INFLUENCE OF NITRATE SUPPLEMENTATION ON *IN VITRO* METHANE EMISSION, MILK PRODUCTION, RUMINAL FERMENTATION, AND MICROBIAL METHANOTROPHS IN DAIRY COWS FED AT TWO FORAGE LEVELS

Majid Sharifi¹, Akbar Taghizadeh^{1*}, Ali Hosseinkhani¹, Valiollah Palangi², Muhlis Macit², Abdelfattah Z.M. Salem^{3*}, Mona M.M.Y. Elghndour³, Soheila Abachi⁴

¹Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

²Department of Animal Science, Agricultural Faculty, Ataturk University, 25240, Erzurum, Turkey

³Faculty of Veterinary Medicine and Animal Science, Autonomous University of the State of Mexico, Toluca, State of Mexico, Mexico

⁴Institute of Nutrition and Functional Foods (INAF), Université Laval, Quebec, QC G1V 0A6, Canada

*Corresponding authors: A.Z.M. Salem: asalem70@yahoo.com; salem@uaemex.mx;

A. Taghizadeh: a_taghizadeh@tabrizu.ac.ir

Majid Sharifi: <https://orcid.org/0000-0002-1546-7864>

Akbar Taghizadeh: <https://orcid.org/0000-0002-9120-1662>

Ali Hosseinkhani: <https://orcid.org/0000-0001-5553-8404>

Valiollah Palangi: <https://orcid.org/0000-0001-6470-2608>

Muhlis Macit: <https://orcid.org/0000-0002-5055-1156>

Abdelfattah Z. M. Salem: <http://orcid.org/0000-0001-7418-4170>

Soheila Abachi: <http://orcid.org/0000-0002-9807-3632>

Abstract

Modifying the chemical composition of a diet can be a good strategy for reducing methane emission in the rumen. However, this strategy can have adverse effects on the ruminal microbial flora. The aim of our study was to reduce methane without disturbing ruminal function by stimulating the growth and propagation of methanotrophs. In this study, we randomly divided twenty multiparous Holstein dairy cows into 4 groups in a 2×2 factorial design with two forage levels (40% and 60%) and two nitrate supplementation levels (3.5% and zero). We examined the effect of experimental diets on cow performance, ruminal fermentation, blood metabolites and changes of ruminal microbial flora throughout the experimental period (45-day). Additionally, *in vitro* methane emission was evaluated. Animals fed diet with 60% forage had greater dry matter intake (DMI) and milk fat content, but lower lactose and milk urea content compared with those fed 40% forage diet. Moreover, nitrate supplementation had no significant effect on DMI and milk yield. Furthermore, the interactions showed that nitrate reduces DMI and milk fat independently of forage levels. Our findings showed that nitrate can increase ammonia concentration, pH, nitrite, and acetate while reducing the total volatile fatty acids concentration, propionate, and butyrate in the rumen. With increasing nitrate, methane emission was considerably decreased possibly due to the stimulated growth of *Fibrobacteria*, *Proteobacteria*, type II methanotrophs, and *Methanoperedense nitroreducens*, especially with high forage level. Overall, nitrate supplementation could potentially increase methane oxidizing microorganisms without adversely affecting cattle performance.

Key words: dairy cows, forage, methanotrophs, microbiota, milk yield, nitrate, ruminal fermentation

Graphical abstract

Imperfect fermentation of feedstuff in the rumen, with methane as an end product, results in a loss of up to 12 percent of feed's gross energy (Sharifi and Khadem, 2012). Moreover, approximately 81% of greenhouse gas emitted by livestock industry is associated with ruminal methane production. Nonetheless, few methane emission mitigation strategies are commonly practiced: (1) decreasing the population of ruminal methanogens, (2) abatement of H_2 production, and (3) providing alternative H_2 sink (Johnson and Johnson, 1995; Feldewert et al., 2020). Despite the negative association of methanogen microorganisms with energy retention in livestock (Yang et al., 2016), their presence (e.g., such as archaea dominant group in methanogen by $\leq 3.3\%$ of total microorganism) plays an important ecological role in the rumen (Danielsson et al., 2017). Generally, methanogens maintain a low partial pressure of hydrogen, promoting the inhibition of NADH oxidoreductase. A disruption in NADH oxidation and subsequent reduction in NAD^+ concentrations have been shown to inhibit microbial fermentation (Hegarty and Gerdes, 1999). Conversely, the reduction in methane production could reduce energy lost through methanogenesis (Latham et al., 2016; Chen et al., 2019; Sharifi et al., 2021). Various studies have investigated the potential of unsaturated fatty acids, nitrate, sulphate, and fumarate as electron acceptors for the methane production reduction in livestock (Zeitz et al., 2013; Latham et al., 2016; Li et al., 2018). From a thermodynamic perspective, nitrate has been shown to be more effectual among the others (Veneman et al., 2015). On the one hand, converting nitrate to ammonia, is energetically more favorable than converting carbon dioxide to methane (Sharifi et al., 2021). On the other hand, due to the presence of other electron acceptors in the rumen, utilization of nitrate is facing to performance limitation (Hulshof et al., 2012; Clough et al., 2020). Lee et al. (2017) reported that utilization of encapsulated nitrate not only could reduce methane emission but could also reduce meta-hemoglobin (met-Hb) levels in comparison with the control group in the beef cow. However, Hulshof et al. (2012), Veneman et al. (2015) and Zhao et al. (2015) found that nitrate alters the ruminal bacterial flora based on duration of adaptation and nitrate intake. Few studies have reported that nitrate could inhibit the *in vitro* growth of *F. succinogenes* (Hulshof et al., 2012; Zhao et al., 2015). The mechanism of action of nitrate is not completely understood yet. It is noteworthy that the flora of rumen is highly affected by pH and chemical composition of feed (Sharifi and Khadem, 2012; Sharifi et al., 2016 b). Therefore, changing forage to concentrate ratio of the diet can alter pH and available nitrogen thus affecting the ruminal flora (Sharifi et al., 2019 a; Chen et al., 2020).

