

EFFECT OF REPLACING RAPESEED MEAL WITH CALCIUM NITRATE ON RUMEN FERMENTATION AND RUMEN MICROBIOME IN DAIRY COWS

Veronika Stejskalová^{1,3}, Kristýna Králová¹, Mariana Vadroňová^{1,2},
Alena Výborná², Michaela Beranová², Miroslav Joch^{1,2}

¹*Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, Czech Republic*

²*Department of Nutrition and Feeding of Farm Animals, Institute of Animal Science, Přátelství 815, 104 00 Prague, Czech Republic*

³*Department of Genetics and Breeding of Farm Animals, Institute of Animal Science, Přátelství 815, 104 00 Prague, Czech Republic*

Corresponding email address: stejskalovav@af.czu.cz

ABSTRACT

Cattle farming is a major agricultural source of methane emissions originating from rumen fermentation. Methane has a high global warming potential, and its production represents an energy loss for ruminants. Nitrate supplementation is a promising mitigation strategy, as nitrates act as alternative hydrogen acceptors in the rumen, reducing methanogenesis and producing ammonia that can serve as a source of non-protein nitrogen. This study aimed to assess the effect of replacing rapeseed meal with calcium nitrate on rumen fermentation and the composition of the rumen microbiome in dairy cows under in vivo conditions. We hypothesized that calcium nitrate would alter the ratio of volatile fatty acids in rumen fluid, increase the concentration of ammoniacal nitrogen in rumen fluid, and influence the structure of the rumen microbial community. Eight Holstein dairy cows were fed two diets in a crossover design for two 21-day periods (14 days of adaptation and 7 days of sampling). The treatments were: 1) silage-based basal diet + rapeseed meal (40 g/kg TMR) (CONTROL) and 2) silage-based basal diet + 100 g nitrate per cow/d (Bolifor CNF 1%) (NITRATE). Nitrate supplementation did not affect dry matter intake ($P = 0.521$) or any fermentation parameters: VFA ($P = 0.342$); pH ($P = 0.421$); $\text{NH}_3\text{-N}$ ($P = 0.761$). Nitrate supplementation affected alpha diversity in the Chao1 richness index ($P = 0.0164$), and the number of ASVs ($P = 0.0153$); other indices (Shannon and Simpson diversity indices and Pielou evenness index) were not statistically significant. At the phylum level, Firmicutes ($P = 0.844$) and Bacteroidota ($P = 0.945$) were dominant with no significant changes



in their relative abundances. No significant differences were observed in the relative abundance of any major bacterial families, including Methanobacteriaceae (Control 6.2 %, Nitrate 3.9 %, $P = 0.039$). In conclusion, calcium nitrate supplementation affected bacterial diversity and the Methanobacteriaceae family.

Keywords: nitrate; methane; emissions; volatile fatty acids; ruminant nutrition

INTRODUCTION

In recent years, the approach to reducing emissions from ruminants has shifted from a narrow focus on enteric methane production to a broader perspective on optimizing the entire feed ration. Modern strategies aim not only to reduce greenhouse gas emissions but also to increase nutrient utilization efficiency and improve animal performance. Methane (CH_4) production during enteric fermentation in dairy cows represents a significant energy loss for the animal, as approximately 6% of the gross energy in the diet is lost in the form of methane; at the same time, it is a major source of greenhouse gas emissions (Ellis et al., 2008). Reducing methane production is therefore considered an effective way to not only lower greenhouse gas emissions from the dairy industry but also to increase the energy efficiency of dairy cows (Johnson & Johnson, 1995). The goal is to balance environmental sustainability with the economic efficiency of livestock production, while maintaining or improving production parameters such as feed intake, nutrient digestibility, and milk or meat production.

Enteric methane originates primarily from the microbial fermentation of feed in the rumen. Here, plant polysaccharides are primarily broken down to form volatile fatty acids, which represent the main source of energy for ruminants. At the same time, however, hydrogen is produced and utilized by methanogens, with methane formed as a byproduct of fermentation (Mizrahi et al., 2021). Various factors influence its production, but adjusting the feed ration composition using feed additives appears to be the most effective strategy for reducing methane emissions (Hristov et al., 2013).

One promising option is the use of nitrates, which serve as an alternative electron acceptor in the rumen. When nitrates are reduced, competition arises with methanogenic archaea for available hydrogen, leading to a redirection of hydrogen from methanogenesis to nitrate reduction. The final product of this reduction is ammonia, which microorganisms can use to synthesize microbial biomass. Their significance, therefore, also lies in partially replacing the nitrogen component of the feed ration (Latham et al., 2016).

The addition of nitrate to the diet may affect not only methane production but also rumen fermentation, the profile of volatile fatty acids, nitrogen metabolism, and the composition of the rumen microbiome (Olijhoek et al., 2016). In particular, the impact on the microbial community remains poorly understood, and changes in its composition can significantly affect not only the intensity of methanogenesis

but also nutrient utilization efficiency and the stability of the rumen environment (Veneman et al., 2015).

This study examined three hypotheses. It was hypothesized that calcium nitrate would alter the ratio of volatile fatty acids in rumen fluid, increasing the proportion of propionic acid at the expense of acetic acid. Furthermore, it was hypothesized that calcium nitrate would increase the concentration of ammoniacal nitrogen in rumen fluid. Finally, it was expected that calcium nitrate would influence the rumen microbial community structure, specifically by suppressing methanogenic archaea and promoting the growth of bacteria involved in nitrate reduction. This study aimed to describe the effects of replacing rapeseed meal with calcium nitrate on rumen fermentation and rumen microbiome composition in dairy cows.

