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NITRATE SUPPLEMENTATION AT TWO FORAGE LEVELS IN DAIRY COWS FEEDING: MILK PRODUCTION AND COMPOSITION, FATTY ACID PROFILES, BLOOD METABOLITES, RUMINAL FERMENTATION, AND HYDROGEN SINK

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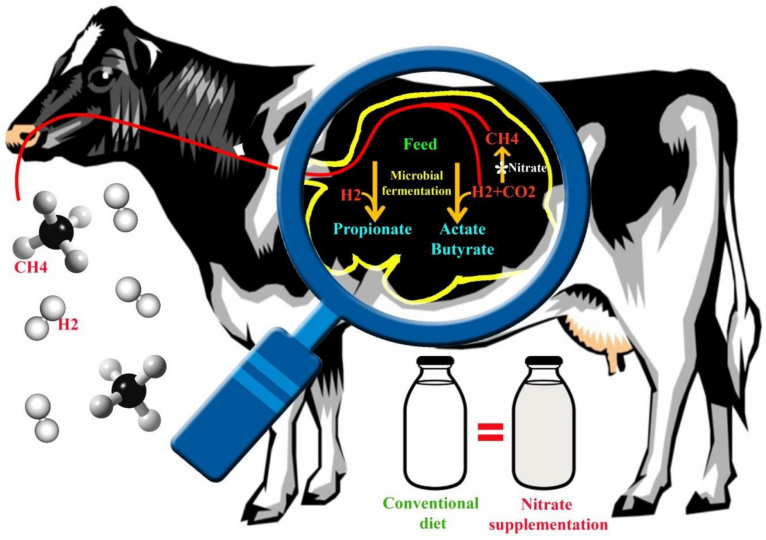
Abstract

Nitrate may reduce the ruminal methane emission by competing methanogenesis to achieve more hydrogen. For this purpose, twenty Holstein lactating cows were examined using a 2×2 factorial design in 4 groups for 60 days with two forage levels (40% and 60%) and supplemental nitrate 0% (F40 and F60) and 3.5% (F40N and F60N) of diet dry matter (DM). Then, the effect of nitrate and forage levels on cow performance, ruminal fermentation, methane emission, and metabolic hydrogen sink were evaluated. The nitrate supplementation did not significantly affect milk yield and ECM/DMI, while milk urea nitrogen was increased. Lowest quantity of milk vitamins (A and E) was observed in nitrate groups. The nitrate supplementation increased c9-C18:1, unsaturated fatty acids, and n-6/n-3 contents of the milk. Blood parameters were affected by nitrate supplementation. Blood met-Hb concentration was increased, while blood glucose was decreased in nitrate groups. High forage and nitrate fed animals (F60N) had higher ruminal acetate and lower propionate concentration, and higher acetate+butyrate to propionate ratio than other groups. Nitrite and NH_3-N concentrations were higher in the rumen of nitrate fed animals. Nitrate supplementation inhibited gas volume and methane emission without affecting volatile fatty acids at 12 and 24 h of incubation. The H_2 balance, H_2 production and consumption, and recovery percentage were significantly lower in F60N group. In conclusion, nitrate supplementation can be employed as an alternative strategy for improving ruminal fermentation, milk quality and methane inhibition.

Key words: blood parameters, methane emission, milk composition, milk fatty acids, nitrate

Abbreviations: blood urea nitrogen (BUN); dry matter intake (DMI); energy-corrected milk (ECM); feed efficiency (ECM/DMI); milk urea nitrogen (MUN); somatic cell count (SCC); volatile fatty acids (VFA).

Graphical abstract:



Despite the importance of transforming plants into useful products such as milk, the gas emission by ruminant accounts for approximately 7 to 18% of total global anthropogenic activities (greenhouse gas emissions) (Ungerfeld, 2015; Difford et al., 2018). The release of methane produced in the rumen to the atmosphere is 2–12% of ruminant gross energy intake (Palangi and Macit, 2021). Molecular H_2 is formed from the reducing equivalents by H_2-producing microbes and utilized by methanogens to generate methane (Wang et al., 2016; Duthie et al., 2018). In general, methane inhibition occurs with the accumulation of H_2-generated, which can change the production pattern of volatile fatty acids (VFA) and H_2 balance (Rooke et al., 2014; Capelari, 2018). To date, numerous methane production inhibitors have been investigated for their methanogenesis reducing potentials (Patra et al., 2017; Sharifi et al., 2019; Yin et al., 2020). Nevertheless, mitigating the methane production by these compounds would lead to increased H_2 and decreased acetate accumulations along with compensatory increases in accumulations of fermented acids such as propionate or butyrate (Oh et al., 2017; Zhang et al., 2018; Lan and Yang, 2019).

