measured. Finally, 50 mL was subsampled for microbial studies, 10 mL for determination of VFA concentration, 10 mL for ammonia concentration, and 5 mL for the count of ciliated protozoa. The samples for microbial studies were lyophilized and stored at −70 °C, and the samples for ruminal fermentation were acidified by the addition of 100 μL of 99% sulfuric acid and kept at −20 °C until their use. Samples for protozoa count were conserved in a solution of 10% formaldehyde (vol/vol), at room temperature.

2.5. Determinations of the ruminal fermentation parameters

Concentrations of VFA were determined by gas chromatography using Konik 5000B equipment (KONIK Group, Miami, USA) and according to the procedures described by Friggens et al. (1998). The concentration of total VFA was expressed in mM, and the fermentation profiles of acetate, propionate, iso-butyrate, butyrate, isovalerate, and valerate were expressed in percentage of total VFA. Ruminal NH3–N concentration was determined using the colorimetry technique using the uremia kit (Lab Wiener, Rosario-Argentina) according to the manufacturer’s instructions. The pH of the ruminal samples was analyzed with a potentiometer (Corning Ltd, Halstead, Essex, UK).

2.6. DNA extraction, microbial quantification of specific populations and counting of protozoa

Total genomic DNA was extracted from 100 mg of the lyophilized ruminal sample using the commercial Mini Kit DNA Stool QIAamp extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. The DNA concentration was analyzed qualitatively and quantitatively using electrophoretic
runs on 0.8% agarose gels and fluorometry (Qubit 2.0, Qubit dsDNA Broad Range Assay Kit, Life Technologies, Oregon, USA).

The absolute quantification of specific microbial populations was determined using real-time PCR (qPCR) by comparison with serial dilutions (10^3 to 10^8) of specific plasmid DNA standards. The quantitative qPCR was performed using the StepOnePlus Real-Time System (TermoFisher Scientific, USA). A total of 2 μl of genomic DNA (10 ng/μl) was added to the amplification reaction containing 20 pmol of each primer, 4 μl of 5X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia), and DNA/RNA free water adjusting to the total volume (20 μl) in duplicate for each sample. The primers used for the amplification of the 16S rRNA and methyl coenzyme-M reductase (mcrA) gene region were obtained from the bibliographical reference (Appendix Table 2).

The amplification of the fragments was previously fine-tuned (Ortiz-Chura et al., 2018). The procedure briefly consisted of a cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s for denaturation; hybridization at 60 °C for 30 s, but which varied according to the primer, and 72 °C for 1 min for the extension. The quantification was based on the construction of regression curves of the standard plasmid with already known concentrations. In each reaction, the linear regression values of the standard curve were within normal limits (R² = 0.99, slope = −3.2 to −3.6 and efficiency = 95% to 110%).

The ruminal ciliated protozoa count was performed according to the methodology described by Dehority (1993). This procedure consisted of mixing the previously filtered fluid samples with a formalin saline solution. Then, the samples were stained with methyl green and the count of the number of ciliated protozoa was performed using an optical microscope (Nikon eclipse E200MV, Nikon Tokyo, Japan).

2.7. Microbial analysis by 16S rRNA amplicon sequencing

In order to analyze the meta-taxonomic temporal changes of ruminal bacteria, a total of 30 samples of genomic DNA of ruminal content were evaluated, consisting of 5 samples randomly chosen from each treatment group, at 3 separate evaluation times. The samples for 16S rRNA amplicon sequencing were sent to the Genomics and Sequencing Service of the Research Center in Veterinary and Agronomic Sciences of INTA, Argentina. The processing of samples for sequencing is briefly described below. A PCR was used to amplify the V3 to V4 regions of the 16S rRNA gene for bacteria. For this purpose, 4 primers were used: 2 specific: 341f/805r (341F: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATCTAATCC) + 2 that contain the adapter sequences P5/P7 for the indexed flow cells (Klindworth et al., 2012). The reactions were kept at 95 °C for 3 min to denature the DNA, proceeding to amplification for 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; a final extension of 10 min at 72 °C was added to ensure complete amplification.

The expected fragment length of the PCR products was verified with agarose gel electrophoresis (1%) with ethidium bromide, and the size of the amplicon was estimated comparing it with a scale of 1 kb plus DNA (1 kb plus the scale of DNA, Invitrogen, Carlsbad, CA, USA). In addition, the PCR fragments were purified using the Agencourt AMPure XP kit (Beckman Coulter, USA) following the manufacturer’s instructions. Finally, to validate the library, the size of the DNA fragment was verified using the 5200 Fragment Analyzer System (Agilent Technology Inc. CA, USA). The amplicons were sequenced with the Illumina MiSeq platform (Illumina Inc., San Diego, USA). Raw data of the sequencing from our samples are available upon request.

2.8. Bioinformatic analysis

Sequencing of the bacterial 16S rRNA amplicon yielded 3,178,366 paired-end reads with a mean length of 464 pb, which merged using MOTHUR’s Make. Contig command (Schloss et al., 2009). Further, sequences were quality trimmed and those that did not meet the following criteria were excluded from analysis: length 200 to 600 bp, ambiguous bases > 6, Phred score > 25, homopolymer < 8, mismatches in primers < 4. Operational taxonomic units (OTU) picking was performed using the Uclust algorithm with GreenGenes v13.8 database as a reference and 97% of similarity threshold (Seedorf et al., 2014). Representative sequences were aligned using PyNAST (Caporaso et al., 2009), and chimeric sequences were searched using the Chimera. Uchime command from the Mothur package. The taxonomy was assigned using the GreenGenes v13.8 database.

2.9. Statistical analysis

Data for feed intake and CH4 emission were analyzed using a general linear model (GLM) SAS Software (13.1, SAS Institute SAS, 2013, Cary, NC, USA) as a randomized complete block design (Block = Sex), according to the following model I:

\[ Y_{ij} = \mu + \text{Sex}_i + \text{Treat}_j + e_{ij}, \]

where: \( Y_{ij} \) = response variable; \( \mu \) = general mean of the experiment; \( \text{Sex}_i \) = sex \( (i = 2) \); \( \text{Treat}_j \) = dietary treatments, CTR versus NIT \( (j = 2) \); \( e_{ij} \) = experimental error. Data for pH, NH3−N, VFA, ciliated protozoa, the concentration of bacteria, methanogens and fungi by qPCR, and the alpha-diversity indices (Chao 1 and ACE estimators and Shannon and Simpson diversity indices) were compared between dietary treatments and sampling times by a repeated measures ANOVA using the PROC MIXED of the SAS Software, according to the model II:

\[ Y_{ijkl} = \mu + \text{Sex}_i + \text{Treat}_j + \text{Anim}_k + \text{Diet}_l + \text{Sex} \times \text{Treat} + \text{Sex} \times \text{Diet} + \text{Treat} \times \text{Diet} + e_{ijkl}, \]

where: \( Y_{ijkl} \) = response variable; \( \mu \) = general mean of the experiment; \( \text{Sex}_i \) = sex \( (i = 2, block) \); \( \text{Treat}_j \) = dietary treatments, CTR versus NIT \( (j = 2) \); \( \text{Anim}_k \) = is the repeated effect of the kth calf within the jth dietary treatments \( (k = 18) \); \( \text{Treat} \) = sampling times \( (l = 3) \); \( \text{Treat} \times \text{Time} = \) interaction; \( e_{ijkl} \) = experimental error.