MATERIAL AND METHODS

The animals were treated in accordance with applicable European and Czech legislation (Directive 2010/63/EU on the protection of animals used for scientific purposes, European Union 2010). All procedures involving animals were approved by the Ethics Committee of the Institute of Animal Science (Prague, Czech Republic). The cows were housed in the experimental barn of the Institute of Animal Science (Netluky, Prague, Czech Republic).

Animals, experimental design, and treatment

Eight Holstein dairy cows were included in the experiment (mean $\pm$ standard deviation: 567 $\pm$ 70 kg body weight, parity 3 $\pm$ 1). The experiment was designed as a crossover trial with two experimental periods, each lasting 21 days. Each experimental period included a 14-day adaptation phase to the diet, followed by 7 days of data and sample collection. The cows were sorted by parity and weight, then randomly assigned to one of two controlled-airflow chambers, each designed for 4 animals (4 open stalls simulating standard housing in a loose-housing system on dairy farms). The chambers were then randomly assigned to one of two dietary treatments.

The cows in the control group were fed ad libitum a total mixed ration (TMR) three times a day (at 5:00 a.m., 11:00 a.m., and 5:00 p. m.). The control total mixed ration contained alfalfa silage (490 g/kg), corn silage (230 g/kg), straw (160 g/kg), wheat (80 g/kg), and rapeseed meal (40 g/kg). The experimental group (nitrate) was fed the same diet, with rapeseed meal replaced by calcium nitrate, and the amount of wheat increased. The experimental diet contained alfalfa silage (490 g/kg), corn silage (230 g/kg), straw (160 g/kg), wheat (110 g/kg), and the commercial product Bolifor CNF 1% containing calcium nitrate. Both diets had the same protein and energy content. In the experimental diet, the dairy cows consumed approximately 100 g of nitrates per head per day. The chemical composition of the substrate (DM basis) was as follows: organic matter (OM) 915 g/kg, crude

protein (CP) 130 g/kg, crude fat 19 g/kg, neutral detergent fiber (NDF) 501 g/kg, and acid detergent fiber (ADF) 326 g/kg.

Sample collection

Feed intake was determined in the respiratory chambers as the difference between the amount of feed offered and the amount of feed remaining over the last 7 days of each experimental period. Samples of the experimental diets and their residues were collected daily and stored at -20 °C. These samples were subsequently thawed and combined into composite samples for each chamber within the individual experimental periods. Samples of the individual components of the basal diet were collected once during the last 7 days of each experimental period. All samples were dried at 60 °C for 48 hours (forced-air oven), then ground to a particle size passing through a 1-mm mesh sieve (Retsch GmbH, Haan, Germany) and analyzed for dry matter (DM), ash, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), starch, and gross energy (GE) according to the methodology described previously (Joch et al., 2022).

Rumen fluid samples (250 ml) were collected from all cows on the 18th day of each experimental period, 3 hours after the morning feeding, using an oral rumen probe. The tip of the oral rumen probe, fitted with a strainer, was inserted to a depth of approximately 180 cm to reach the central part of the rumen, and the sample was collected using a vacuum pump into an Erlenmeyer flask (500 ml). To minimize contamination with saliva, the first 200 ml of rumen fluid was discarded. The samples were placed on ice immediately after collection and transported to the laboratory. The samples were stored at -80 °C for subsequent microbiome analysis. In the laboratory, the pH of the rumen fluid was determined using a portable pH meter (WTW) with a WTW Sentix 41 electrode (WTW, Weilheim, Germany).

To determine volatile fatty acids (VFA) and ammonia nitrogen (NH₃-N), 0.5 ml of rumen fluid was acidified by adding 10 µl of 9 M H₂SO₄. The concentrations of volatile fatty acids (VFA) were determined by gas chromatography by comparison with a calibration mixture of standards (Supelco Volatile Fatty Acid Standard Mix; Merck, Missouri, USA). The analysis was performed on a gas chromatograph (GC-82F, Labio, Czech Republic) equipped with a flame ionization detector (FID) and a Stabilwax capillary column (15 m × 0.53 mm ID × 0.5 µm; Restek, USA) with hydrogen as the carrier gas (Joch et al., 2022). The concentration of NH₃-N was determined using the phenol-hypochlorite reaction (Weatherburn, 1967), with absorbance measured at 625 nm (Libra S22, Biochrom, Cambridge, UK).

DNA extraction, polymerase chain reaction (PCR), and analysis of amplicon sequence variants (ASVs)

DNA extraction was performed using a modified method involving mechanical cell disruption (bead-beating) and column purification. DNA was isolated from 200 µl of a homogenized rumen fluid sample (0.5 ml). The quantity and quality of the DNA were verified using a spectrophotometer (Tecan Group, Männedorf,

Switzerland). The isolated DNA was subsequently adjusted to a uniform molecular concentration (5 ng/μl) and stored at -20 °C until polymerase chain reaction (PCR) was performed.