There is ample amount of data about ruminal methane production; however, information about methane consumption in the rumen is scarce. Methanotrophs can oxidize methane and use it as carbon and energy source in anaerobic conditions with relatively high methane

pressures (Parmar et al., 2015). Methanotrophic bacteria, based on carbon assimilation pathway and the intracellular membranes arrangement, are classified into two types (Sharifi and Khadem, 2012; Bhattarai et al., 2019). Type I and II methanotrophs belong to g- and a-subclass of Proteobacteria, respectively (Kumar et al., 2016). A study showed that about 20% of the net global methane flux is oxidized before entering atmosphere (Finn et al., 2012). Research is rather focused on type II anaerobic methanotrophs since type I methanotrophs are incompetent to survive and grow in anaerobic conditions of the rumen. Studies have shown that type II methanotrophs (e.g., *Methylobacterium oxyfera* and *Methanoperedens nitroreducens*) can reduce nitrate to nitrite in anaerobic environments that would subsequently be converted to nitrogen (Cui et al., 2015). *M. nitroreducens* and *M. oxyfera* are found in methane-rich anaerobic environment such as freshwater sludge, and wastewater (Stultiens et al., 2020). Since the anaerobic methane oxidation in the rumen can be expected (Kajikawa et al., 2003), the rumen microorganisms are compatible with nitrate supplementation (Zhou et al., 2012), and nitrate supplementation can be used to increase the anaerobic methanotrophic population to reduce methane generation. However, to the knowledge of authors the topic has not yet been studied.

As there is a greater potential of nitrate toxicity with diets containing less rapid fermentable energy, we tested nitrate supplementation at different levels of forage on (1) methanotrophs and microbial population in rumen fluid, (2) methane emission parameters through gas technique, and (3) performance of dairy cows.

Material and methods

Animals, diets, and experimental design

The study was carried out at the SEPID DAM farm in Tehran Province, Iran. The experiments were performed in accordance with the ethical standards approved by Tabriz University (Sharifi et al., 2016 b). Twenty Holstein cows, with an initial body weight of 550 ± 22.5 kg and 122 ± 10 days in milk, were transferred from free stall to individual stall for adaptation (15 days). Then the cows were kept in individual stall for 30 days to run the experimental period. The study was carried out as a 2×2 factorial design with four treatments ($n=20$) as follows: 1) forage 40% (F40, with no nitrate supplementation), 2) forage 40% with 3.5% nitrate (F40N); 3) forage 60% (F60, with no nitrate supplementation); and 4) forage 60% with 3.5% nitrate (F60N). The source and chemical structure of the nitrate used included $5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$; 75% NO_3 in DM. The forage was a mixture of alfalfa hay and maize silage by 1 to 1 ratio (on dry matter basis). All cows were fed a total mixed ration *ad libitum* (TMR; Table 1) and Orts were allowed at 10% of daily feed supply.

Table 1. Ingredient and chemical composition of experimental diets

Item	Treatments ¹			
	F40	F40N	F60	F60N
Ingredient (g/kg of DM)				
alfalfa hay	200	200	300	300
maize silage	200	200	300	300
barley grain	120	120	81	81
corn grain	120	120	59	59
soybean meal	67	67	55	54
meat and bone meal	16	17	10	10
sugar beet pulp	69	69	30	30
wheat bran	99	99	59	59
molasses (beet sugar)	35	35	34	34
fat supplementation	20	20	20	20
dicalcium phosphate	5	5	4	4
limestone	10	1	10	1
salt	2	2	3	3
urea	25	0	25	0
calcinit ²	0	35	0	35
mineral-vitamin mix ³	7	5	5	5
sodium bicarbonate	5	5	5	5
Chemical composition (g/kg of DM)				
dry matter (DM)	615	612	561	559
total digestion nutrient (TDN) ⁴	669	673	641	644
crude fiber	98	97	124	123
crude protein	165	166	164	165
crude fat	34	34	33	33
ash	99	101	102	105
nitrate	0.05	18.1	0.09	17.5
neutral detergent fiber (NDF)	298	292	317	315
acid detergent fiber (ADF)	173	169	206	202
non-fiber carbohydrate (NFC) ⁵	414	417	394	392
net energy for lactation (NEL) ⁶	1.51	1.52	1.45	1.45

¹F40: forage 40% without nitrate supplementation, F40N: forage 40% with nitrate supplementation, F60: forage 60% without nitrate supplementation and F60N: forage 60% with nitrate supplementation.

²Nitrate source with molecular formula: $5\text{Ca}(\text{NO}_3)_2 \cdot \text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$; 75% NO_3 in DM.

³Mineral composition (mg/kg): Ca: 195 000; P: 90 000; Mg: 20 000; Na: 55 000; Cu: 280; Zn: 3 000; Mn: 2 000; I: 100; Co: 100; Se: 1. Vitamin composition (IU/kg): vitamin A: 600 000; vitamin D₃: 120 000 and vitamin E: 1 300.

⁴TDN (%) = $\text{tdNFC} + \text{tdCP} + (\text{tdFA} \times 2.25) + \text{tdNDF} - 7$.

⁵NFC = $100 - (\text{Fat}\% + \text{NDF}\% + \text{CP}\% + \text{Ash}\%)$.

⁶NEL (Mcal/kg DM) = $0.0245 \times \text{TDN}(\%) - 0.12$.