The OTU tables and raw counts per taxonomic level were used to compute the relative abundance of bacterial phyla and genera. The data of abundance were analyzed according to model II using PROC MIXED of SAS Software, either for data with normal distribution or for those data that were transformed to normalize them using the square root function. Prior to the alpha and beta-diversity analysis, the data from the OTU table of the samples were normalized using the rarefy function of the vegan package in R Studio 3.6.1 (R Core Team, 2019), which is applied as an ad hoc medium to normalize the microbiome counts that have resulted from libraries of very different sizes.

For the beta-diversity analysis, the ordering graphs of the non-metric dimension scale (NMDS) were applied using the Bray–Curtis dissimilarity distance in the R ggplot2 package (Wickham, 2016) and the meta MDS function in the R vegan statistical package (Oksanen et al., 2019), in which the OTU were filtered to the lowest sequence number. For statistical analysis of beta-diversity, we performed the permutational multivariate analysis of variance (PERMANOVA) using Adonis function in R. In addition, for each set of beta-diversity data, the differences were compared using the group dispersion homogeneity test using the
betadisper function in the R vegan package (Anderson, 2006). All differences were significant when $P < 0.05$.

Spearman’s correlation analysis was used to determine the degree of association between enteric CH$_4$ emission, ruminal fermentation, and the relative abundance of bacterial communities from sequencing results. The analyses were performed using the corplot function in R.

3. Results

3.1. Feed intake and CH$_4$ emission

Feed intake, initial and final weights did not differ ($P > 0.05$) between dietary treatments. Compared to the CTR diet, feeding the NIT diet decreased both CH$_4$ emission (g/d; $P = 0.009$) and CH$_4$ yield (g/kg DMI; $P = 0.041$), by 27% and 21%, respectively (Table 1).

3.2. Temporal changes of ruminal fermentation parameters

Ruminal pH and total VFA concentration did not differ ($P > 0.05$; Table 2) between dietary treatments. There was no Diet × Time (i.e., sampling time) interaction effect on pH ($P = 0.136$). However, pH changed as a function of sampling time ($P = 0.001$). The initial pH was close to neutral, but 4 h post-feeding, this value decreased significantly in both dietary treatments; then the pH value tended to increase until reaching an average value of 6.8 in both treatments. There were Diet, Time, and Diet × Time interaction effects ($P < 0.05$) on the concentration of NH$_3$–N. The concentrations of NH$_3$–N did not differ ($P > 0.05$) at 8 h post-feeding period, but for the pre-feeding period it was higher for the CTR than for the NIT diet; whereas at 4 h post-feeding it was higher for the NIT than for the CTR diet (11.8 vs. 5.2 mg/dL, respectively) (Fig. 1A).

Total VFA concentration showed no Diet × Time interaction ($P = 0.092$). However, it changed with sampling time ($P = 0.036$), concentrations being higher at 4 h post-feeding than pre-feeding and 8 h post-feeding periods (Fig. 1B). The fermentation profile analysis showed Diet × Time interaction ($P < 0.05$) for the molar proportion of acetate, propionate, and for the acetate to propionate (A/P) ratio, whereas the molar proportions of the other VFA showed no Diet × Time interaction ($P > 0.05$) (Table 2). No effects of sampling time on the molar proportions of VFA were observed ($P > 0.05$). Moreover, there were diet effects on the molar proportion of acetate, propionate, valerate, and on the A/P ratio ($P < 0.05$; Table 2). The molar proportion of acetate increased between 0 h and 4 h for the NIT diet, whereas in the same period it decreased for CTR diet, hence at 4 h post-feeding, the acetate molar proportion was much higher for the NIT than for the CTR diet (Fig. 1C). For the same period of sampling, the molar proportion of propionate showed opposite changes than those for acetate, hence at 4 h post-feeding, propionate molar proportion was higher for the CTR than the NIT diet (Fig. 1D). The A/P ratio in the NIT diet increased from 0 h to 4 h post-feeding, hence, at 4 h post-feeding it was much higher than for the CTR diet ($P < 0.05$).

3.3. Temporal changes of ruminal microbiota using qPCR

Feeding the NIT diet led to a significant decrease in the bacterial population ($P = 0.001$; Fig. 2A). Likewise, a time sampling effect was observed ($P = 0.002$), but no Diet × Time interaction effect was observed ($P = 0.353$) (Table 3). The methanogens population showed a Diet × Time interaction ($P < 0.013$) (Table 3). Compared to the CTR diet, the NIT feeding decreased the methanogens population, by 0.31 and 0.42 log$_{10}$ at 4 and 8 h post-feeding, respectively (Fig. 2B). The total fungal population showed a Diet × Time interaction ($P = 0.003$) (Table 3). The fungal population increased at 4 h and 8 h post-feeding of the NIT diet in comparison to the CTR diet (Fig. 2C). In addition, feeding the NIT diet increased significantly the population of ciliated protozoa ($P = 0.001$; Fig. 2D) and Fibrobacter succinogenes ($P = 0.001$; Fig. 3A) compared to the CTR diet. However, neither Diet × Time interaction nor time sampling effects were observed ($P > 0.05$).

The populations of Ruminococcus albus, Ruminococcus flavefaciens, and Veillonella parvula showed no Diet × Time interaction ($P > 0.05$). However, feeding the NIT diet decreased the populations of R. albus and R. flavefaciens compared to the CTR diet ($P < 0.05$; Fig. 3B and C). No differences were observed between dietary treatments for the population of V. parvula ($P = 0.349$) and no sampling time effect was observed either ($P = 0.110$). The population of Selenomonas ruminantium showed a Diet × Time interaction ($P = 0.046$), the population was much higher for the CTR than for the NIT diet at the pre-feeding and 4 h post-feeding periods (Fig. 3D).