The V4 region of 16S rRNA was used for amplification using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The number of PCR cycles was the same for all samples and was kept as low as possible to prevent the formation of chimeric sequences. The success of PCR amplification was verified by gel electrophoresis using a 1% agarose gel stained with SYBR™ Green I Nucleic Acid Gel Stain (Thermo Fisher, Waltham, MA, USA) and subsequently documented using the Gel Doc XR+ System (BioRad, Hercules, CA, USA). All samples were amplified in triplicate. The PCR products were sequenced on the MiniSeq platform (Illumina, San Diego, CA, USA). Subsequent analysis of the resulting amplicons was performed using the DADA2 pipeline and the SILVA database (v128). The obtained data were normalized to the sample with the lowest sequencing depth (30,000 sequences/sample).

Visualization, assessment, and statistical analysis of microbial communities

The relative abundance of bacteria was visualized at various taxonomic levels (taxa with a relative abundance > 0.1% of the total number of sequences) in Python using the “plotly” package. Differences in relative abundance were determined using the Wilcoxon signed-rank test. Differences with $p < 0,05$ were considered statistically significant. Alpha diversity indices (Chao1, Pielou, Shannon, and Simpson) were calculated based on a table of standardized (rarefied) amplicon sequence variants (ASVs) and subsequently analyzed using a paired t-test. Box plots illustrating differences in relative abundance at the family level were created in R software (version 4.3.2) using the “ggplot2”, “ggpubr”, and “ggsignif” packages.

Statistical analysis of fermentation parameters was performed using the PROC MIXED procedure in SAS, with the cow considered as the experimental unit, according to the following model:

$$Y_{(ijkl)} = \mu + T_i + P_j + W(C)_{kl} + e_{(ijkl)}$$

where Y represents the dependent variable, μ the grand mean, T_i the treatment effect, P_j the period effect, $W(C)_{kl}$ is the within-pen cow effect, and $e_{(ijkl)}$ is the residual error. The within-pen cow effect was considered random, while the other effects were fixed. Differences were considered statistically significant at the $P < 0,05$ level.

RESULTS AND DISCUSSION

Fermentation parameters

No statistically significant difference was found between the group of dairy cows fed the control diet (Control) and the group of dairy cows fed the nitrate-supplemented diet (Nitrate) for any of the monitored parameters ($P > 0.05$). Cows fed the nitrate diet consumed 0.3 kg more dry matter per day, but this difference was not statistically significant. The effect of nitrates on dry matter intake and fermentation parameters is shown in Table 1. Nitrate supplementation resulted in an approximately 12% reduction in methane production; however, these data are not presented in this study because methane production measurements were not among the main objectives. The obtained values should be considered indicative only, as methane was measured across the entire respiratory chamber, and the number of replicates was insufficient for a robust statistical analysis.

Tab. 1: *Feed intake and rumen fermentation parameters in dairy cows fed a control diet (Control) and a diet containing nitrates (Nitrate)*

	Parameters	Control	Nitrate	SEM	P-value
	DMI (kg/day)	17.7	18.0	0.33	0.521
Rumen	VFA (mmol/l)	113	104	4.5	0.342
	Acetic acid	60.8	61.3	0.98	0.769
	Propionic acid	21.8	20.1	1.31	0.510
	Butyric acid	17.0	18.3	0.40	0.135
	Lactic acid	0.50	0.34	0.059	0.165
	pH	6.67	6.74	0.041	0.421
	NH ₃ -N (mg/100 ml)	14.03	14.54	1.365	0.761

SEM – standard error of the mean; DMI – dry matter intake; VFA – volatile fatty acids; NH₃-N – ammonia nitrogen; $P < 0.05$

The administration of calcium nitrate did not alter the ratio of volatile fatty acids (VFA) in rumen fluid, nor did it increase the proportion of propionic acid at the expense of acetic acid, which refutes the first hypothesis. Although there was a numerical decrease in VFA concentration, it was not statistically significant. This result is consistent with the study by Villar et al. (2020), which also failed to demonstrate an effect of nitrates on VFA concentration. On the other hand, Sharifi et al. (2022) report a decrease in VFA concentration with nitrate supplementation, which may be due to reduced dry matter intake at higher dietary nitrate doses (Newbold et al., 2014). However, a decrease in VFA concentration was also observed under in vitro conditions, where the substrate concentration is constant; therefore, the decrease could not be attributed to lower dry matter intake (Guo et al., 2009). One possible explanation is the inhibitory effect of nitrite, an intermediate product of nitrate reduction, on rumen microorganisms, which may reduce fermentation activity and TMK production.

Dry matter intake (DMI) was also not statistically significantly affected by nitrate supplementation, which is consistent with Olijhoek et al. (2016), who found that nitrates do not appear to have a consistent effect on dry matter intake but may increase the animals' susceptibility to a potential decrease in dry matter intake. These differences may be related to feeding strategies and the amount of nitrate in the diet, with a decrease in dry matter intake observed up to 2.4 % dry matter (Newbold et al., 2014). According to Meller et al. (2019), dry matter intake is negatively affected by reduced diet palatability and the metabolic consequences of higher nitrate levels.

The molar proportion of the individual VFA was not affected by nitrate supplementation. The percentage of propionic acid remained unchanged, even though its absolute concentration decreased numerically. In contrast, the proportions of acetic and butyric acids were numerically higher; however, these differences were not statistically significant. Thus, the anticipated shift in the fermentation profile toward an increase in the proportion of propionic acid at the expense of acetic acid was not confirmed. These results are also supported by the studies by Ntokome et al. (2026) and Olijhoek et al. (2016), which also failed to demonstrate a significant effect of nitrates on the VFA profile. However, the meta-analysis by Abdelbagi et al. (2023) describes changes in the VFA profile following nitrate supplementation, specifically an increase in the proportion of acetic acid accompanied by a decrease in the proportion of propionic acid. This discrepancy in results may be related to the fact that during their reduction, nitrates compete for hydrogen not only with methanogenesis but also with propionogenesis, thereby reducing their availability for propionic acid production (Van Zijderveld et al., 2010).