It has been previously demonstrated that feeding nitrate as alternative electron acceptor can effectively decrease methane production in sheep (Wang et al., 2016; de Raphelis-Soissan et al., 2017) and lactating dairy cows (Lee et al., 2017; Granja-Salcedo et al., 2019; Ku-Vera et al., 2020). Nitrate acts as a hydrogen receptor and effectively prevents the production and emission of methane in the rumen (Wang et al., 2016). Nitrate, in the presence of H_2, would reduce to nitrite ($NO^{3-}+H_2 \rightarrow NO^{2-}+H_2O$) and then to ammonium ($NO^{2-}+3H_2+2H^+ \rightarrow NH_4^+ +2H_2O$) leading to the accumulation of hydrogen (Ungerfeld, 2015). According to the Gibbs free energy hypothesis, the reduction of nitrate to nitrite and then ammonium produces more energy than methanogenesis (Dolfing and Hubert, 2017). Consumption of H_2 by nitrate is highly competitive compared to methane production by carbon dioxide, thus can considerably reduce methane production. Moreover, release of H_2 from the rumen increases with nitrate intake (Wang et al., 2016). In a recent meta-analysis, Lee and Beauchemin (2014) showed that high concentrations of dietary nitrate would result in a linear decrease in methane emission. Furthermore, Vlaeminck and Fievez (2005) suggested that milk fatty acids (FA) may be used as markers of ruminal microbial activity since those have a strong relationship with molar proportions of VFA in the rumen which are related to methane production (Van Gastelen et al., 2017; van Lingen et al., 2019; Williams et al., 2019). The VFA profile in rumen fluid may shift toward more acetate with nitrate intake. Nonetheless, propionate production, an H_2-consuming process, would decrease methane production (Guyader et al., 2016; Doreau et al., 2018). Accordingly, mitigation of methane emission should not have a negative effect on the milk FAs. The possibility of restoring the milk fat content and its profile in cows with dietary nitrate has been the subject of studies since there is a correlation between ruminal acetate and milk fat production (Sharifi et al., 2016; Khachlouf et al., 2018). Nonetheless, problems such as met-hemoglobin (met-Hb) and increasing urea in milk with nitrate utilization are very significant (Klop et al., 2016). In addition, Guyader et al. (2016) revealed that toxic effect of nitrate may inhibit the growth and propagation of fibrolytic bacteria and methanogens (Latham et al., 2018). Undesirable change of microorganism population can affect the digestibility of nutrients in the rumen due to changes in VFA concentration with increasing NH_3-N (Olijhoek et al., 2016; Sharifi et al., 2021). Also, reports indicate adverse effects of increased nitrate on the performance of dairy cows (Veneman et al., 2015; Klop et al., 2016). Nitrate toxicity can be reduced and or prevented in dairy cows using various techniques (e.g., gradually consuming nitrate for adaptation), but increasing the rapid fermentation energy of the diet by enhancing concentrate-to-forage ratio is one of the most important solutions (Iwamoto et al., 2001). The aim of our study was (1) to investigate the effect of nitrate on cow performance, methane emission, H_2 sink, and (2) to determine the potential of nitrate as an alternate electron acceptor in the ruminal fermentation. In this study we used high forage to reduce the rapid fermentation energy.

Material and methods

Animals, diets, and experimental design

This study was conducted at the SEPID DAM farm, in Tehran Province, Iran. Twenty Holstein lactating cows (with initial body weight of 520.5±19.6 kg, multiparous 36–40 months old, average milk yield of 23.5±1.1 kg and 122±9 days in milk) were used in the study. The adaptation and trial periods were 15 and 60 days, respectively. Lactating cows were housed individually in tie-stall facilities and had free access to water. Cows were milked 3 times daily (05:30, 13:30, and 21:30). The experiment had a 2×2 factorial design with 4 treatments and cows were randomly distributed into the experimental groups. The treatments were (1) F40; forage 40%, (2) F60; forage 40% without nitrate supplementation, (3) F40N; forage 40% and (4) F60N; forage 60% with nitrate supplementation. The diets, formulated as isonitrogenous (Table1), were offered three times daily (06:00, 14:00 and 22:00) right after milking. The feed amount offered was adjusted daily to obtain approximately 10% of orts (as-fed basis). Approximately 40% of the diet was offered at 06:00 h and the remaining 60% at 14:00 and 22:00 h. The study was in accordance with the guidelines of Animal Care Committee of the College of Agriculture of University of Tabriz.