3.4. Diversity and composition of bacterial communities

A total of 2,783,124 bacterial and archaeal sequences were obtained as a result of the filtering analysis from 30 samples. The results revealed that the richness indices (Chao1 and ACE) and alpha-diversity estimators (Shannon and Simpson) of the ruminal bacteria did not differ between the dietary treatments ($P > 0.05$) or during the 3 evaluation times ($P > 0.05$) (Appendix Table 3). The beta-dispersion analysis showed differences between dietary treatments (Appendix Fig. A). However, the beta-diversity analysis of bacterial communities revealed significant differences between the dietary treatments ($P < 0.001$); and showed a distinct

---

**Table 1**

Feed dry matter intake (DMI), average liveweight (LW) and methane emissions from Holstein calves fed a control diet (CTR) and a nitrate-containing diet (NIT).

| Item                      | Diet  | SEM | P-value |
|---------------------------|-------|-----|---------|
|                          | CTR   | NIT |         |
| DMI $^{1}$, kg of DM/d    | 8.8   | 8.2 | 0.23    | 0.117   |
| Initial LW, kg            | 213.9 | 214.2| 4.76   | 0.960   |
| Final LW, kg              | 267.6 | 265.8| 5.81   | 0.832   |
| DMI $^{1}$, kg of DM/d    | 8.0   | 7.3 | 0.37    | 0.086   |
| CH$_4$ emission, g/d      | 242.7 | 178.0| 15.33  | 0.009   |
| CH$_4$ yield, g/kg of DMI | 30.8  | 24.4 | 2.16   | 0.041   |

$^{1}$ DMI = dry matter.

$^{2}$ DMI ad libitum.

$^{3}$ DMI restricted (95%) during the methane measurement period.

---

**Table 2**

Effects of diet, time of collection of sample and their interaction on ruminal pH and fermentation parameters in ruminal fluid collected from Holstein calves fed a control diet (CTR) and a nitrate-containing diet (NIT).

| Item                      | Diet  | SEM | P-value |
|---------------------------|-------|-----|---------|
|                          | CTR   | NIT |         |
| pH                        | 6.8   | 6.8 | 0.03    | 0.753   |
| NH$_3$–N, mg/dL           | 8.5   | 10.2| 0.35    | 0.047   |
| Total VFA, mmol/L         | 236.2 | 228.4| 8.23   | 0.253   |
| Fermentation profile, %   |       |     |         |
| Acetate                   | 69.6  | 75.3| 0.55    | 0.009   |
| Propionate                | 20.9  | 14.9| 0.49    | 0.100   |
| Butyrate                  | 6.2   | 6.9 | 0.19    | 0.198   |
| Iso-butyrate               | 1.3   | 0.8 | 0.13    | 0.115   |
| Valerate                  | 0.6   | 0.4 | 0.02    | 0.037   |
| Iso-valerate              | 1.3   | 1.4 | 0.05    | 0.748   |
| A/P ratio                 | 3.7   | 5.4 | 0.14    | 0.005   |

D × T = Diet × Time interaction; NH$_3$–N = ammonia nitrogen; VFA = Volatile fatty acids; A/P = acetate to propionate ratio.

---
separation in the analysis of NMDS plot between the dietary treatments (Fig. 4). As regards the time factor, no separation was observed between the 3 levels, and no significant differences were found ($P = 0.996$).

A total of 24 bacterial and archaeal phyla were identified. The 12 most abundant bacterial phyla found in both treatments and in the 3 evaluation times were those summarized in Table 4 and Fig. 5. The Euryarchaeota phyla was the only one identified within the...
archaeal community, and the sequences that were not classified under any phylum (other) in the dietary treatments and during the 3 evaluation times were on average (7.6 ± 0.96)% and (7.4 ± 1.54)% (mean ± standard deviation) in the CTR diet and the NIT diet, respectively. The Firmicutes and Bacteroidetes phyla were the most abundant ones, accounting for 84.1% and 85.7% of the total bacterial phyla in the CTR diet and the NIT diet, respectively. Likewise, changes in the relative abundance of Firmicutes and Bacteroidetes phyla were variable among animals within each dietary treatment, i.e., the coefficient of variation (CV) in the pre-feeding period ranged from 5.9% to 13.9%. In contrast, in the 4 h and 8 h post-feeding periods, the CV ranged from 6.1% to 26.3% (Fig. 5). However, the relative abundance of these dominant phyla, that of the Tenericutes, WPS-2, Cyanobacteria, Euryarchaeota phyla, and the Firmicutes and Bacteroidetes ratio (F/B) did not result in a Diet × Time interaction and neither did they differ between dietary treatments (P > 0.05).

In turn, no interaction was observed for the Proteobacteria, Spirochaetes, and Synergistetes phyla (P > 0.05), but their abundance decreased as a result of feeding the NIT diet (P < 0.05). The TM7 and Fibrobacteres phyla did not show any interaction either (P > 0.05), but their abundance increased as a result of feeding the NIT diet compared to the CTR diet. Moreover, a significant effect of sampling times was observed on the relative abundance of TM7, Cyanobacteria, and Euryarchaeota (P < 0.05). However, the Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, Tenericutes, WPS-2, Synergistetes, and Fibrobacteres phyla and the F/B ratio did not differ among the sampling times (P > 0.05). An interaction of Diet × Time (P < 0.05) was shown for Actinobacteria and Verrucomicrobia. Compared to the CTR diet, feeding the NIT diet increased the relative abundance of Actinobacteria at 0 h (P < 0.05), whereas, the relative abundance of Verrucomicrobia was increased at 4 and 8 h post-feeding (P < 0.05) (Table 4).

Feeding the NIT diet reduced the ruminal abundance of Succinivibrio, Butyryrivibrio, and Mogibacterium genera, compared to the CTR diet (P < 0.05). This contrasts with the abundance of Atopobium, Erysipelotrichaceae_L7A_E11, and Fibrobacter, which increased in the NIT diet (P < 0.05). However, no differences were found between dietary treatments for the Prevotella, Ruminococcus, Butyryrivibrio, Clostridium, Oscillospira, Bulleidia, Moryella, Streptococcus, Schwartia, Lactobacillus, and Mogibacterium genera (P > 0.05). On the other hand, significant changes were observed as a result of the sampling times, as the abundance of the genera Pseudobutyryrivibrio, Butyryrivibrio, Clostridium, and Mogibacterium decreased and then recovered. In contrast, the abundance of the genus Ruminococcus increased at 4 h post-feeding (P < 0.05). Furthermore, a Diet × Time interaction was observed (P < 0.05) for the Atopobium and Erysipelotrichaceae L7A_E11 genera. The abundance of Atopobium was higher for the NIT diet than for the CTR diet.