The pH of the rumen fluid did not differ statistically significantly between the groups, given the constant concentration and profile of VFA (6.67 vs. 6.74). Fluctuations in rumen fluid pH reflect changes in the relative contributions of hydrogen flux and individual volatile fatty acids (Latham et al., 2016). Similar results are reported in studies by Van Zijderveld et al. (2011) and Olijhoek et al. (2016), which recorded only a slight numerical increase in pH. Latham et al. (2016) further report that the form of the nitrate salt influences the rates of nitrate reduction and nitrite accumulation, as well as the efficiency of methane reduction, with sodium and potassium salts being more soluble than calcium nitrates. The addition of calcium nitrate may therefore lead to a more uniform reduction process and fewer fluctuations in fermentation activity.

The concentration of ammoniacal nitrogen ($\text{NH}_3\text{-N}$) was not affected by calcium nitrate supplementation, which is inconsistent with the second hypothesis of this study, which predicted an increase in $\text{NH}_3\text{-N}$. This prediction is based on the fact that bacteria in the rumen convert nitrates to nitrites and then to ammonia. At the same time, they serve as an alternative electron acceptor, thereby reducing methane production (Datta et al., 2017). Olijhoek et al. (2016) observed an increase in $\text{NH}_3\text{-N}$ concentration upon isonitrogenous replacement of urea with nitrates, which they attributed to incomplete reduction of microbial protein synthesis by

rumen microorganisms. The study by Wang et al. (2018), however, describes a 33.1% reduction in $\text{NH}_3\text{-N}$ concentration during a similar replacement of the nitrogen source with nitrates. Such an increase in ammonia utilization can redirect more nitrogen, electrons, and energy toward microbial protein synthesis at the expense of methane emissions (Wang et al., 2018). In this study, there was no increase in the concentration of ammoniacal nitrogen in rumen fluid when rapeseed meal was isonitrogenously replaced with nitrate. Nitrates can thus serve as a source of non-protein nitrogen, a finding confirmed by Rebelo et al. (2019), who replaced nitrogen from protein feeds with nitrates and found that isonitrogenous replacement of soybean meal with nitrates did not increase ammonia concentration or adversely affect rumen fermentation. The rumen microbiota in this study can effectively utilize nitrogen derived from nitrates. However, the relatively low nitrate content in the diet (approximately 0.6% of dry matter) may also contribute to this. The measured $\text{NH}_3\text{-N}$ values (approximately 14 mg/100 ml) fall within the optimal range for microbial protein synthesis (approximately 5–20 mg/100 ml). Within this range, sufficient nitrogen is available to microorganisms without excessive accumulation. In conclusion, the availability of ammonia and fermentable energy is particularly crucial for microbial protein synthesis, whereas the nitrogen source itself is of secondary importance (Rebelo et al., 2019). At an isonitrogenous feed ration, the addition of nitrates in the diet represents more of an opportunity to utilize an alternative form of nitrogen than a process leading to a change in the concentration of ammoniacal nitrogen.

Rumen microbiome

Nitrate supplementation affected the alpha diversity of rumen fluid as measured by the Chao1 richness index and the number of ASVs ($P < 0.05$). For the other indices examined—the Shannon and Simpson diversity indices and the Pielou evenness index—no statistically significant difference was found between the groups ($P > 0.05$). The effect of nitrates on alpha diversity is shown in Table 2.

The effect of nitrates on the rumen microbiome of dairy cows is shown in Table 3 and Figure 1. At the phylum level (Figure 1A), the rumen microbiome was not statistically significantly affected by nitrate supplementation. In rumen fluid samples (both Control and Nitrate groups), Firmicutes and Bacteroidota were dominant, while Euryarchaeota, Spirochaetota, and Proteobacteria were present

Tab. 2: *Effect of Nitrates on Alpha Diversity – Diversity Indices, Species Richness*

Index	Control	Nitrate	SEM	P-value
Shannon diversity index	8.71	8.90	0.063	0.0791
Simpson diversity index	0.994	0.0791	0.0004	0.1834
Chao1 richness index	999.4	1102.0	31.69	0.0164*
Pielou evenness index	0.877	0.883	0.0054	0.4700
ASV	986.0	1088.3	30.60	0.0153*

SEM – standard error of the mean; ASV – amplicon sequence variants

in lower relative abundances. A trend toward a decrease in the relative abundance of the Euryarchaeota phylum was observed in the group of dairy cows fed a diet containing nitrate ($P = 0.078$). At the family level, a statistically significant difference was observed only for the family Methanobacteriaceae, whose relative abundance was lower in the nitrate group (control 6.2% vs. nitrate 3.9%, $P = 0.039$).