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	71	71
Corn grain	130	130	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	10	10	30	30
Di-calcium 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 digestible nutrients (TDN)[4]	514	518	448	453
Crude fiber	98	97	124	123
Crude protein	165	166	164	165
Crude fat	28	29	43	42
Ash	99	101	110	112
Nitrate	0.05	18.1	0.09	17.5
Neutral detergent fiber (NDF)	281	282	317	315
Non fiber carbohydrate (NFC)[5]	414	417	366	365
Net energy for lactation (NEL)[6]	1.38	1.39	1.22	1.23
Fatty acids (g/100 g of total fat)				
Saturated fatty acids	50.99	51.02	55.29	55.00
Unsaturated fatty acids	49.01	48.98	44.71	45.00
Monounsaturated fatty acids	22.17	22.36	19.52	19.97
Polyunsaturated fatty acids	26.73	26.48	25.13	24.96
c9-C18:1	21.17	21.39	18.02	17.84
c9,c12-C18:2	23.04	22.86	21.97	21.76
c6,c9,c12-C18:3	1.69	1.62	1.16	1.20

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]Nitrate source with molecular formula: $5Ca(NO_3)_2\_NH_4NO_3\_10H_2O$; 75% NO_3 in DM.

[3]Mineral composition (mg/kg): Ca: 195000; P:90000; Mg:20000; Cu:280; Na:55000; Zn:3000; Mn:2000; I:100; Co:100; Se:1. Vitamin composition (IU/kg): vitamin A:600000; vitamin D_3:120000 and vitamin E:1300.

[4]TDN (%) = tdNFC + tdCP+(tdFA × 2.25) + tdNDF–7 (NRC, 2001).

[5]NFC = 100 – (Fat% + NDF% + CP% + Ash%) (NRC, 2001).

[6]NEL (Mcal/kg DM) = 0.0245 × TDN(%) – 0.12 (NRC, 2001).

Data collection

Feed samples were evaluated according to the method of Association of Official Agricultural Chemists (AOAC, 2005). The body weight of cows was recorded at 10-day intervals prior to the morning feed allotment. Dry matter intake (DMI) was recorded during trial. Furthermore, milk yield was recorded as the average milk produced in each day of the entire 60-day experimental period. Additionally, milk fat and protein contents, somatic cell count (SCC), milk urea nitrogen (MUN), cholesterol and vitamins A and E were analyzed every 6 days. The Lacti-Check ultrasound milk analyzer (Hopkinton, MA) was used to measure the fat and protein contents, while the SCC and MUN were determined using Fossomatic 90. The vitamins (A and E) were quantified using gas chromatography as described by Bae and Kratzsch (2018) and Levêques et al. (2019). An additional milk sample (from each of the animals) was collected, extracted, methylated and further analyzed for its FA content using gas chromatography as described by Klop et al. (2016). Individual milk FA were expressed as a fraction of total FA. Feed efficiency (ECM/DMI) was estimated by dividing energy-corrected milk (ECM (kg))=[0.327×(milk (kg))]+[12.96×(fat (kg))]+[7.2×(protein (kg))] by daily DMI of cows. For blood metabolite and urea nitrogen, blood samples (20 ml) were collected from the tail vein of the cows at 6 days using evacuated tubes containing EDTA at a level of 1.8 g/l of blood. The samples were kept on ice for 15 minutes after collection and then centrifuged at 1000×g for 20 minutes. Plasma was harvested and stored in polyethylene tubes at –20°C until further analysis. Beta-hydroxyl butyric acid (BHBA) concentration was measured by enzymatic analysis using an auto-analyzer (Ranbut, Randox, Crumlin, United Kingdom). Moreover, determination of met-Hb was carried out according to the method of Kiss et al. (2018). Rumen fluid samples (200 ml) were collected from each cow three hours after morning feedings at 6-day intervals, using an oral probe. The ruminal samples were filtered with two layers of cheesecloth. 5 mL of the strained rumen fluid samples were stored at –20°C after adding 1 ml of 25% meta-phosphoric acid solution for VFA analysis. To measure the ruminal NH_3-N, 10 ml of the rumen fluid were stored at –20°C. For the VFA measurement of rumen fluid, frozen samples were thawed at room temperature and centrifuged at 6000g for 10 min. The VFA was determined by gas chromatography (model no. CP-9002, Delft, The Netherlands) as outlined by Sharifi et al. (2016). NH_3-N was measured by the colorimetric phenol-hypochlorite method of Darabighane et al. (2020). Supernatant of the samples was used for nitrite measurements according to the method of Sakthivel et al. (2012).