Data collection and samples analysis

Forage and feedstuff were evaluated for dry matter, crude protein, crude fibre, ether extract, ash and neutral detergent fibre according to the procedure of the Association of Official Analytical Chemists method (AOAC, 2005). Measurements of daily dry matter intake (DMI) and milk yield (MY) were recorded from start to the end of study. Additionally, content of fat, protein and lactose of milk, and milk urea nitrogen (MUN) were analysed every 3 days by infrared spectroscopy (MilkoScanTM, Foss Electric, Hillerød, Denmark). Furthermore, we es-

timated the feed efficiency (FE) by dividing MY and fat and protein corrected milk (FPCM: equation 1) by daily DMI using the following equation:

$$\text{Equation 1: } FPCM \text{ (kg)} = [(0.337 + 0.116 \times \text{fat } (\%) + 0.06 \times \text{protein } (\%)) \times MY \text{ (kg/d)}]$$

For haemoglobin (Hb) and MUN measurements, blood samples (20 ml) were collected from the tail vein into EDTA coated tubes of cows at 3-day intervals (1.8 g/l of blood). The MUN was determined according to a

previously described colorimetric phenol-hypochlorite method (Naderi et al., 2016). The Hb and meta-Hb determinations were carried out by optical spectrophotometric method of Abhilash (2019).

Rumen fluid samples were taken (200 ml) from each cow 3 h after morning feedings at 3-day intervals, using a Geishauser oral probe (Keefe and Ogilvie, 1997; Khadem et al., 2009). For volatile fatty acid (VFA) determinations of the rumen fluid, the samples were thawed at 25°C and centrifuged at 6000g for 10 min. The individual VFA was determined by gas liquid chromatography (model no. CP-9002, Delft, The Netherlands) as outlined by Filípek and Dvořák (2009). Ammonia was measured by a previously described colorimetric phenol-hypochlorite method (Cho et al., 2018). For estimation of nitrate and nitrite, fermented medium (2 ml) was mixed with 2 ml of 75 mM ZnSO₄ and 2.5 ml of 55 mM NaOH. After 15 min, samples were centrifuged at 2500g for 10 min at 4°C. Supernatant, free from any solid particles, was recovered and analysed for nitrate and nitrite content using the cadmium-reduction procedure of MacDonald et al. (2017).

Total gas production and methane production

Gas production was measured using the method of Rymer et al. (2005). Twenty millilitres of strained rumen fluid and McDougall's buffer solution mixture were added to each bottle under continuous CO₂ flow (1:2 ratio of rumen fluid to McDougall's buffer). The rumen fluid was obtained 3 h after morning feeding from the rumen of animals from each group and filtered through 4 layers of cheesecloth. All serum bottles were heated to 39°C and subsequently flushed with O₂-free CO₂ prior to the addition of inoculums. Gas production was recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h using a water displacement apparatus. The gas volume was calculated using the following equation.

$$\text{Equation 2: } V = ((Vt - Vb) \times 100 / W)$$

Where V is the net volume of gas produced per gram of DM of diet sample, Vt is the total gas produced in sample bottles (ml), Vb is the average total gas produced in blank bottles (ml), and W is the weight of sample on DM basis (g).

The gas production profiles were fitted with equation 3 of Kamalak et al. (2005) using the NLIN procedure of SAS (SAS Institute, Inc., Cary, North Carolina, USA).

$$\text{Equation 3: } y = a + b(1 - e^{-ct})$$

Where y is the volume of gas produced at time (t) (ml/g DM), a is the gas produced from immediately soluble fraction (ml), b is the gas production from the insoluble fraction (ml), c is the gas production rate constant, $(a+b)$ is the potential gas production (ml), and t is the incubation time (h).

For methane estimation, 100 µl of gas from the serum bottles was injected into gas-liquid chromatography as described by Agarwal et al. (2008). A mixture of H₂, methane, and CO₂ (100, 201, and 101 ml/l, respectively) was used as the standard.

Molecular identification and quantitation of the rumen microbial population

The quantitative Polymerase Chain Reaction (qPCR) technique was applied for the quantitative analysis of the ruminal microbes. Microbial cells were kept in 2 ml RNAlater reagent (Qiagen, Germany). In the process of separating, 200 µl of lysozyme solution (10 mg/ml), was added to the collected microbes, and incubated for 5 minutes at 5°C for lysing the cell wall. Next, DNA was elicited by a QIAamp DNA Stool Mini Kit (Qiagen, Germany). The DNA condensation was evaluated by applying the NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA) at the absorbance of 280 nm and elicited DNA quality was determined with 1% agarose gel electrophoresis (100 v, 40 min). The DNA specimens were kept at -20°C until further analysis.

To measure the population of microorganisms in samples from the SYBR, green-based absolute real-time qPCR was used. To provide the qPCR DNA standard, a general PCR was carried out by applying the particular bacteria primer (Patra and Yu, 2014). Then, the PCR products were cloned into PCR2.1-TOPO TA cloning vector (Invitrogen, CA, USA) and transformed into chemically competent *Escherichia coli* TOP10 cells. The best positive clone was cultured, and then the plasmid recombinant DNA in the cultured clone was elicited by TIANprep Mini Plasmid Kit (Tiangen, China) and applied as the qPCR DNA standard. The standard DNA condensation was diluted to 30 ng/µl, and then series of dilutions were used to create a calibration curve. The diluted DNA standard copy number was estimated according to the equation described by Yu et al. (2005) via the recombinant DNA average molecular weight and the mass concentration. The population size of bacteria in each sample with 30 ng genomic DNA were obtained using calibration curve and its Ct value. To calculate the number of bacteria in one millilitre of ruminal fluid, initial volume of elicited DNA was multiplied by its dilutions. Afterwards, the microorganism's relative proportions to the total bacteria were calculated by quantitative competitive PCR. The relative proportion values were estimated by applying the $2^{-\Delta\Delta C_t}$ as explained by Chini et al. (2007). The PCR reaction mixture was composed of 10 µl of SYBR Premix Ex Taq (Takara Biotechnology Co., China), 0.4 µM of each primer and 30 ng of the elicited bacterial genomic DNA. The qPCR assay for each sample was carried out in triplicate. The primers applied for recognizing the microbes are listed in Table 2.