### Table 3

Effect of diet, time collection and their interaction on ruminal microbiota (copies log10/g) in ruminal fluid collected from Holstein calves fed a control diet (CTR) and a nitrate-containing diet (NIT).

| Item                      | Diet   | SEM   | P-value |
|---------------------------|--------|-------|---------|
| Total bacteria            | CTR    | 11.8  | 0.03    | 0.001   | 0.002   | 0.353   |
| Methanogens               | CTR    | 8.4   | 0.03   | 0.048   | 0.001   | 0.013   |
| Fungi                     | CTR    | 4.8   | 0.12   | 0.001   | 0.122   | 0.003   |
| Protozoa, log10/mL.       | CTR    | 4.8   | 0.04   | 0.001   | 0.057   | 0.140   |
| Fibrobacter succinogenes  | CTR    | 6.4   | 0.07   | 0.001   | 0.460   | 0.747   |
| Ruminococcus albus        | CTR    | 7.4   | 0.06   | 0.001   | 0.003   | 0.182   |
| Ruminococcus flavefaciens | CTR    | 9.1   | 0.05   | 0.001   | 0.051   | 0.061   |
| Selenomonas ruminantium   | CTR    | 8.5   | 0.06   | 0.001   | 0.160   | 0.046   |
| Veillonella parvula        | CTR    | 5.3   | 0.08   | 0.349   | 0.110   | 0.800   |

**D × T** – Diet × Time interaction.

---

**Fig. 3.** Temporal changes of ruminal microbial populations (mean ± standard error) in ruminal fluid collected from Holstein calves fed a control diet (CTR; grey line) and a nitrate-containing diet (NIT; black line). (A) Fibrobacter succinogenes; (B) Ruminococcus albus; (C) Ruminococcus flavefaciens; (D) Selenomonas ruminantium. 0 h: Pre-feeding; 4 h: 4 h post-feeding; 8 h: 8 h post-feeding. Significance level: *, P ≤ 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
only in the pre-feeding period \((P < 0.05)\); whereas, the abundance of genus \(L7A_E11\) from the \(Erysipelotrichaceae\) family was higher at 0 and 4 h post-feeding periods for the NIT diet than for the CTR diet \((P < 0.05)\).

3.5. Correlation between methane emission, ruminal fermentation, and ruminal microbiota

In order to estimate the degree of association between enteric \(\text{CH}_4\) emission, ruminal fermentation and ruminal microbiota, a correlation analysis was performed for both dietary treatments at 0 h (pre-feeding period). In the CTR diet (Fig. 6A), the \(\text{CH}_4\) emission and yield were not significantly correlated with any member of the ruminal microbiota. The proportion of acetate was positively correlated with the \(\text{Tenericutes}\) phylum \((r = 0.90, P < 0.05)\) and negatively correlated with the \(\text{F/B}\) ratio \((r = -0.90, P < 0.05)\), \(\text{Shuttleworta}\) \((r = -0.90, P < 0.05)\) and \(\text{Selenomon}\) \((r ≥ -0.90, P < 0.001)\). Propionate was negatively correlated with \(\text{Cyanobacteria}\) \((r = -0.90, P < 0.05)\) and \(\text{Erysipelotrichaceae L7A_E11}\) \((r = -0.90, P < 0.05)\) and positively correlated with \(\text{Actinobacteria}\) \((r = 0.90, P < 0.05)\) and \(\text{Fibrobacter}\) \((r = -0.90, P < 0.05)\). Methane yield was positively correlated with \(\text{TM7}\) \((r = 0.90, P < 0.05)\) and \(\text{Succinivibrio}\) \((r = -0.90, P < 0.05)\) and negatively correlated with \(\text{Prevotella}\) \((r = -0.90, P < 0.05)\) and \(\text{Succinivibrio}\) \((r = -0.90, P < 0.05)\). The total VFA concentration was positively correlated with \(\text{Succinilasticum}\) \((r = 0.90, P < 0.05)\) and negatively correlated with \(\text{Shuttleworta}\) \((r = -0.90, P < 0.05)\). The \(\text{NH}_3-N\) concentration was positively correlated with \(\text{Sporochaete}\) \((r = 0.90, P < 0.05)\) and \(\text{Treponema}\) \((r = 0.90, P < 0.05)\). Ruminal pH was positively correlated with \(\text{Bacteroides}\) \((r ≥ 0.90, P < 0.001)\) and \(\text{Prevotella}\) \((r = 0.90, P < 0.05)\), and negatively correlated with the \(\text{F/B}\) ratio \((r = -0.90, P < 0.05)\), \(\text{Coprocooc}\) \((r ≥ -0.90, P < 0.001)\) and \(\text{Erysipelotrichaceae L7A_E11}\) \((r = -0.90, P < 0.05)\).

4. Discussion

4.1. Feed intake, methane emission, andmethanogen population

Dry matter intake was not affected when nitrate was included in the diet of dairy calves at 15 g/kg of DM. This is in agreement with findings from previous studies (Hulshof et al., 2012; Olijhoek et al., 2016). However, recent studies by Meller et al. (2019) and Rebole et al. (2019) reported a decrease of around 8.0% in DMI for...
inclusions of nitrate at similar levels of inclusion. This decrease in DMI was attributed, initially, to nitrate toxicity (evidenced by increased blood methemoglobin level \(>20\%\) of total hemoglobin), when animals received diets >1% (on DM basis) of nitrate and without considering a scheme of dietary acclimatization (Bruning-Fann and Kaneene, 1993). Likewise, when the methemoglobinemia incidence was controlled, by gradual acclimatization or by using protected nitrate, the reduction in DMI was explained mainly due to the organoleptic properties of nitrate (bitter taste), both in cattle (Newbold et al., 2014; Lee et al., 2015) and in sheep (Li et al., 2012). Therefore, it seems that a gradual acclimatization scheme to nitrate is key to maintain DMI levels without affecting animal performance (van Zijderveld et al., 2011b) and without compromising animal health (Ortiz-Chura et al., 2021).

Methane yield was reduced by 21% when nitrate was included in the diet of dairy calves. This result agrees with findings from Fig. 5.

Table 5

| Item                      | Diet   | Time | SEM | P-value | Diet | Time | D × T |
|---------------------------|--------|------|-----|---------|------|------|-------|
| Group of diminished bacteria by NIT diet |        |      |     |         |      |      |       |
| Succinivibrio             | 1.90   | 0.59 | 1.19| 1.16    | 1.39 | 0.069| 0.004 | 0.354 | 0.132 |
| Treponema                 | 1.31   | 0.66 | 1.10| 0.89    | 0.97 | 0.315| 0.015 | 0.167 | 0.848 |
| Shuttleworthia            | 1.07   | 0.20 | 0.68| 0.56    | 0.65 | 0.117| 0.007 | 0.052 | 0.591 |
| Succinivibrio             | 0.84   | 0.01 | 0.34| 0.57    | 0.37 | 0.106| 0.006 | 0.603 | 0.279 |
| Pseudobutyrvibrio         | 0.58   | 0.04 | 0.41| 0.16    | 0.36 | 0.036| 0.002 | 0.016 | 0.056 |
| Sharpes                   | 0.32   | 0.04 | 0.14| 0.22    | 0.18 | 0.032| 0.010 | 0.642 | 0.185 |
| Selenomonas               | 0.22   | 0.10 | 0.13| 0.13    | 0.21 | 0.023| 0.024 | 0.270 | 0.271 |
| Group of increased bacteria by NIT diet |        |      |     |         |      |      |       |
| Atopobium                 | 0.18   | 0.52 | 0.55| 0.36    | 0.13 | 0.052| 0.023 | 0.015 | 0.008 |
| Erysipelotrichaceae_L7A_E11| 0.08  | 0.21 | 0.20| 0.10    | 0.13 | 0.011| 0.010 | 0.005 | 0.010 |
| Fibrobacter               | <0.01  | 0.08 | 0.04| 0.02    | 0.06 | 0.009| 0.003 | 0.240 | 0.192 |
| Group of bacteria unchanged by NIT diet |        |      |     |         |      |      |       |
| Prevotella                | 17.16  | 16.68| 15.79| 17.40   | 17.58| 0.583| 0.856 | 0.409 | 0.778 |
| Ruminococcus             | 6.18   | 7.34 | 4.71| 9.66    | 5.90 | 0.530| 0.483 | 0.004 | 0.511 |
| Butyrivibrio             | 3.55   | 4.59 | 5.08| 3.42    | 3.72 | 0.254| 0.207 | 0.037 | 0.065 |
| Ruminococcace_Clostridium| 0.92   | 0.99 | 0.96| 0.89    | 1.02 | 0.110| 0.804 | 0.011 | 0.823 |
| Oscillospira              | 0.79   | 0.80 | 0.74| 0.80    | 0.84 | 0.093| 0.971 | 0.920 | 0.828 |
| Butyricella               | 0.65   | 0.42 | 0.49| 0.77    | 0.35 | 0.143| 0.886 | 0.133 | 0.164 |
| Moryella                 | 0.13   | 0.19 | 0.19| 0.12    | 0.18 | 0.018| 0.132 | 0.277 | 0.572 |
| Streptococcus             | 0.07   | 0.07 | 0.07| 0.08    | 0.06 | 0.005| 0.642 | 0.185 | 0.262 |
| Schwartzia               | 0.07   | 0.02 | 0.05| 0.03    | 0.06 | 0.004| 0.065 | 0.088 | 0.417 |
| Lactobacillus             | 0.07   | 0.02 | 0.05| 0.04    | 0.04 | 0.016| 0.090 | 0.535 | 0.067 |
| Mogibacterium            | 0.04   | 0.06 | 0.07| 0.05    | 0.03 | 0.005| 0.256 | 0.020 | 0.184 |