Gas production and *in-vitro* fermentation characteristics

Gas production was measured according to the method of Fedorah and Hrudey (1983). Briefly, samples (0.25 g of finely ground TMR (1mm)) were weighed and transferred into 50 ml sterile serum bottles (triplicates of each treatment combination). The rumen fluid was obtained 2 h after morning feeding from the rumen of total mixed ration fed cow (Table 1). Rumen contents were filtered through 4 layers of cheesecloth into a warm flask containing CO_2 before transferring to the laboratory. All vials were warmed to 39°C and flushed with O_2-free CO_2 prior to the addition of inoculums. The vials were sealed and affixed to a rotary shaker platform (120 rpm). Additionally, triplicate blanks (vials containing no substrate) were incubated for each time point for correcting the gas produced from the inoculum. Gas production was recorded at 12 and 24 h using a water displacement apparatus. The gas volume was calculated using the following formula.

$$V = (Vt–Vb)\times 100/W$$

where:

V (mL) is the net volume of gas produced per g DM of diet sample,

Vt (mL) is the volume of total gas produced in sample bottles,

Vb (mL) is the average volume of total gas produced in blank bottles,

W (g) is the total weight of sample.

For estimation of methane, 100 µl of gas from headspace of the syringe was injected into gas-liquid chromatography as described by Danielsson et al. (2017). Fermentation processes were stopped after 12 and 24 h of incubation and subsequently the substrate was separated and oven-dried at 105°C. The DM disappearance was determined based on the difference in DM weights between before and after incubation. Liquid samples were collected at the start and at the end of incubation for NH_3-N, and VFA measurements. Amounts of fermented hexose, generated and consumed H_2 as well as H_2 recovery were calculated using the equation described by Demeyer (1991).

Hexose fermented=0.5×acetate+0.5×propionate+butyrate+valerate.

H_2 generated=2×acetate+propionate+4×butyrate+2×valerate+4×iso-butyrate+2×iso-valerate.

H_2 consumed=4×methane+2×propionate+2× butyrate +valerate.

H_2 recovery %= (H_2 consumed/H_2 generated)×100.

Fermentation efficiency was calculated using the following equation.

Fermentation efficiency = ((0.62×acetate+1.09×propionate+0.78×butyrate)÷(acetate+ propio-nate+butyrate))×100. The equation is based on the heats of combustion of glucose and the respective VFA (Latham et al., 2018).

Statistical analyses

Statistical analysis was performed as repeated measures data using the MIXED PROC model of SAS software 9.4 (2018). The model included the fixed effects of nitrate supplementation, forage levels, and their interac-

tion. We analyzed the *in-vitro* gas production data by analysis of variance (ANOVA), the general linear model (GLM) for a 2×2 factorial design. All treatments were conducted in triplicates.

Results

Dry matter intake, milk yield and milk composition

Effect of nitrate supplementation (*i.e.,* F40N and F60N cows) on DMI, milk yield, ECM, milk fat and protein contents, and ECM/DMI, as presented in Table 2, was insignificant. However, its effects were stimulatory on MUN and SCC (P<0.05). Higher forage levels increased SCC (P<0.05), whereas MUN and ECM/DMI were decreased (P<0.05) by the same diet. Vitamins A and E decreased (P<0.05) when diets were supplemented with nitrate (*i.e.,* F40N and F60N cows) (P<0.05). However, high forage increased (P<0.05) milk vitamins A and E while decreasing its cholesterol (P<0.05) (Table 2). Nitrate supplementation (F40N and F60N) increased C4:0, *cis-9* $C_{18:1}$; unsaturated FAs and n-6/n-3 in milk fat, although nitrate (*i.e.,* F40N and F60N cows) decreased *trans-10* C18:1 and C14:0 in milk fat. Furthermore, $C_{4:0}$, $C_{10:0}$, *cis-9 cis-12* $C_{18:2}$, saturated FAs, n-6/n-3, and atherogenicity index (AI) were decreased (P<0.05) in F60 cows. High forage fed cows (*i.e.,* F60) had the highest concentrations of *trans-10* $C_{18:1}$ as well as $C_{14:1}$ (P<0.01), unsaturated FAs and mono-unsaturated FAs (P<0.05) compared with other group (Tables 3).