Statistical analyses

The ANOVA for averages of DMI, MY, FPCM, percentage of milk fat and protein, and FE, as well as MUN

concentration were analysed according to a completely randomized design with the repeated measures using PROC MIXED procedure of SAS (SAS Institute, 2018). The model included the fixed effects of nitrate supplementation, forage levels, days, and their interaction. The cow within treatments was used as a random effect. The covariance structure that yielded the smaller Akaike's information criterion was used as described by Cavanaugh and Neath (2019). All parameters of fermentation in the rumen, blood metabolites and the population of microorganisms were analysed by analysis of variance (ANOVA) based on a 2×2 factorial model that accounted for the main effects of nitrate supplementation and forage level in a complete randomized design. Differences among diets were considered significant at $P<0.05$. The gas production data and its parameters were analysed by analysis of variance using the general linear model for a randomized complete block design. All *in vitro* measurements were conducted in triplicates for each treatment.

Results

Intake and milk production

Our findings showed that increasing forage to concentrate ratio from 40% to 60% could augment DMI while reducing FPCM ($P<0.05$). Nevertheless, nitrate supplementation (*i.e.*, F40N and F60N) had no effect on DMI, MY and FPCM. Moreover, interaction effects indicated that the use of nitrate supplementation could reduce DMI with 60% forage level. Likewise, the changes in feed efficiency during the trial period were negatively correlated to DMI fluctuation ($P<0.01$). Same trend was observed for MY ($P<0.05$). Furthermore, despite the reduction in milk fat percentage with nitrate supplementation (*i.e.*, F40N and F60N), increasing levels of forage (*i.e.*, F60N) reduced ($P<0.05$) the negative impact of nitrate supplementation. However, increasing the level of forage plus dietary nitrate supplementation significantly reduced ($P<0.05$) MUN. Therefore, the interaction effects between nitrate addition and the forage level indicated that the level of forage strongly influences milk quality and quantity (Table 3).

Table 2. PCR primer sets used in the experiment

Item	Sequence 5'→3'	References
General bacteria	F ¹ : CCTACGGGAGGCAGCAG R ¹ : ATTACCGCGGCTGCTGG	Zhao et al. (2015)
<i>Bacteroidetes</i>	F: GGARCATGTGGTTTAATTCGATGAT R: AGCTGACGACAACCATGCAG	Guo et al. (2008)
<i>Prevotella</i>	F: ACTGCAGCGCGAACTGTCAGA R: ACCTTACGGTGGCAGTGTCTC	Fernando et al. (2010)
<i>Firmicutes</i>	F: GGAGYATGTGGTTTAATTCGAAGCA R: AGCTGACGACAACCATGCAC	Guo et al. (2008)
<i>Ruminococcus</i>	F: ATGCCGTAAAGGAAGCG R: CACACCTATCGCCTGATA	Christopherson et al. (2014)
<i>Fibrobacteria</i>	F: GGTATGGGATGAGCTTGC R: GCCTGCCCTGAACTATC	Fernando et al. (2010)
<i>Proteobacteria</i>	F: GAGTTTGATCMTGGCTCAG R: CAKAAAGGAGGTGATCC	Mitsumori et al. (2002)
Type II methanotroph	F: AGAGTTTGATCCTGGCTCAG R: CATCTCTGRCSAYCATACCGG	F: Edwards et al. (2008) R: Wise et al. (1999)
General archaea	F: GCTCAGTAACACGTGG R: GTGCTCCCCGCCAATTCCT	F: Wright and Pimm (2003) R: Watanabe et al. (2007)
<i>Methanobrevibacter ruminantium</i>	F: GGGGCTAATACCGGATAGATGATT R: CGACCTCAAGTTCAACAGTATCAC	Danielsson et al. (2017)
<i>Methanobrevibacter gottschalkii</i>	F: TCCGTAGCCGGTTTAATAAGTCT R: TTCCTCTATTTATCATAGGCGG	Danielsson et al. (2017)
<i>Methanoperedense nitroreducens</i>	F: GCAGCAATCACCAAGAAGAGAGG R: TGCCTCTTTGTGGAGGTACATGGA	Vaksmas et al. (2017)
General protozoa	F: GCTTCGWTGGTAGTGTATT R: ACTTGCCCTCYAATCGTWCT	Sylvester et al. (2004)
Ciliate protozoa	F: CAYGTCTAAGTATAAATAACTAC R: CTCTAGGTGATWWGRTTTAC	Kittelmann et al. (2015)

¹F: Forward and R: Reverse.

Table 3. Effect of nitrate (N) and forage level (F) on milk yield and feed efficiency in mid-lactation dairy Holstein cows

Item	Treatments ¹				±SE ²	P-values		
	F40	F40N	F60	F60N		N	F	N×F
DMI (dry matter intake: kg/d)	19.6 b	19.4 b	21.1 a	20.8 a	0.45	0.15	0.04	0.07
Milk yield (kg/d)	23.3 b	24.1 a	22.7 c	23.2 bc	0.25	0.08	0.06	0.05
FPCM ³ (kg/day)	22.0	22.4	21.8	21.7	0.34	0.19	0.06	0.08
Milk fat (%)	3.53 ba	3.41 c	3.60 a	3.50 b	0.04	0.04	0.02	0.03
Milk protein (%)	3.27	3.22	3.21	3.17	0.08	0.23	0.06	0.21
Lactose (%)	4.58 a	4.52 ab	4.45 bc	4.36 c	0.04	0.65	0.04	0.05
Milk urea nitrogen (mg/dl)	15.60 b	16.00 a	14.57 d	15.03 c	0.11	0.03	0.04	0.04
Feed efficiency								
milk/DMI	1.19 a	1.24 a	1.08 b	1.11 b	0.04	0.31	0.009	0.04
FPCM/DMI	1.12 a	1.15 a	1.03 b	1.04 b	0.03	0.27	0.008	0.05

¹F40: forage 40% without nitrate supplementation, F40N: forage 40% with nitrate supplementation, F60: forage 60% without nitrate supplementation and F60N: forage 60% with nitrate supplementation.