Tab. 3: *Relative abundance of strains and associated families in the rumen fluid of dairy cows fed the control diet (Control) and the nitrate-containing diet (Nitrate) ($P > 0.05$)*

Phylum	Family	Control	Nitrate	P-value
Firmicutes		57.2	61.7	0.844
	Lachnospiraceae	18.2	18.7	0.844
	Christensenellaceae	9.6	11.1	0.148
	Ruminococcaceae	5.8	8.0	0.195
	Anaerovoracaceae	5.6	4.3	0.460
	Oscillospiraceae	4.8	5.7	0.109
	Hungateiclostridiaceae	2.0	2.1	0.641
	Erysipelotrichaceae	1.9	2.2	0.742
	Eubacterium coprostanoligenes group	1.4	1.5	0.313
Bacteroidota		34.4	31.9	0.945
	Prevotellaceae	24.0	22.3	0.844
	Rikenellaceae	6.8	6.5	0.641
	Bacteroidales RF16 group	1.4	1.2	0.945
Euryarchaeota		5.7	3.72	0.078
	Methanobacteriaceae	6.2	3.9	0.039*
Spirochaetota		1.3	1.6	0.547
	Spirochaetaceae	1.4	1.6	0.742
Proteobacteria		1.3	1.1	0.641
Unclassified	F082	2.2	2.2	0.547

The third hypothesis posited that nitrate supplementation would alter the structure of the rumen microbiome, specifically decreasing the proportion of methanogenic archaea and increasing the abundance of bacteria involved in nitrate reduction.

Alpha diversity of the microbial community was not significantly affected by either the Simpson diversity index or the Pielou evenness index, suggesting no change in the dominance of individual bacterial taxa or in the evenness of their representation. A similar result is reported by Granja-Salcedo et al. (2019), who observed no changes in any of the alpha diversity indices; however, this is inconsistent with the results of this study, which showed changes in the indices of bacterial community species richness—specifically the Chao1 index and the number

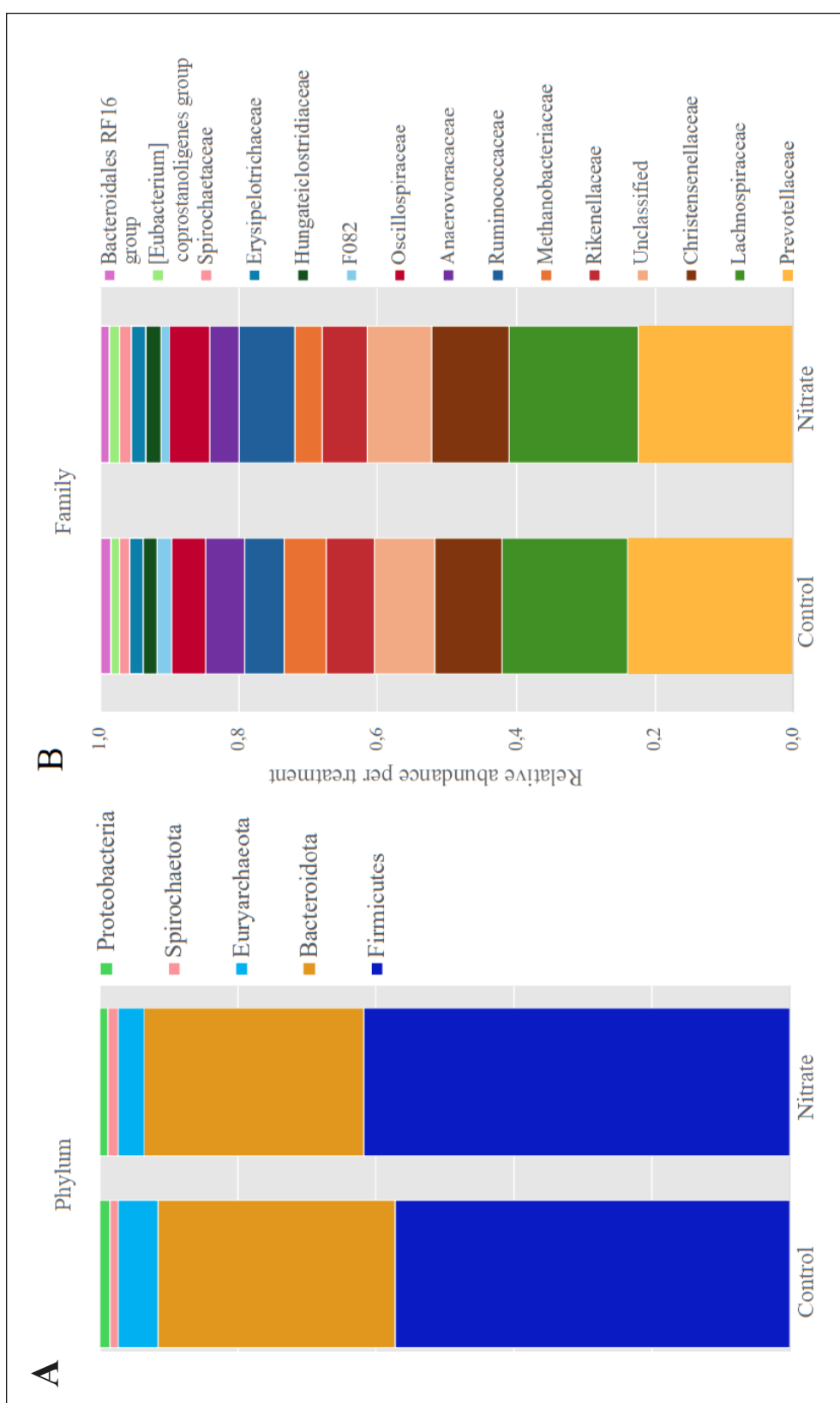


Fig. 1: The effect of nitrate supplementation on the relative abundance of bacteria at the Phylum (A) and Family (B) levels in the rumen fluid of dairy cows. The experimental groups included a control diet (Control; n = 8) and a diet supplemented with nitrate (Nitrate; n = 8)