Table 2. Effect of nitrate (N) and forage level (F) on quality of milk in mid-lactation dairy Holstein cows

Item	Treatments[1]				SEM	P-values		
	F40	F40N	F60	F60N		N	F	N×F
Milk yield (kg/d)	23.3	24.1	22.7	23.2	1.05	0.08	0.06	0.06
ECM[2] (kg/day)	23.8	24.2	23.6	23.5	1.06	0.22	0.06	0.09
ECM/DMI[2]	1.22 a	1.25 a	1.12 b	1.13b	0.04	0.29	0.007	0.01
Milk fat (g)	826.3	824.8	829.2	815.7	9.87	0.46	0.15	0.34
Milk protein (g)	764.1 a	779.4 a	738.7 b	739.3 b	8.05	0.18	0.006	0.01
SCC[2] (10^3 cells/ml)	105.4 c	115.6 b	113.4 b	119.2 a	13.9	0.008	0.009	0.01
Milk urea nitrogen (mmol/l)	0.865 b	0.888 a	0.808 d	0.834 c	0.03	0.05	0.03	0.04
Cholesterol (mg/dl)	15.94 a	15.67 ab	15.47 bc	15.20 c	0.21	0.36	0.07	0.09
Vitamin A (μg/dl)	40.3 b	37.3 c	44.3 a	41.6 b	1.01	0.02	0.009	0.01
Vitamin E (μg/dl)	91.0 b	88.6 c	95.3 a	92.8 b	0.97	0.009	0.005	0.007
Vitamin E: Cholesterol	26.6 ab	25.1 c	27.2 a	25.7 bc	0.61	0.04	0.08	0.05

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]Content: ECM, Energy-corrected milk=[0.327×(milk kg)]+[12.96×(fat kg)]+[7.2×(protein kg)] (Thanh and Suksombat, 2015); DMI, Dry matter intake; SCC, Somatic cell count.

a–d – least square means with different letters are different at P<0.05.

Table 3. Effect of nitrate (N) and forage level (F) on fatty acids profile in milk in mid-lactation dairy Holstein cows

Fatty acids (FA) (g/100 g milk fat)	Treatments[1]				SEM	P-values		
	F40	F40N	F60	F60N		N	F	N×F
1	2	3	4	5	6	7	8	9
$C_{4:0}$	2.42 b	2.57 a	2.15 c	2.40 b	0.07	0.05	0.03	0.04
$C_{6:0}$	1.06	1.09	1.01	1.04	0.12	0.47	0.34	0.41
$C_{8:0}$	0.80	0.85	0.75	0.80	0.18	0.62	0.51	0.55
$C_{10:0}$	2.59 a	2.51 a	2.32 b	2.24 b	0.17	0.43	0.04	0.05
$C_{12:0}$	2.75	2.82	2.64	2.71	0.29	0.46	0.51	0.52
$C_{14:0}$	11.25 a	10.86 b	11.15 a	10.74 b	0.15	0.008	0.33	0.01
$C_{14:1}$	2.19 b	2.13 b	2.43a	2.37 a	0.11	0.28	0.03	0.04
$C_{16:0}$	31.75	31.83	31.54	31.62	0.25	0.49	0.55	0.54
$C_{16:1}$	1.88	1.80	2.13	2.05	0.19	0.38	0.08	0.09
$C_{18:0}$	13.25	13.21	13.23	13.19	0.28	0.52	0.42	0.47
c9-$C_{18:1}$	22.42 c	22.94 ab	22.70 bc	23.24 a	0.21	0.03	0.08	0.05
t10-$C_{18:1}$	0.82 bc	0.68 c	0.98 a	0.83 ab	0.05	0.04	0.007	0.03
*t*11-$C_{18:1}$	1.04	1.02	1.23	1.21	0.17	0.21	0.07	0.10

Table 3 – contd.

1	2	3	4	5	6	7	8	9
$C_{18:2}$	3.42	3.35	3.35	3.27	0.29	0.36	0.09	0.27
*c*9,*c*12-$C_{18:2}$	2.17 a	2.15 a	1.59 b	1.57 b	0.23	0.17	0.05	0.01
*c*9,*t*11-$C_{18:2}$	0.73	0.71	0.89	0.87	0.11	0.32	0.06	0.09
*t*10,*c*12-$C_{18:2}$	0.039	0.037	0.047	0.045	0.009	0.63	0.55	0.59
$C_{18:3}$	0.50	0.48	0.53	0.51	0.16	0.47	0.41	0.45
*c*6,*c*9,*c*12-$C_{18:3}$	0.23	0.21	0.18	0.15	0.06	0.30	0.07	0.11
Other LCFA[2]	0.54	0.52	0.57	0.55	0.04	0.28	0.24	0.29
FA groups[3]								
SFA	67.14 a	66.93 a	66.05 ab	65.81 b	0.65	0.15	0.04	0.05
UFA	32.85 b	33.06 a	33.94 a	34.18 a	0.59	0.03	0.05	0.05
MUFA	28.37 c	28.69 bc	29.48 ab	29.83 a	0.37	0.24	0.04	0.05
PUFA	4.47	4.37	4.46	4.34	0.15	0.36	0.41	0.40
Indices								
UFA/SFA	0.48	0.49	0.51	0.51	0.03	0.54	0.44	0.49
MUFA/SFA	0.42	0.42	0.44	0.45	0.02	0.41	0.42	0.46
PUFA/SFA	0.066	0.065	0.067	0.065	0.004	0.33	0.29	0.32
n-6/n-3	9.49 bc	10.03 a	8.97 c	9.64 ab	0.21	0.03	0.04	0.04
AI[4]	2.42 a	2.36 ab	2.32 ab	2.26 b	0.04	0.08	0.04	0.05
TI[5]	3.49	3.45	3.45	3.41	0.08	0.45	0.52	0.49