**Fig. 5.** Ruminal relative abundance of the most abundant phyla in individual ruminal fluid collected from Holstein calves fed a control diet (CTR; \(n = 5\)) and a nitrate-containing (NIT; \(n = 5\)) diet. During 3 sampling times; 0 h: pre-feeding; 4 h: 4 h post-feeding; 8 h: 8 h post-feeding.
previous studies showing a reduction of between 16% and 25% with inclusion levels between 13 and 21 g of NO₃/kg of DM (Klop et al., 2016; Lund et al., 2014; Olijhoek et al., 2016; van Zijderveld et al., 2011a). In contrast, Meller et al. (2019) and Rebelo et al. (2019) reported that although the CH₄ emission was decreased as a result of dietary nitrate, no differences in CH₄ yield were observed. This lower CH₄ emission and null effect on CH₄ yield were mainly attributed to lowered DMI by nitrate effect because feed intake is positively associated with enteric CH₄ emissions (Hristov et al., 2018). However, in the present study, the lower CH₄ emission is not related to DMI variation, as it did not differ significantly among dietary treatments. Therefore, the lower CH₄ emission would be mainly related to the mode of action of nitrate at the ruminal level.

In the rumen, the reduction of NO₃ to NH₃ is energetically more favorable than carbon dioxide reduction. Stoichiometrically, the addition of 1 mol of NO₃ reduces by 1 mol the CH₄ production, which is equivalent to 28.4 g of CH₄/100 g of NO₃ (Latham et al., 2016). Thus, assuming a complete reduction of NO₃, the addition of 15 g of calcium nitrate (equivalent to 11.3 g NO₃/kg of DM) would have a reduction potential of 2.9 g CH₄/kg of DM. However, the CH₄ reduction observed in the present study (6.4 g/kg of DM) was much higher than the stoichiometric expectation. Reported CH₄ reduction efficiencies greater than 100% of those from the stoichiometric expected value (Zhang et al., 2019), and increasing H₂ concentration (gaseous and dissolved) after feeding dietary nitrate (Guyader et al., 2015; Olijhoek et al., 2016), suggest that nitrate may not act only as an effective H₂ sink. In this sense, the toxic effects of nitrate-reducing metabolites (e.g., nitrite, nitrate esters, nitric oxide, and nitrous oxide) on ruminal methanogen growth seem to be more important because this inhibitory effect was previously reported in vitro (Iwamoto et al., 2002) and in vivo studies (Asanuma et al., 2015). In addition, some studies suggest that hydrogenotrophic methanogens such as Methanobrevibacter spp. would be very sensitive to dietary nitrate (Bowen et al., 2020). Although the direct and indirect effects on methanogens and other members of the ruminal microbiota remain unclear, these inhibitory effects may result from the oxidizing nature of nitrite in relation to its antimicrobial properties (Cammack et al., 1999; Marais et al., 1988).

In the present study, the decrease in CH₄ emissions and CH₄ yield as a result of NIT diet feeding seems not only due to thermodynamic competition for H₂ but also to the inhibition of the methanogen population (mcrA; a conserved gene involved in methanogenesis) observed at 4 and 8 h post-feeding. Similar results in mcrA copy reduction were reported in dairy cows fed nitrate (Veneman et al., 2015). In the same line, Granja-Salcedo et al. (2019) and Bowen et al. (2020) revealed that lower CH₄ emissions in beef cattle fed nitrate were associated with a smaller Euryarchaeota to bacteria ratio and a lower abundance of M. spp.

4.2. Ruminal fermentation and microbiota

Ruminal pH usually decreases after feeding and then gradually increases due to VFA absorption, rumination, and salivation (Aschenbach et al., 2011). This pattern of change in pH was also observed in the present study, for a diet low in forage to
concentrate ratio (20:80). It is well known (e.g., van Lingen et al., 2017) that following feeding, the molar proportion of acetate decreases and then recovers, whereas the molar proportion of propionate increases and then decreases. In this study, the described patterns of change in molar proportions of VFA were observed in the CTR diet. On the other hand, feeding the NIT diet altered the fermentation profile in a contrasting pattern; i.e., an increase in the molar proportion of acetate and a decrease in propionate molar proportion (at 4 h post-feeding period). This latter profile was also observed in in vitro (Lee et al., 2017) and in vivo (Hulshof et al., 2012; Olijhoek et al., 2016; Troy et al., 2015) studies, when nitrate was included in the diet.

Propionate synthesis is thermodynamically considered a more favorable pathway under high H₂ pressure conditions (typically after feeding) (Janssen, 2010), and can sometimes be stimulated when methanogenesis is inhibited (Wang et al., 2018). In this study, although the concentration of dissolved H₂ was not evaluated, the results suggest that the available H₂ was not incorporated into the propiogenes either, as the proportion of propionate decreased when fed the NIT diet. Therefore, it might be suggested that changes in the fermentation profile might be more related to the toxic effects of the nitrate-reduction metabolites on bacterial populations involved in propionate synthesis than on changes in metabolic hydrogen flow.