²SE: Standard error.

³FPCM (fat and protein corrected milk)=[(0.337+0.116×fat(%)+0.060×protein(%))×milk yield (kg/d)].

a, b, c – letters are used to indicate statistical significance (P<0.05) among different experimental diets.

Table 4. Effect of nitrate (N) and forage level (F) on blood metabolites and ruminal parameters in mid-lactation dairy Holstein cows

Item	Treatments ¹				±SE ²	P-values		
	F40	F40N	F60	F60N		N	F	N×F
Blood metabolite								
blood urea nitrogen (mmol/l)	6.40 c	6.53 b	6.63 b	6.74 a	0.05	0.007	0.02	0.009
hemoglobin (Hb) (g/l)	98.46	98.72	100.2	99.94	1.08	0.54	0.47	0.52
methemoglobin (% of Hb)	1.28 c	2.40 a	1.43 b	2.42 a	0.09	0.001	0.07	0.006
Rumen parameters								
pH	6.17 c	6.33 ab	6.28 bc	6.44 a	0.06	0.005	0.03	0.009
ammonia (mmol/l)	0.785 d	0.852 c	0.885 b	0.957 a	0.05	0.004	0.009	0.007
nitrate (µM)	5.60 b	103.05 a	5.67 b	103.58 a	0.85	0.008	0.12	0.001
nitrite (µM)	4.39 b	94.38 a	4.46 b	94.73 a	0.55	0.007	0.34	0.009
total VFA ³ (mmol/l)	115.4 a	114.3 b	113.8 bc	112.9 c	0.30	0.04	0.03	0.04
Individual VFA (% of total VFA)								
acetate	72.17 d	74.13 b	73.32 c	75.28 a	0.13	0.005	0.006	0.005
propionate	17.05 a	15.19 c	16.19 b	14.99 c	0.19	0.007	0.008	0.007
butyrate	7.26 a	7.17 ab	6.97 b	6.01 c	0.11	0.09	0.04	0.03
iso-butyrate	0.75	0.72	0.69	0.67	0.22	0.34	0.41	0.08
valerate	1.00	0.97	1.04	1.01	0.13	0.54	0.28	0.07
iso-valerate	0.61	0.63	0.62	0.64	0.14	0.67	0.45	0.59
Acetate:propionate ratio	4.23 c	4.88 a	4.53 b	4.96 a	0.19	0.04	0.06	0.04

¹F40: forage 40% without nitrate supplementation, F40N: forage 40% with nitrate supplementation, F60: forage 60% without nitrate supplementation and F60N: forage 60% with nitrate supplementation.

²SE: Standard error.

³Total VFA: total volatile fatty acids.

a, b, c, d – letters are used to indicate statistical significance (P<0.05) among different experimental diets.

Blood metabolites and rumen parameters

Per our results, daily intake of nitrate supplementation and forage level increased plasma concentration of BUN (P<0.01) but did not affect the plasma Hb concentrations. Nonetheless, meta-Hb concentration at 3 h post feeding was affected by nitrate supplementation (Table 4). Although, ruminal pH (measured at 3 h after feeding) was different (P<0.01) among groups, feeding nitrate and higher forage level increased (P<0.01) rumen NH₃-N and pH. Ruminal total VFA concentration (P<0.05) and propionate (P<0.01) along with butyrate (P<0.05) were

decreased by adding nitrate to the diets, and these differences were more noticeable with high forage level. Concentration of iso-butyrate and valerate were affected neither by dietary nitrate supplementation nor by forage level. Nitrate supplementation increased (P<0.001) concentrations of nitrate (103.2 µM) and nitrite (94.56 µM) in the rumen, whereas no forage level effect was found for these parameters (Table 4). Nevertheless, unlike forage level nitrate supplementation could significantly increase the ratio of acetate:propionate (P<0.05).

***In vitro* fermentation, total gas, and methane emission**

According to the results, nitrate inclusion was only effective on the VFA concentration at the forage level of 60% (from the beginning to 36 hours of incubation) ($P < 0.01$). Furthermore, the nitrate supplement and high forage significantly decreased total gas production ($P < 0.01$). Nitrate supplementation also resulted in lower ($P < 0.05$) methane production during 96 h of incubation. Rate of gas reduction and methane production were more

intense by nitrate inclusion at 60% forage level. On the one hand, forage level seemed to be inversely correlated with gas production potential (a+b) as well as b parameter, while nitrate inclusion did not affect the same factors. On the other hand, neither forage level nor nitrate supplementation were effective on gas production rate (c). Nevertheless, increasing forage level and nitrate inclusion had a more negative effect on a+b and b parameters (Table 5).

Table 5. Effects of nitrate (N) and different forage (F) levels on *in vitro* total volatile fatty acids, and total gas and methane production