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]LCFA: long chain fatty acids.

[3]FA group: SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[4]AI: Atherogenecity index=($C_{12:0}$+4($C_{14:0}$)+$C_{16:0}$)/(MUFA+PUFA) (Thanh and Suksombat, 2015).

[5]TI: Thrombogenecity index=($C_{14:0}$+$C_{16:0}$+ $C_{18:0}$)/(MUFA+n–6)/2+3(n–3)+(n–3/n–6) (Thanh and Suksombat, 2015).

a–c – least square means with different letters are different at P<0.05.

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

Item	Treatments[1]				SEM	P-values		
	F40	F40N	F60	F60N		N	F	N×F
Blood metabolite								
Glucose (mg/dl)	94.59 a	92.43 b	89.54 c	88.46 d	2.72	0.005	0.007	0.006
BUN[2] (mg/dl)	115.3 c	117.6 b	119.4 b	121.4 a	3.03	0.009	0.04	0.01
BHBA[2] (mg/dl)	17.47 d	18.19 c	19.09 b	19.81 a	0.69	0.02	0.008	0.009
Cholesterol (mmol/l)	8.89	8.83	8.81	8.76	0.05	0.19	0.23	0.22
met-Hemoglobin (umol/dl)	0.195 c	0.356 a	0.224 b	0.367 a	0.11	0.001	0.08	0.01
Vitamin A (µg/dl)	41.3 b	39.1 c	44.1 a	41.9 b	1.03	0.005	0.007	0.006
Vitamin E (µg/dl)	92.1 a	89.9 b	92.6 a	90.2 b	1.21	0.006	0.11	0.05
Rumen fermentation								
NH_3-N (mg/dl)	14.15 d	15.36 c	15.95 b	17.25 a	0.25	0.008	0.01	0.009
NO_2^- (mg/dl)	0.049 b	1.066 a	0.051 b	1.070 a	0.25	0.0007	0.65	0.001
Acetate (mM)	83.29 b	84.76 a	83.49 b	85.05 a	1.05	0.005	0.04	0.01
Propionate (mM)	19.67 a	17.36 c	18.43 b	17.16 c	0.12	0.008	0.03	0.009
Butyrate (mM)	8.38 a	8.09 ab	7.93 bc	6.79 c	0.14	0.09	0.03	0.04
Iso-butyrate (mM)	0.87	0.83	0.78	0.75	0.05	0.42	0.06	0.09
Valerate (mM)	1.14	1.11	1.18	1.13	0.02	0.35	0.07	0.10
Iso-valerate (mM)	0.70	0.72	0.71	0.72	0.03	0.51	0.47	0.55
A+B/P ratio[2]	4.66 c	5.36 ab	4.96 b	5.42 a	0.15	0.01	0.05	0.03

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]Content: BUN, Blood urea nitrogen; BHBA, beta–hydroxyl butyric acid; A+B/P ratio, acetate plus butyrate divided to propionate.

a–d – least square means with different letters are different at P<0.05.

Table 5. Effects of nitrate (N) and forage level (F) on fermentation characteristics during 12 and 24 h batch culture