Relative abundances of Succinivibrio, Coprococcus, Treponema, Shuttleworthia, Succinivibrio, Pseudobutyribivibrio, Sharpea, and Selenomonas were decreased when nitrate was included in the diet of dairy calves. These findings are unprecedented because, on the one hand, previous studies that reported a reduction in propionate synthesis did not evaluate the effects of nitrate feeding on the ruminal microbiota. On the other hand, studies that evaluated the effects of nitrate on the ruminal microbiota did not show changes in propionate synthesis. Furthermore, our results suggest that this detrimental effect on bacterial abundance could be associated with the lower propionate production because the conversion of pyruvate to propionate involves the acrylate and the succinate pathways. For instance, genes encoding these metabolic pathways were identified in the Succinivibrio dextrinosolvens genome (Hackmann et al., 2017; Hailemariam et al., 2020), which participates in the active synthesis of succinate (O’Herrin and Kenealy, 1993). In addition, this study and another study conducted by Ren et al. (2019) observed that Succinivibrio was positively correlated with propionate.

Another bacteria which could participate in the succinate pathway through the production of succinate, formate, and acetate is Treponema bryanti (Stanton, 1984), but in this study, the Treponema genus was not correlated to the production of propionate. Concomitantly, genes that participate in the synthesis of propionate through the succinate pathway were identified in the genome of some species of the Selenomonas genus (Hackmann et al., 2017; Wang et al., 2020). In turn, Succinivibrio ruminantis has been identified as an active participant in the conversion of succinate to propionate (van Gyllswyk, 1995). In addition, this study revealed a strong correlation between the abundance of Selenomonas and Succinivibrio and the production of propionate. Furthermore, it was determined that the Sharpea spp. (Kumar et al., 2018) and Pseudobutyribivibrio xylanovorans bacteria (Palevich et al., 2020) produce lactate, formate, and butyrate as a final product of glucose fermentation. It was also determined that Coprococcus participates in the production of propionate through the acrylate pathway using lactate (Reichardt et al., 2014). However, in the present study, no strong correlations were found between Coprococcus and propionate production.

On the other hand, the relative abundance of bacteria of the genus Atopobium (from Actinobacteria phyla) increased in response to the feeding the NIT diet. Also, the genus Atopobium was found to be a prevalent member of the human gut microbiota or ruminal microbe (Harmsen et al., 2000). In the rumen, the genus Atopobium was described as a member of the epimural community (Chen et al., 2011), and their abundance increase under high-concentration diets (>70%) (Mao et al., 2013; Petri et al., 2013). In turn, the L7a_E11 genus was phylogenetically categorized within the Erysipelotrichaceae family. The genome of this bacterium was recently sequenced from the metagenome of beef cattle (Stewart et al., 2018). Although very little is known about these bacteria, the relative abundance of the Erysipelotrichaceae family has been associated with the energy and protein metabolism of animal gut microbiota (Bermingham et al., 2017). Some previous studies on beef cattle showed a positive correlation between the abundance of L7a_E11 and total VFA concentration (Bi et al., 2018), and with the level of muscle marbling (Kim et al., 2020). However, their role in the rumen remains unknown.

The ruminal population of R. albus and R. flavafaciens decreased, whereas the population of F. succinogenes increased when nitrate was included in the diet of dairy calves. However, these results were contradictory to the findings of previous studies (Iwamoto et al., 2002; Asanuma et al., 2015) because these studies revealed a reduction in the populations of these fibrolytic bacteria, whereas other studies showed no change, except for F. succinogenes, which increased (Granja-Salcedo et al., 2019). The reason for these contradictory findings could be related to the technique used in the different studies (in vitro vs. in vivo), sampling times (pre- and post-feeding periods), ruminal sampling technique (ruminal cannula vs. stomach tubing), DNA extraction method, ruminal fraction used (liquid, solid or mixture), among others. Similarly, the basal diet used in previous studies seems to play a crucial role because, in recent studies, when nitrate was used as a non-protein nitrogen source in low-quality diets, it was shown that higher fiber digestibility was associated with higher R. albus and total protozoa population (Zhang et al., 2019). However, our findings could partly explain the lower CH₄ emissions for the NIT diet because the genus Ruminococcus has been considered an H₂ producer, but this is not the case for R. succinogenes (Chaucheys-Durand et al., 2010). Furthermore, F. succinogenes was found in higher density in low CH₄-emitting beef cattle phenotypes (Tapio et al., 2017) and, in the present study, it was negatively correlated with CH₄ production, suggesting an indirect effect on CH₄ emission for the NIT diet.

The population of the genus Selenomonas and the species S. ruminantium decreased, but there was no change in the population of V. parvula in response to the NIT diet. The results were contradictory with findings from previous studies (Granja-Salcedo et al., 2019; Iwamoto et al., 2002; Zhao et al., 2018), although another study showed no changes in the populations of nitrate-reducing bacteria when nitrate was included in the diet (Popova et al., 2017). The reason for these differences might be related to the methodology applied in the different studies (in vitro vs. in vivo), and sampling times (pre- and post-feeding), among others. We recommend furthering these studies using metatranscriptomic tools to more deeply understand the nitrate- and nitrite-reduction process in the rumen.

Ciliated protozoa populations increased significantly as a result of feeding the NIT diet, which is in line with the results obtained in goats (Zhang et al., 2019) and in vitro studies (Lin et al., 2011). Furthermore, in a recent in vivo experiment with defaunated and faunated sheep supplemented with 1.8% of nitrate (on a DM basis), it was reported that nitrate metabolizes more rapidly in the presence of ruminal protozoa, and defaunated sheep may have an increased risk of poisoning due to nitrite accumulation in the rumen (Villar et al., 2020). In contrast, Iwamoto et al. (2001) observed that nitrite inhibited the growth of protozoa, and
recently it was determined that nitrite interfered with the motility and chemotaxis of protozoa (mainly on *isotrichids*) in *in vitro* cultures (Roman-García et al., 2019). In other studies, in sheep (van Zijderveld et al., 2010) and cattle (Popova et al., 2017) the protozoan population was not influenced by nitrate supplementation.

Previous studies suggested a smaller relationship between the fungal population and nitrate metabolism (Lin et al., 2011; Zhang et al., 2010) and cattle (Popova et al., 2017) the protozoan population was not in adults due to the status of rumen maturity, and the development and establishment of ruminal microbiota (Jami et al., 2013).

The results confirmed the hypothesis that nitrate feeding induced changes in the populations of total bacteria, methanogens, ciliate protozoa, total fungi, fibrobacteria, and bacteria involved in the succinate and acrylate pathways, thus, these changes were associated with decreased enteric CH$_4$ emission and altered ruminal fermentation profiles.

5. Conclusions

This study, in dairy calves, confirmed the ability of inclusion of nitrate in the diet to reduce enteric CH$_4$ emission and CH$_4$ yield without significantly affecting feed intake. The lower enteric CH$_4$ emission in response to nitrate feeding was over the stoichiometrically predicted response potential and also associated with a lower density of total methanogen population, suggesting a predominantly direct effect on ruminal methanogenesis. The decrease in the molar proportion of propionate in ruminal fluid in response to nitrate was associated with a reduction in the relative abundance of the genera *Succinivibrio, Coprococcus, Treponema, Shuttleworthia, Succinivibrion, Pseudobutyribrio, Sharpea, and Selenomonas* genera.