of ASV (amplicon sequence variants)—as well as a tendency toward an increase in the Shannon index. Methodological differences can explain these differences, as the ASV-based approach enables high-resolution detection of microbial sequences at the single-nucleotide level, whereas OTU (Operational Taxonomic Unit)-based analyses cluster sequences using a predetermined identity threshold (Chiarello et al., 2022). Changes in the Chao1 index and the number of ASV in this study may thus reflect an increase in microbial community species richness, implying a broadening of the spectrum of microbial taxa while maintaining the dominance of major microbial populations.

According to Belanche et al. (2026), such an increase in rumen microbial complexity can lead to positive effects, particularly better adaptation to changes in diet composition. Promoting greater rumen microbial diversity is thus desirable for preventing digestive disorders (Belanche et al., 2026).

The composition of bacterial strains in rumen fluid reflects the dynamics of the rumen microbiome, with Firmicutes and Bacteroidota strains often the most abundant across various conditions (Mao et al., 2015), consistent with this study's results. At a lower taxonomic level, it was further found that within the Firmicutes phylum, the families Lachnospiraceae, Christensenellaceae, and Ruminococcaceae were primarily represented. Within the Bacteroidota phylum, the Prevotellaceae family was dominant.

Among the less abundant phyla, Euryarchaeota, Spirochaetota, and Proteobacteria were particularly well represented, which is generally consistent with the literature (Mao et al., 2015). Granja-Salcedo et al. (2019), however, demonstrated a statistically significant increase in the Proteobacteria phylum following nitrate supplementation. In contrast, this study observed only a slight numerical decrease, without statistical significance. Sharifi et al. (2022) report that an increase in the abundance of the Proteobacteria phylum is associated with reduced methane emissions due to competition for hydrogen (Zhao et al., 2015). However, the differing results also suggest that the response of the Proteobacteria phylum to nitrate supplementation may not be uniform. The slight numerical decrease, without statistical significance, observed in this study may also be explained by the taxonomic composition of the microbiome at the family level, as families typically classified within the phylum Proteobacteria and associated with nitrate reduction (e.g., Succinivibrionaceae or Desulfovibrionaceae) were not detected. This result may also be related to the presence of unclassified taxa (F082), which may include nitrate-reducing bacteria. Zhao et al. (2015) also note that nitrate-reducing bacteria have been reported across various bacterial families, including Selenomonadaceae, Veillonellaceae, Helicobacteraceae, Campylobacteraceae, and Pasteurellaceae, and are thus not confined to a single taxon. Furthermore, these bacteria are present in the rumen in low numbers and at lower taxonomic levels (Zhao et al., 2015; Granja-Salcedo et al., 2019).

A statistically significant decrease was observed in one of the most abundant methanogen families in the rumen, the Methanobacteriaceae, consistent with

findings reported by Granja-Salcedo et al. (2019). In contrast, Zhao et al. (2018) report that the addition of nitrates did not significantly affect the dominant genus *Methanobrevibacter*. However, the abundance of the least prevalent genera, *Methanosphaera* and *Methanimicrococcus*, was significantly affected. Patra & Yu (2013) report that significant changes in methanogen composition may not accompany a reduction in methane production.

Zhao et al. (2015) and Latham et al. (2016) attribute the decline in the *Methanobacteriaceae* family primarily to competition for hydrogen, whereby nitrate is reduced to ammonia—a thermodynamically more favorable reaction—thereby limiting the availability of hydrogen for hydrogenotrophic methanogens. This explanation is also supported by Van Zijderveld et al. (2010), who additionally suggest a possible role for nitrite, a toxic intermediate product of nitrate reduction. However, a direct inhibitory effect on methanogens has not been fully confirmed (Yang et al., 2016). Given the absence of significant changes at the genus and family levels in this study, we can conclude that there was likely no significant accumulation of nitrite, as its accumulation is associated with the inhibition not only of methanogenic archaea but also of other microbial groups (Yang et al., 2016).

In conclusion, the hypothesis regarding a change in the structure of the rumen microbiome was only partially confirmed. A decrease in methanogenic archaea was demonstrated, while an increase in bacteria involved in nitrate reduction was not confirmed.

CONCLUSION

Our study demonstrated that calcium nitrate did not alter the ratio of volatile fatty acids in rumen fluid; thus, the first hypothesis regarding a change in the volatile fatty acid profile was not confirmed, as neither the total concentration nor the relative ratios were statistically significantly affected by nitrate. The second hypothesis regarding an increase in the concentration of ammoniacal nitrogen in rumen fluid was also not confirmed. Given the stable concentration of ammoniacal nitrogen, it can be assumed that the supply of nitrogen to the rumen microbiome via calcium nitrate was sufficient. The third hypothesis regarding the structure of the microbial community in the rumen was only partially confirmed, as suppression of methanogenic archaea was observed. However, there was no change in the overall structure of the microbial community, nor was it possible to confirm or refute the growth of bacteria involved in nitrate reduction.

The observed effect of calcium nitrate on rumen fermentation parameters and the microbiome may represent a potentially effective mitigation strategy for ruminants. Replacing part of the nitrogen in the diet with nitrates offers a solution without a significant economic burden for producers and could yield economic benefits.