Item	Incubation time (h)	Treatments ¹				±SE ²	P-values		
		F40	F40N	F60	F60N		N	F	N×F
Total VFA ³ (mmol/l)	2	28.17 a	27.28 ab	25.98 b	25.20 b	0.79	0.27	0.008	0.04
	4	34.90 a	33.33 ab	31.71 bc	30.72 c	0.85	0.31	0.006	0.03
	6	38.44 a	36.71 ab	35.26 bc	33.19 bc	0.93	0.42	0.01	0.05
	8	43.99 a	41.91 ab	40.77 ab	40.01 b	0.88	0.35	0.009	0.03
	12	51.61 a	50.80 ab	50.36 ab	49.32 b	0.51	0.51	0.04	0.05
	16	60.21 a	59.28 ab	59.04 ab	57.96 b	0.62	0.46	0.07	0.06
	24	66.88 a	66.32 ab	65.61 bc	64.54 c	0.74	0.67	0.08	0.09
	36	69.50 a	68.90 ab	68.30 ab	67.29 b	0.95	0.38	0.42	0.41
	48	71.15	70.23	69.97	68.99	0.99	0.32	0.41	0.39
	72	73.78	72.83	72.55	71.60	1.03	0.39	0.37	0.40
	96	76.08	75.51	74.88	73.82	1.02	0.55	0.46	0.49
Total gas (ml)	2	25.6 a	23.0 b	24.7 a	22.9 b	0.52	0.05	0.22	0.05
	4	45.8 a	43.3 b	45.0 a	45.2 a	0.49	0.04	0.35	0.05
	6	71.1 a	67.9 c	69.3 b	66.8 d	0.39	0.006	0.009	0.008
	8	90.3 a	87.1 b	86.6 b	84.0 c	0.37	0.005	0.009	0.007
	12	103.0 a	99.9 b	98.6 c	95.9 d	0.46	0.008	0.008	0.008
	16	116.9 a	113.2 b	113.6 b	109.7 c	0.65	0.004	0.009	0.006
	24	129.5 a	126.4 b	126.0 b	122.2 c	0.76	0.005	0.006	0.007
	36	140.7 a	136.9 b	136.2 b	132.5 c	0.69	0.003	0.007	0.006
	48	154.2 a	150.7 b	149.9 b	145.0 c	0.72	0.008	0.008	0.009
	72	169.8 a	165.9 b	166.4 c	161.7 d	0.51	0.007	0.006	0.007
	96	186.8 a	182.9 c	184.0 b	179.2 d	0.69	0.006	0.008	0.007
Methane (ml)	2	3.6	3.1	3.4	3.2	0.18	0.07	0.24	0.09
	4	6.8	5.9	6.4	6.0	0.32	0.08	0.35	0.09
	6	11.4 a	10.6 b	11.0 ab	10.0 c	0.24	0.04	0.08	0.05
	8	15.8 a	13.9 cd	14.7 c	13.5 d	0.37	0.03	0.07	0.04
	12	19.7 a	17.3 b	17.5 b	16.1 c	0.54	0.007	0.009	0.008
	16	22.8 a	20.5 b	21.1 b	19.2 c	0.39	0.005	0.03	0.008
	24	25.9 a	23.2 c	24.0 b	22.8 d	0.26	0.007	0.05	0.009
	36	29.0 a	26.5 c	28.1 b	25.6 d	0.39	0.004	0.02	0.007
	48	33.1 a	31.7 b	31.3 b	29.8 c	0.57	0.005	0.006	0.006
	72	37.3 a	35.8 b	35.5 b	33.9 c	0.45	0.007	0.009	0.008
	96	44.5 a	41.2 c	43.3 b	40.3 d	0.48	0.008	0.04	0.009
Estimated parameters ⁴									
a	—	17.91 b	16.51 b	19.51 a	19.08 a	0.56	0.19	0.005	0.008
b	—	153.65 a	152.35 a	149.87 b	145.68 c	0.75	0.003	0.004	0.004
c	—	0.061	0.061	0.057	0.056	0.003	0.46	0.41	0.43
(a+b)	—	171.56 a	168.85 b	169.37 b	164.76 c	0.42	0.009	0.007	0.008
RSD	—	10.83	10.71	10.77	10.59	0.11	0.33	0.37	0.36

¹F40: forage 40% without nitrate supplementation, F40N: forage 40% with nitrate supplementation, F60: forage 60% without nitrate supplementation and F60N: forage 60% with nitrate supplementation.

²SE: Standard error.

³Total VFA: total volatile fatty acids.

⁴Estimated parameters: a, gas production (ml) from quickly soluble fraction, b, gas production (ml) from insoluble but fermentable fraction, c, gas production rate, (a+b), potential gas production and RSD, residual standard deviation.

a, b, c, d – letters are used to indicate statistical significance ($P < 0.05$) among different experimental diets.

Table 6. Effect of nitrate (N) and forage levels (F) on microbial population in mid-lactation dairy Holstein cows

Item (per ml of rumen content)	Treatments ¹				±SE ²	P-values		
	F40	F40N	F60	F60N		N	F	N×F
General bacteria, Log ₁₀ copies, 16S	11.05 a	10.63 b	10.45 b	10.13 c	0.15	0.04	0.05	0.04
<i>Bacteroidetes</i> , % of bacteria	55.90 a	54.96 a	50.99 b	50.06 b	0.85	0.19	0.007	0.03
<i>Prevotella</i> , % of <i>Bacteroidetes</i>	26.4 a	23.8 b	21.1 c	18.3 d	0.67	0.006	0.005	0.005
<i>Firmicutes</i> , % of bacteria	31.5 a	29.5 b	29.1 b	28.0 b	0.75	0.04	0.34	0.05
<i>Ruminococcus</i> , % of <i>Firmicutes</i>	1.64 b	0.95 c	2.28 a	1.44 b	0.16	0.003	0.005	0.005
<i>Fibrobacteres</i> , % of bacteria	0.81 b	1.57 a	0.67 b	1.24 a	0.35	0.006	0.26	0.03
<i>Proteobacteria</i> , % of bacteria	3.02 b	5.27 a	1.33 d	2.44 c	0.24	0.008	0.009	0.007
Type II methanotroph, % of <i>Proteobacteria</i>	0.20 d	0.66 c	1.23 b	1.63 a	0.18	0.02	0.01	0.03
General Archaea, Log ₁₀ copies, 16S	7.20 c	7.10 c	7.76 a	7.38 b	0.08	0.08	0.008	0.009
<i>Methanobrevibacter ruminantium</i> , % of Archaea	34.08	33.84	33.53	33.35	0.15	0.45	0.56	0.49
<i>Methanobrevibacter gottschalkii</i> , % of Archaea	35.65	35.48	36.21	36.01	0.35	0.41	0.45	0.43
<i>Methanoperedense nitroreducens</i> , % of Archaea	0.000 c	0.005 b	0.002 b	0.036 a	0.01	0.008	0.01	0.009
General protozoa, Log ₁₀ copies, 18S	7.11 a	6.99 b	6.89 c	6.83 c	0.04	0.06	0.04	0.05
Ciliate protozoa, % of total protozoa	73.02 a	70.09 b	69.24 b	67.54 c	0.44	0.006	0.005	0.005

¹F40: forage 40% without nitrate supplementation, F40N: forage 40% with nitrate supplementation, F60: forage 60% without nitrate supplementation and F60N: forage 60% with nitrate supplementation.