Author contributions

**Abimael Ortiz-Chura**: Conceptualization, Methodology, Investigation, Software, and Writing- Original draft preparation. **Gisela Marcoppido**: Investigation, Resources. **Gustavo Depetri**: Investigation, Methodology. **José Gere**: Formal analysis, Data curation, Funding acquisition. **Claudia Faverin**: Investigation, Software. **Cesar Pinares-Patino**: Writing, Review, and Editing. **Silvio Cavero**: Resources, Visualization. **Ángel Cataldi**: Supervision, Validation. **María E. Cerón-Cucchi**: Investigation, Funding acquisition, Writing, Review, and Editing.

Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This study was supported by a grant from the FonCyT-Argentina: PICT 2015-294, PID MS-UTNBA-0004540 and INTA 1058. We wish to thank Dra Milka Popova (from INRAE-France) for the support in the bioinformatics analysis. We also thank Eng. Ricardo Bualo and Ricardo Arias for their help and technical support in methane emission measurements.

Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.07.003.

References

Anderson MJ. Distance-based tests for homogeneity of multivariate dispersions. Biometrics 2006;62(1):245–53.

AOAC. Official methods of analysis, 15 ed. 1990. Washington, USA.

Armstrong DG, Blaxter KL. The utilization of acetic, propionic and butyric acids by fattening sheep. Br J Nutr 1957;11(4):413–25.

Asanuma N, Yokoyama S, Hino T. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to Selenomonas ruminantium having the ability to reduce nitrate and nitrite. Anim Sci J 2015;86(4):384–378.

Aschenbach JR, Penner GB, Stumpfl F, Gabel G. Ruminant Nutrition Symposium: role of fermentation acid absorption in the regulation of ruminal pH. J Anim Sci 2011;89(4):1092–107.

Beauchemin KA, Ungerfeld EM, Eckard BJ, Wang M. Review: fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. Animal 2020. https://doi.org/10.1002/anim.1216.

Bermingham EN, Maclean P, Thomas DG, Cave NJ, Young W. Key bacterial families (*Clostridiaceae*, *Erysipelotrichaceae* and *Bacteroidaceae*) are related to the digestion of protein and energy in dogs. PeerJ 2017. https://doi.org/10.7717/ peerj.3019.

Bi Y, Zeng S, Zhang R, Diao Q, Tu Y. Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. BMC Microbiol 2018;18(1):59.

Bowen JM, Cormican P, Lister SJ, McCabe MS, Duthie CA, Roehe R, et al. Links between the rumen microbiota, methane emissions and feed efficiency of finishing steers offered dietary lipid and nitrate supplementation. PLoS One 2020. https://doi.org/10.1371/journal.pone.0231759.

Bruning-Fan San CS, Kaneene JB. The effects of nitrate, nitrite, and N-nitrosocompounds on animal health. Vet Hum Toxicol 1993;35(3):237–53.

Cammack R, Joannou CL, Cui XY, Torres Martinez C, Maraj SR, Hughes MN. Nitrite and nitrosyl compounds in food preservation. Biochim Biophys Acta Bioenerg 1999;1411(2):475–88.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 2009;26(2):266–7.

Chaucheyras-Durand F, Masséglia S, Fonty G, Forano E. Influence of the composition of the cellulolytic flora on the development of hydrogenotrophic microorganisms, hydrogen utilization, and methane production in the rumens of gnotobiotically reared lambs. Appl Environ Microbiol 2010;76(24): 7937–7931.

Chen Y, Penner GB, Li M, Oba M, Guan LL. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. Appl Environ Microbiol 2011;77(16):5781/5770.

Dehority BA. Laboratory manual for classification and morphology of rumen ciliate Protozoa. 1st ed. CRC Press; 1993.

FAOSTAT. Food and agriculture organization of the united nations (FAO). 2020. http://www.fao.org/faostat. [Accessed 20 August 2020].

Friggens NC, Oldham JD, Dewhurst RJ, Horgan G. Proportions of volatile fatty acids in relation to the chemical composition of feeds based on grass silage. J Dairy Sci 1998;81(5):1331–44.

Gerber PJ, Steinfeld H, Henderson B, Mottet A, Opio C, Dijkman J, et al. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. Rome: Food and Agriculture Organization of the United Nations (FAO); 2013a.

Gerber PJ, Hirabayashi AN, Henderson B, Makkar H, Oh J, Lee C, et al. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 2013b;7(2):220–34.

Gere J, Gratton R. Simple, low-cost fly monitoring and slaughter sampling and other applications. Latin American Applied Research Pesquisa Aplicada Latino Americana Investigación Aplicada Latinoamericana 2010;40:377–81.

Gere JI, Bualó RA, Perini AL, Arias RD, Ortega FM, Wulf AE, et al. Methane emission factors for beef cows in Argentina: effect of diet quality. N Z J Agric Res 2019: 9–1.

Granja-Salcedo YT, Fernandes RM, de Araujo RC, Kishi LT, Berchielli TT, de Resende FD, et al. Long-term encapsulated nitrate supplementation modulates rumen microbial diversity and rumen fermentation to reduce methane emission in grazing steers. Front Microbiol 2019;10:614.

Guyader J, Eugène M, Meunier B, Doreau M, Morgavi DP, Silberberg M, et al. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J Anim Sci 2015;93(7):3564–77.
Lee C, Araujo RC, Koenig KM, Beauchemin KA. In situ and in vitro evaluations of a Latham EA, Anderson RC, Pinchak WE, Nisbet DJ. Insights on alterations to the MacRae JC, Armstrong DG. Enzyme method for determination of Lund P, Dahl R, Yang HJ, Hellwing ALF, Cao BB, Weisbjerg MR. The acute effect of Newbold JR, van Zijderveld SM, Hulshoff RBA, Fokkink WB, Leng RA, Terencio P, et al. Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methano- Lin M, Schaefer DM, Guo WS, Ren LP, Meng QX. Comparisons of in vitro nitrate Iwamoto M, Asanuma N, Hino T. Effect of protozoa on nitrate and nitrite reduction Hailemariam S, Zhao S, Wang J. Complete genome sequencing and transcriptome analysis of nitrogen metabolism of Succinivibrio dextrinosolvens strain Z6 isolated from dairy cow rumen. Front Microbiol 2020. https://doi.org/10.3389/ Harmens HJ, Wildboer-Veloo AC, Grijspeer J, Knol J, Degener JE, Welling GW. Development of 16S rDNA-based probes for the Coriobacteriaceae group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces: development of antibodies for different age groups. Appl Environ Microbiol 2000;66(10):4523–7.

Hellwing ALF, Lund P, Mogensen L, Vestergaard M. Growth, feed intake, methane emissions and carbon footprint from Holstein bull calves fed four different diets. J Appl Anim Sci 2018;24(1):51–61.