Item	Incubation time (h)	Treatments[1]				SEM	P-values		
		F40	F40N	F60	F60N		N	F	N×F
pH	12	6.44	6.50	6.48	6.55	0.05	0.35	0.31	0.34
Ammonia (µmol/ml)		6.01 c	7.36 b	5.93 c	7.61 a	0.10	0.008	0.09	0.01
Disappearance (%)		36.2	35.4	34.5	34.5	2.32	0.08	0.06	0.07
Gas volume (ml)		103.0 a	99.91 b	98.61 c	95.91 d	3.06	0.007	0.005	0.006
Acetate (µmol/ml)		37.42 b	37.64 b	38.88 a	37.63 b	1.15	0.10	0.03	0.05
Propionate (µmol/ml)		7.60 a	6.87 b	6.09 c	5.91 c	0.28	0.29	0.009	0.01
Butyrate (µmol/ml)		4.02 a	3.63 b	3.40 b	3.46 b	0.18	0.06	0.25	0.05
Iso-butyrate (µmol/ml)		0.29	0.27	0.27	0.25	0.01	0.45	0.49	0.47
Valerate (µmol/ml)		0.51	0.48	0.51	0.48	0.04	0.57	0.55	0.59
Iso-valerate (µmol/ml)		0.27	0.26	0.25	0.23	0.04	0.61	0.53	0.59
A+B/P ratio[2]		5.46 b	6.01 b	6.94 a	6.96 a	0.27	0.17	0.04	0.05
pH	24	6.28 b	6.37 a	6.32 ab	6.42 a	0.04	0.06	0.25	0.05
Ammonia (µmol/ml)		7.90 c	9.33 b	7.87 c	9.81 a	0.21	0.007	0.06	0.01
Disappearance (%)		42.9 a	41.9 ab	41.2 bc	39.5 c	1.59	0.05	0.008	0.01
Gas volume (ml)		129.5 a	126.4 b	126.0 b	122.2 c	2.96	0.005	0.006	0.006
Acetate (µmol/ml)		47.49 c	49.14 b	50.65 a	49.25 b	1.29	0.29	0.04	0.009
Propionate (µmol/ml)		11.63 a	10.49 b	9.08 c	9.22 c	0.18	0.04	0.008	0.01
Butyrate (µmol/ml)		6.08 a	5.43 b	4.39 c	4.62 c	0.29	0.06	0.01	0.03
Iso-butyrate (µmol/ml)		0.35	0.33	0.33	0.30	0.02	0.57	0.52	0.49
Valerate (µmol/ml)		0.67	0.64	0.67	0.65	0.02	0.47	0.53	0.58
Iso-valerate (µmol/ml)		0.33	0.31	0.30	0.27	0.03	0.39	0.42	0.41
A+B/P ratio[2]		4.69 c	5.20 b	6.06 a	5.84 a	0.19	0.35	0.03	0.05

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]Content: A+B/P ratio, acetate plus butyrate divided to propionate.

a–d – least square means with different letters are different at P<0.05.

Table 6. Metabolic hydrogen sinks of different treatments

Item	Treatment[1]				SEM	P-values		
	F40	F40N	F60	F60N		N	F	N×F
1	2	3	4	5	6	7	8	9
H_2 balance at 12 h (µmol/ml)								
H_2	0.25 b	0.58 a	0.19 b	0.31 b	0.10	0.03	0.06	0.04
CH_4	7.30 a	6.75 b	6.82 b	6.31 c	0.19	0.04	0.05	0.05
Hexose fermented	27.04	26.36	26.39	25.72	2.81	0.31	0.08	0.10
H_2 generated	101.2	99.24	100.0	97.44	3.95	0.04	0.11	0.09
H_2 consumed	53.53 a	49.02 b	47.31 b	44.96 c	2.13	0.006	0.009	0.01
via CH_4	29.20 a	27.00 b	27.28 b	25.24 c	1.49	0.04	0.01	0.03
via VFA[2]	24.33 a	22.02 b	20.03 c	19.72 c	1.31	0.05	0.009	0.01
Recovery (%)	52.9 a	49.4 b	47.3 c	46.1 c	1.86	0.03	0.04	0.05
Fermentation efficiency (%)	70.6	69.9	69.1	69.1	2.29	0.07	0.10	0.09
H_2 balance at 24 h (µmol/ml)								
H_2	0.32 b	0.63 a	0.24 b	0.37 b	0.12	0.04	0.16	0.05
CH_4	9.76 a	9.25 bc	9.38 b	8.90 c	0.17	0.04	0.05	0.04
Hexose fermented	36.81 a	35.89 a	34.93 b	34.50 b	0.89	0.35	0.04	0.04
H_2 generated	136.3 a	133.7 b	131.2 bc	129.2 c	2.37	0.03	0.06	0.05
H_2 consumed	75.84 a	70.14 b	65.80 c	64.54 c	1.54	0.009	0.006	0.007

Table 6 – contd.