ACKNOWLEDGEMENT

This study was supported by the Ministry of Agriculture of the Czech Republic (MZERO0723).

REFERENCES

- Abdelbagi M., Ridwan R., Fitri A., Nahrowi N., Jayanegara A. (2023): Performance, Methane Emission, Nutrient Utilization, and the Nitrate Toxicity of Ruminants with Dietary Nitrate Addition: A Meta-analysis from *In Vivo* Trials. *Tropical Animal Science Journal*, 46:74–84. Available from <https://journal.ipb.ac.id/index.php/tasj/article/view/41001> (accessed June 25, 2026).
- Belanche A, Palma-Hidalgo J.M., Yáñez-Ruiz D.R. (2026): Increasing Rumen Microbial Diversity in Goats Favours the Adaptation to High-Concentrate Diets With Minor Effects on Feed Utilization. *Journal of Animal Physiology and Animal Nutrition*, 110:148–168. Available from <https://onlinelibrary.wiley.com/doi/10.1111/jpn.70024> (accessed June 25, 2026).
- Datta M., Jha P., Arumbaka S. (2017): Effects of Nitrate Supplementation on Nutrition, Performance and Methane Mitigation in Ruminants: A Review. *International Journal of Livestock Research*, 7(9): 19–29. Available from <https://ejmanager.com/fulltextpdf.php?mno=265848> (accessed June 25, 2026).
- Ellis J.L., Dijkstra J., Kebreab E., Bannink A., Odongo N.E., McBride B.W., France J. (2008): Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. *The Journal of Agricultural Science*, 146:213–233. Available from https://www.cambridge.org/core/product/identifier/S0021859608007752/type/journal_article (accessed June 25, 2026).
- Granja-Salcedo Y.T., Fernandes R.M., Araujo R.C.D., Kishi L.T., Berchielli T.T., Resende F.D.D., Berndt A., Siqueira G.R. (2019): Long-Term Encapsulated Nitrate Supplementation Modulates Rumen Microbial Diversity and Rumen Fermentation to Reduce Methane Emission in Grazing Steers. *Frontiers in Microbiology*, 10:614. Available from <https://www.frontiersin.org/article/10.3389/fmicb.2019.00614/full> (accessed June 25, 2026).
- Guo W.S., Schaefer D.M., Guo X.X., Ren L.P., Meng Q.X. (2009): Use of Nitrate-nitrogen as a Sole Dietary Nitrogen Source to Inhibit Ruminal Methanogenesis and to Improve Microbial Nitrogen Synthesis *In vitro*. *Asian-Australasian Journal of Animal Sciences*, 22:542–549. Available from <http://ajas.info/journal/view.php?doi=10.5713/ajas.2009.80361> (accessed June 25, 2026).
- Hristov A.N., Ott T., Tricarico J., Rotz A., Waghorn G., Adesogan A., Dijkstra J., Montes F., Oh J., Kebreab E., Oosting S.J., Gerber P. J., Henderson B., Makkar H.P.S., Firkins J.L. (2013): SPECIAL TOPICS — Mitigation of methane and nitrous oxide emissions from animal operations: III. A review of animal management mitigation options. *Journal of Animal Science*, 91:5095–5113. Available from <https://academic.oup.com/jas/article/91/11/5095/4731330> (accessed June 25, 2026).

- Chiarello M., McCauley M., Villéger S., Jackson C.R. (2022): Ranking the biases: The choice of OTUs vs. ASVs in 16S rRNA amplicon data analysis has stronger effects on diversity measures than rarefaction and OTU identity threshold. PLOS ONE, 17:e0264443. Available from <https://dx.plos.org/10.1371/journal.pone.0264443> (accessed June 25, 2026).
- Johnson K.A., Johnson D.E. (1995): Methane emissions from cattle. Journal of Animal Science, 73:2483–2492. Available from <https://academic.oup.com/jas/article/73/8/2483-2492/4632901> (accessed June 25, 2026).
- Joch M., Vadroňová M., Výborná A., Jochová K. (2022): Inhibition of *in Vitro* Rumen Methane Production by Three Statins. Annals of Animal Science, 22:271–282. Available from <https://reference-global.com/article/10.2478/aoas-2021-0022> (accessed June 25, 2026).
- Latham E.A., Anderson R.C., Pinchak W.E., Nisbet D.J. (2016): Insights on Alterations to the Rumen Ecosystem by Nitrate and Nitrocompounds. Frontiers in Microbiology, 7:228. Available from <http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00228/abstract> (accessed June 25, 2026).
- Mao S., Zhang M., Liu J., Zhu W. (2015) Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. Scientific Reports, 5:16116. Available from <https://www.nature.com/articles/srep16116> (accessed June 25, 2026).
- Meller R.A., Wenne, B.A., Ashworth J., Gehman A.M., Lakritz J., Firkins J.L. (2019): Potential roles of nitrate and live yeast culture in suppressing methane emission and influencing ruminal fermentation, digestibility, and milk production in lactating Jersey cows. Journal of Dairy Science, 102:6144–6156. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0022030219303832> (accessed June 25, 2026).
- Mizrahi I., Wallace R.J., Morais S. (2021): The rumen microbiome: balancing food security and environmental impacts. Nature Reviews Microbiology, 19:553–566. Available from <https://www.nature.com/articles/s41579-021-00543-6> (accessed June 25, 2026).
- Newbold J.R., Van Zijderveld S.M., Hulshof R.B.A., Fokkink W.B., Leng R.A., Terencio P., Powers W.J., Van Adrichem P. S.J., Paton N.D., Perdok H.B. (2014): The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. Journal of Animal Science, 92:5032–5040. Available from <https://academic.oup.com/jas/article/92/11/5032/4703708> (accessed June 25, 2026).
- Ntokome K., Obitsu T., Hisadomi S., Inabu Y., Sugino T. (2026): Effect of Nitrate Supplementation on Digestion, Rumen Fermentation, Plasma Metabolites, and Nitrogen Use in Sheep Fed a High-Forage Diet. Animal Science Journal, 97:e70145. Available from <https://onlinelibrary.wiley.com/doi/10.1111/asj.70145> (accessed June 25, 2026).