Hristov AN, Kneebread E, Niu M, Oh J, Bannink A, Bayat AR, et al. Symposium review: uncertainties in enteric methane inventories, measurement techniques, and prediction models. J Dairy Sci 2018;101(7):6609–618.

Hulshof RBA, Berndt A, Gerrits WJJ, Dijkstra J, van Zijderveld SM, Newbold JR, et al. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J Anim Sci 2012;90(7):2317–23.

Iwamoto M, Asanuma N, Hino T. Ability of Selenomonas ruminantium, veillonella parvula, and wolinella succinogenes to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. Anaerobe 2002;8(4):209–15.

Iwamoto M, Asanuma N, Hino T. Effect of protozoa on nitrate and nitrite reduction on ruminal microflora. Kanto J Anim Sci 2001;51:9–25.

Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J 2013;7(6):1069–79.

Janssen PH. Influence of inulin on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics.Anim Feed Sci Technol 2010;160(1):1–22.

Johnson K, Huyler M, Westberg H, Lamb B, Zimmerman P. Measurement of methane emissions from ruminant livestock using a sulfur hexaferrilu trace ene. Environ Sci Technol 1994;28(2):359–62.

Kim M, Park T, Jeong JY, Baek Y, Lee HJ. Association between rumen microflora and marbling score in Korean native beef cattle. Animals 2020;10(4):712.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2012;41(1):e1–.

Kloep G, Hatve B, Bannink A, Dijkstra J. Feeding nitrate and dicrocaxyenoacetic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows. J Dairy Sci 2016;99(2):1611–72.

Kumar S, Treolar BP, The KH, McKenzie CM, Henderson G, Attwood GT, et al. Sharpea and Kandleria are lactic acid producing rumen bacteria that do not affects enteric methane production and milk fatty acid composition in lactating Holstein steers and performance in Nelore bulls 1. J Anim Sci 2014;92(11):5032–5.

Latham EA, Anderson RC, Pinchak WE, Nisbet DJ, Insights on alterations to the rumen ecosystem by nitrates and nitrocompounds. Front Microbiol 2016;7: 228.

Lee C, Araujo RC, Koenig KM, Beaucahnkin KA. In situ and in vitro evaluations of a slow release form of nitrate for ruminants: nitrate release rate, ruminant nitrate metabolism and the production of methane, hydrogen, and nitrous oxide. Anim Feed Sci Technol 2015;190(1–3):107–10.

Lee C, Araujo RC, Koenig KM, Beaucahnkin KA. Effects of feed consumption rate of beef cattle offered a diet supplemented with nitrate ad libitum or restrictively on potential toxicity of nitrate. J Anim Sci 2015;93(10):4956–60.

Li L, Davis J, Nolan AG, Sharp AN. A new investigation into rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. Anim Prod Sci 2012;52(7):853–5.

Lin M, Scholz DM, Gao WS, Res LP, Meng QX. Comparisons of in vitro nitrate reduction, methanogenesis, and fermentation acid profile among rumen bacteria. Protopalossil and fungal fractions. Asia-Australas J Anim Sci 2011;24(4):471–8.

Lupi D, Pehle R, Yang HJ, Hellwing ALF, Cao BB, Weisbjerg MR. The acute effect of addition of nitrate on in vitro and in vivo methane emission in dairy cows. Anim Prod Sci 2014;54(9):1432–5.

MacRae JC, Armstrong DG. Enzyme method for determination of α-linked glucose molecules in biological materials. J Sci Food Agric 1968;19(10):578–81.

Mao SY, Zhang RY, Wang DS, Zhu WY. Impact of subacute ruminal acidosis (ARA) adaptation on rumen microflora in dairy cow using pyrosequencing. Anaerobe 2013;24:12–9.

Marais JP, Therion JI, Mackie RI, Kistner A, Denniss C. Effect of nitrate and its reduction products on the growth and activity of the ruminal microbial population. Br J Nutr 1988;59(2):301–13.

Meller RA, Wenner DA, Ashworth J, Gehman AM, Lakritz J, Finkls JK. Potential roles of nitrate and nitrite in enteric methane emissions in ruminal methane emission and improving ruminal fermentation, digestibility, and milk production in lactating Jersey cows. J Dairy Sci 2019;102(7):6144–56.

Morgawi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methano- genesis is in ruminal fermentation and methane mitigation strategies for beef cattle fed two contrasting basal diets 1. J Anim Sci 2015;93(4):1846–57.

Newbold JR, van Zijderveld SM, Hulshoff RBA, Fokkink WB, Leng RA, Terencio P, et al. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nellore bulls 1. J Anim Sci 2014;92(11):5032–40.
van Lingen HJ, Edwards JE, Vaidya JD, van Gastelen S, Saccenti E, van den Bogert B, et al. Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. Front Microbiol 2017. https://doi.org/10.3389/fmicb.2017.00425.

Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74(10):3583–97.

van Zijderveld SM, Gerrits WJJ, Apajalahti JA, Newbold JR, Dijkstra J, Leng RA, et al. Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J Dairy Sci 2010;93(12):5865–66.

van Zijderveld SM, Gerrits WJJ, Dijkstra J, Newbold JR, Hulshof RBA, Perdok HB. Persistence of methane mitigation by dietary nitrate supplementation in dairy cows. J Dairy Sci 2011a;94(8):3094–104.

Veneman JB, Muetzel S, Hart KJ, Faulkner CL, Moorby JM, Perdok HB, et al. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One 2015. https://doi.org/10.1371/journal.pone.0140282.

Villar ML, Hegarty RS, Clay JW, Smith KA, Godwin IR, Nolan JV. Dietary nitrate and presence of protozoa increase nitrate and nitrite reduction in the rumen of sheep. https://doi.org/10.1111/j.1365-2052.2020.12181.

Wang L, Zhang G, Li Y, Zhang Y. Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. Animals 2020. https://doi.org/10.3390/ani10020223.

Wang R, Wang M, Ungerfeld EM, Zhang XM, Long DL, Mao HX, et al. Nitrate improves ammonia incorporation into rumen microbial protein in lactating dairy cows fed a low-protein diet. J Dairy Sci 2018;101(11):9789–99.

Wickham H. ggplot 2: elegant graphics for data analysis. New York: Springer-Verlag; 2016.

Williams SRO, Moate PJ, Hannah MC, Ribaux BE, Wales WJ, Eckard RJ. Background matters with the SF6 tracer method for estimating enteric methane emissions from dairy cows: a critical evaluation of the SF6 procedure. Anim Feed Sci Technol 2011;170(3):265–76.

Zhang X, Medrano RF, Wang M, Beuchemin KA, Ma Z, Wang R, et al. Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats. J Anim Sci Biotechnol 2019. https://doi.org/10.1186/s40104-019-0312-2.

Zimmerman PR. System for measuring metabolic gas emissions from animals. Patent no. 5 265 618. Alexandria, VA: United States Patent and Trademark Office; 1993.