²SE: Standard error.

Data were transformed (log) to obtain homogeneity of variance.

a, b, c, d – letters are used to indicate statistical significance (P<0.05) among different experimental diets.

Microbial community identification and quantitation

Our findings showed that all the identified and characterized ruminal microorganisms were affected (P<0.05) by nitrate supplementation and forage level except for *M. ruminantium* and *M. gottschalkii*. Rumen bacterial 16S gene and protozoa 18S copies number (log₁₀ copies per g DM) decreased with nitrate supplementation (F40N and F60N) versus control groups at both forage levels. Based on the analysis of ruminal bacteria community, nitrate supplementation numerically decreased *Prevotella* genus in the *Bacteroidetes* phylum (P<0.01), and *Firmicutes* (P<0.05). In contrary, nitrate addition increased *Fibrobacteria*, *Ruminococcus* genus in the *Firmicutes* phylum, *Proteobacteria* (P<0.01) and type II methanotroph in the *Proteobacteria* phylum (P<0.05). However, high forage level plus nitrate reduced the population of all bacteria except for *Prevotella* and type II methanotroph (Table 6).

Archaeal 16S gene copy number (Log₁₀ copies) tended to decrease (P=0.08) with nitrate supplementation compared with the control group. Nitrate supplementation at high forage level significantly decreased the *Prevotella* population compared with nitrate-free treatment. Percentages of archaea (*Methanobrevibacter ruminantium* and *M. gottschalkii*) were not affected by nitrate supplementation and forage levels. Furthermore, *Methanoperedense nitroreducens* increased (P<0.01) by the nitrate supplementation and high forage. Protozoa 18S copy number (log₁₀ copies per g DM) and ciliate protozoa were decreased (P<0.05) by nitrate supplementation in the mixed rumen sample. The effect of nitrate addition

on the reduction of protozoa and ciliate protozoa populations were exacerbated at high forage level (Table 6).

Discussion

The impact of nitrate supplementation on DMI and livestock performance has shown to vary from one to another experiment. Olijhoek et al. (2016) and Sharifi et al. (2019 b) demonstrated that DMI was not affected by nitrate supplementation. In disagreement, Hulshof et al. (2012) and Klop et al. (2016) showed that nitrate supplementation could reduce DMI. These differences between the reports could be associated with the feeding strategy (i.e., free nitrate or encapsulated, a single dose or as part of the total mixed ration, and restricted or *ad libitum* feeding). Furthermore, results of FPCM and MUN in the presence of nitrate were in agreement with the reports of Veneman et al. (2015) and Guyader et al. (2016). Van Zijderveld et al. (2011) and Olijhoek et al. (2016) showed that while nitrate addition could reduce milk fat percentage, its effects were insignificant on cow's performance and feed efficiency. The reduction in milk fat could be due to higher MY since FPCMs were rather comparable.

It is noteworthy that utilization of nitrate in ruminant nutrition could pose adverse effects on animal and human health. One of the indicators of health in livestock is the met-Hb level upon nitrate consumption. In spite of a 2.5% increase in met-Hb levels, its amounts in the blood of dairy cows were lower than the values to show clinical signs of methemoglobinemia (Van Zijderveld et al., 2011; Klop et al., 2016). Basically, for the develop-

ment of subclinical methemoglobinemia, the level of met-Hb is reported to be between 25 and 30% (Duthie et al., 2018).

In our experiment, $\text{NH}_3\text{-N}$ concentration in the rumen increased with nitrate addition (*i.e.*, F40N and F60N) (Table 4). The diets in this study were iso-nitrogenous, thus a significant difference in the ruminal $\text{NH}_3\text{-N}$ among groups could be due to the conversion of nitrate to ammonia which can through competition in hydrogen uptake relative to CO_2 ultimately reduce methane emission (Welte et al., 2016). In addition to ammonium, changing the VFA profile can indicate changes in rumen fermentation and microbial activity. Similar to our results, an increase in acetate and a decrease in propionate by the nitrate supplementation have been previously reported by Hulshof et al. (2012), Veneman et al. (2015), Zhao et al. (2015) and Sharifi et al. (2021). Nonetheless, increasing the acetate and reducing the propionate may only illustrate the increase in accumulated hydrogen, not the change in methane production. Reduction of propionate may hinder the full incorporation of accumulated hydrogen into propionogenesis. The collected hydrogen must preferably be addressed to alternative receptors with nutritional value such as propiogenesis (Olijhoek et al., 2016). The total ruminal VFA reduction, in the present study, was in agreement with the findings of Guyader et al. (2016), while others exhibited its insignificant effect on the same parameter (Hulshof et al., 2012; Veneman et al., 2015; Olijhoek et al., 2016). In contrast, Zhao et al. (2015) reported greater total ruminal VFA for nitrate fed cows. Ruminal VFA profile is an indicator of microbial activity (Sharifi and Khadem, 2012; Sharifi et al., 2016 a), thus its reduction by nitrate addition can be due to the effect of supplement on the activity of certain microbial species. Interestingly, nitrate supplementation increased *Fibrobacter* population contributing to augmented acetate production and acetate to propionate ratio (Veneman et al., 2015). On the one hand, higher ruminal pH in the presence of nitrate can make more appropriate environment for *Fibrobacter* activity. On the other hand, the activity of *Prevotella* (a genus known to produce propionate) (Sharifi and Khadem, 2012) is greatly reduced by nitrate supplementation. Correspondingly, the reduction of propionate as well as propiogenesis can be associated with a reduction in the population of *Prevotella*.