- Olijhoek D.W., Hellwing A.L.F., Brask M., Weisbjerg M.R., Højberg O., Larsen M.K., Dijkstra J., Erlandsen E.J., Lund P. (2016): Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *Journal of Dairy Science*, 99:6191–6205. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0022030216303010> (accessed June 25, 2026).
- Patra A.K., Yu Z. (2013): Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen. *Bioresource Technology*, 148:352–360. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0960852413013916> (accessed June 25, 2026).
- Rebello L.R., Lunab I.C., Messanaa J.D., Araujo R.C., Simionia T.A., Granja-Salcedo Y.T., Vitoa E.S., Leed Ch., Teixeiraa I.A.M.A., Rooke J.A., Berchiellia T.T. (2019): Effect of replacing soybean meal with urea or encapsulated nitrate with or without elemental sulfur on nitrogen digestion and methane emissions in feedlot cattle. *Animal Feed Science and Technology*, 257:114293. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0377840118313889> (accessed June 25, 2026).
- Sharifi M., Taghizadeh A., Hosseinkhani A., Palangi V., Macit M., Salem A.Z.M., Elghndour M.M.M.Y., Abachi S. (2022): Influence of nitrate supplementation on *in-vitro* methane emission, milk production, ruminal fermentation, and microbial methanotrophs in dairy cows fed at two forage levels. *Annals of Animal Science*, 22:1015–1026. Available from <https://reference-global.com/article/10.2478/aoas-2021-0087> (accessed June 25, 2026).
- Van Zijderveld S.M., Gerrits W.J.J., Apajalahti J.A., Newbold J.R., Dijkstra J., Leng R.A., Perdok H.B. (2010): Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *Journal of Dairy Science*, 93:5856–5866. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0022030210006375> (accessed June 25, 2026).
- Van Zijderveld S.M., Gerrits W.J.J., Dijkstra J., Newbold J.R., Hulshof R.B.A., Perdok H.B. (2011): Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *Journal of Dairy Science* 94:4028–4038. Available from <https://linkinghub.elsevier.com/retrieve/pii/S0022030211004061> (accessed June 25, 2026).
- Veneman J.B., Muetzel S., Hart K.J., Faulkner C.L., Moorby J.M., Perdok H.B., Newbold C.J. (2015): Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and Animal Productivity in Dairy Cows? *PLOS ONE*, 10:e0140282. Available from <https://dx.plos.org/10.1371/journal.pone.0140282> (accessed June 25, 2026).

- Villar M.L., Hegarty R.S., Clay J.W., Smith K.A., Godwin I.R., Nolan J.V. (2020): Dietary nitrate and presence of protozoa increase nitrate and nitrite reduction in the rumen of sheep. *Journal of Animal Physiology and Animal Nutrition*, 104:1242–1255. Available from <https://onlinelibrary.wiley.com/doi/10.1111/jpn.13365> (accessed June 25, 2026).
- Wang R., Wang M., Ungerfeld E.M., Zhang X.M., Long D.L., Mao H.X., Deng J.P., Bannink A., Tan Z.L. (2018): Nitrate improves ammonia incorporation into rumen microbial protein in lactating dairy cows fed a low-protein diet. *Journal of Dairy Science*, 101:9789–9799. Available from <https://www.elsevier.com/tdm/userlicense/1.0/> (accessed June 25, 2026).
- Weatherburn M.W. (1967): Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39:971–974. Available from <https://pubs.acs.org/doi/abs/10.1021/ac60252a045> (accessed June 25, 2026).
- Yang C., Rooke J.A., Cabeza I., Wallace R.J. (2016): Nitrate and Inhibition of Ruminal Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane Emissions from Ruminant Livestock. *Frontiers in Microbiology*, 7:132. Available from <http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00132/abstract> (accessed June 25, 2026).
- Zhao L., Meng Q., Li Y., Wu H., Huo Y., Zhang X., Zhou Z. (2018): Nitrate decreases ruminal methane production with slight changes to ruminal methanogen composition of nitrate-adapted steers. *BMC Microbiology*, 18:21. Available from <https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-018-1164-1> (accessed June 25, 2026).
- Zhao L., Meng Q., Ren L., Liu W., Zhang X., Huo Y., Zhou Z. (2015): Effects of Nitrate Addition on Rumen Fermentation, Bacterial Biodiversity and Abundance. *Asian-Australasian Journal of Animal Sciences*, 28:1433–1441. Available from <http://ajas.info/journal/view.php?doi=10.5713/ajas.15.0091> (accessed June 25, 2026).