The results of gas technique showed that increasing the level of forage reduces the methane and gas production. While further studies illustrated that greater amount of rapidly fermentable carbohydrates per unit of feed essentially reduces the methane production (Maccarana et al., 2016). Nitrate inclusion along with an increase in forage level, intensified the reduction of gas volume and methane emission, presumably due to successful competition for H_2 uptake by nitrate- and nitrite-reducing microbes. Reducing methane emission by nitrate inclusion agrees with the reports of Lin et al. (2011), Sakthivel et al. (2012), Liu et al. (2017) and Sharifi et al. (2019 b). It

is of note that similar *in vitro* experimental setting with different substrates was used in those studies. The reduction of kinetic parameters in the present study, especially gas production by the soluble and insoluble portions of the diet with nitrate supplementation, was in agreement with those reported by Guo et al. (2009), Lin et al. (2011), Wang et al. (2016) and Sharifi et al. (2019 b). Although nitrate reduces gas volume and methane emission, yet, in accordance with the reports of Lin et al. (2011) and Liu et al. (2017), its effects were insignificant on VFA. Our findings do not agree with those demonstrated by Guo et al. (2009) and Wang et al. (2016) reporting the modulatory effects of methane mitigation agents on VFA profile.

Our results, in concurrence with the reports of Tapio et al. (2017) and Danielsson et al. (2017), showed that the population of general bacteria and protozoa, unlike the archaeal population, decreases with a rising forage level. Likewise, our results, similar to those reported by Veneman et al. (2015) and Zhao et al. (2015), showed that not only nitrate supplementation significantly could alter ruminal microbial flora, but it could also increase the ruminal population of *Fibrobacteria* and *Proteobacteria* (microorganisms known to reduce methane emission via absorbing hydrogen by participating in fumarate reductase activity). Contrarily, our observations illustrated that nitrate supplementation has a negative effect on the population of *Ruminococcus*, which can be due to the low period of habitability (Popova et al., 2019). Observations of Granja-Salcedo et al. (2019) pointed out that the lack of uniformity in the effects of nitrates on cellulolytic bacteria is associated with a short period of habitability. Furthermore, nitrate supplementation and changes in forage level had no effect on *Methanobrevibacter* (from archaeal group) which was positively associated with methane production (Veneman et al., 2015). Despite the high toxicity of nitrate on archaeal and related genus (Danielsson et al., 2017; Granja-Salcedo et al., 2019), its low dose may be safe or less toxic. It is noteworthy that species of archaea respond differently to nitrate addition (based on dose, form, etc.) and changes in H_2 concentration (Kittelmann et al., 2014).

Methane is the principal stable carbon compound in anaerobic environments that can enter the atmosphere without being oxidized by methanotrophs (Parmar et al., 2015; Annika et al., 2017). Nitrate supplementation increased the type II methanotrophs abundance and *Methanoperedense nitroreducens* in high forage diets, leading to methane production reduction in the rumen. However, the relative abundance of *Methanoperedense nitroreducens* was low. Nitrate plus high level of forage improved the *Methanoperedense nitroreducens* population yet high forage diet (with no nitrate) seemed to be more potent than the combination. Mitsumori et al. (2002) stated that *Proteobacteria* and type I methanotrophs were detectable in cattle rumen, but type II methanotrophs were not detected in this study. In agreement with the current study, Finn et al. (2012) and Parmar et al. (2015) reported the presence of type II methanotrophs in the rumen. Addi-

tionally, Finn et al. (2012) reported that the number of *Methylocystis* cells (sub-group of type II methanotrophs) of whole rumen contents across the four animals was 4.38×10^4 cells/ml. In contrary, Liu et al. (2017) could not detect anaerobic methanotrophic archaea in the fresh ruminal fluid and the ruminal fluid culture.

Conclusion

Increasing forage level did not affect blood metabolites but increased DMI, milk fat content, ruminal ammonia, and acetate, and decreased milk urea, lactose, total VFA. Nitrate supplementation suppressed total VFA, propionate and butyrate, and increased acetate concentration, and acetate to propionate ratio. Additionally, nitrate supplementation decreased rumen population of protozoa, archaea, and general bacteria except for *Fibrobacteres*, *Proteobacteria* and methanotrophs. Increasing the level of forage has exacerbated the effects of nitrate on dairy cows' performance and ruminal microbial population. Gas volume and its kinetics parameters, as well as methane emission were affected by nitrate addition. Methane emission reduction seemed to be positively associated with increased methanotrophs. However, it was determined that increasing the population of type II methanotroph was more effective than *Methanopere-dense nitroreducens* on nitrate supplementation. Increasing forage level stimulated the growth and propagation of type II methanotrophs. Therefore, this study demonstrated that methane emission could be decreased without drastic effects on the ruminal microbial population, dry matter intake and or lactational performance of the animal. Overall, according to our results use of 3.5% nitrate supplementation with 60% forage compared with 40% forage not only reduced methane emissions without severe effects on ruminal microbial population, but also did not have a significant negative effect on the performance of dairy cows.

Implications

This study showed that nitrate has the potential to improve rumen fermentation and microbial population in dairy cows, along with reduced methane emissions. Changing the proportions of the ruminal microbial population without altering livestock performance is one of the important factors that should be considered in reducing methane emission. These results suggest that methane production could feasibly be reduced by nitrate without its undesirable effects on ruminal microbial population and livestock performance.

Conflicts of interest

The authors do not have any conflicts of interest to declare.

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