1	2	3	4	5	6	7	8	9
via CH_4	39.04 a	37.00 b	37.52 b	35.60 c	1.75	0.005	0.006	0.006
via VFA	36.80 a	33.14 b	28.28 c	28.94 c	1.82	0.04	0.02	0.04
Recovery (%)	55.6 a	52.5 b	50.2 bc	49.9 c	1.21	0.05	0.03	0.04
Fermentation efficiency (%)	71.7 a	70.9 a	69.8 b	70.0 b	1.55	0.06	0.04	0.05

[1]F40: forage 40% without nitrate supplementation; F40N: forage 40% with nitrate supplementation; F60: forage 60% without nitrate supplementation; F60N: forage 60% with nitrate supplementation.

[2]VFA, volatile fatty acids.

a–c – least square means with different letters are different at P<0.05.

Blood metabolite and *in-vivo* rumen parameters

The effect of experimental diets on blood metabolite and *in-vivo* rumen parameters are presented in Table 4. The increase in forage caused a decrease in blood glucose concentration but did not change plasma concentrations of met-Hb. Increasing forage level, similar to nitrate supplementation, increased the plasma concentrations of BUN and BHBA during the experiment. Higher met-Hb (P<0.001) and lower glucose (P<0.01) were observed in F40N and F60N cows (Table 4). Besides, vitamins A and E were decreased (P<0.05) when diets were supplemented with nitrate. However, using high forage level increased (P<0.05) blood vitamins A and E while decreasing cholesterol (P<0.05). The nitrate supplementation (i.e., F40N and F60N cows) decreased blood vitamin levels (A and E) (P<0.01) yet higher forage consumption (60%) increased the level of vitamin A (ineffective on vitamin E) (Table 4).

The concentration of ruminal NH_3-N was higher in nitrate supplemented and high forage groups (P<0.05). Ruminal nitrite concentrations were affected (P<0.001) by the nitrate addition in the diets (*i.e.,* F40N and F60N cows). Cows consuming high forage and nitrate supplementation (*i.e.,* F60N) had higher ruminal acetate and A+B:P ratio, but lower propionate and VFA concentrations (P<0.05) compared with other treatments (P<0.05). Butyrate, valerate, iso-butyrate, and iso-valerate concentrations were not affected by nitrate supplementation, but iso-butyrate concentration tended to be lower in high forage group (Table 4).

Gas production, *in-vitro* fermentation and H_2 balance

The results of gas production, *in-vitro* fermentation and H_2 balance are presented in Tables 5 and 6. According to the results, gas volume and methane emission of ruminal fluid samples were lower in nitrate supplemented and high forage groups (P<0.05) (Table 5). Consequently, not only the amount of H_2 equivalents of culture fluid increased due to the reduction of methane emission in the nitro-supplemented cultures, but also H_2 accumulations were higher after 12 and 24 h of incubation (P<0.05) than the control group (Table 6). High forage stimulated the acetate concentration (P<0.05) yet the effect of nitrate supplementation on the same parameter was insignificant. In addition, nitrate supplementation at 24 h and high forage at 12 and 24 h decreased propionate concentration, yet being ineffective on iso-butyrate, valerate and iso-valerate concentrations. Butyrate concentration was affected by dietary high forage, but nitrate addition did not affect these values. Adding nitrate to the ruminal batch culture caused an increase in NH_3-N concentration (P<0.01) but increasing the forage level from 40% to 60% could meaningfully increase the concentration of NH_3-N (Table 5). The H_2 balance was altered by nitrate supplementation at 12- and 24-hour incubation times, whereas consumed H_2 and its (P<0.01) recovery percent was decreased (P<0.05). Generated H_2 was decreased (P<0.05) by dietary nitrate at 24 h of incubation, although the value had a tendency towards reduction at 12 h of incubation. Nitrate addition did not affect fermented hexose yet fermentation efficiency was significantly lower at 12 and 24 h of incubation (Table 6).

Discussion

Dry matter intake, milk yield and milk composition

It is often assumed that nitrate supplementation, due to its toxicity potentials, has negative effects on animal performance. Reduced feed intake and milk productions are the anticipated symptoms of cows with mild nitrate-poisoning. Nevertheless, in accordance with earlier studies, nitrate supplementation had no effect on DMI (Veneman et al., 2015; Guyader et al., 2016; Sharifi et al., 2019). Although, Hulshof et al. (2012) and Klop et al. (2016) reported that DMI was decreased with nitrate supplementation. Differences in the reports could be due to the different levels of consumed nitrate and the variety of feed intake. Obtained data by Van Zijderveld et al. (2011) and Olijhoek et al. (2016) for ECM, fat and protein yield, and ECM/DMI in nitrate supplemented diet fed cows are similar to our observations. Furthermore, the increased milk yield, SCC, and MUN in the nitrate fed ruminants are consistent with earlier studies (Veneman et al., 2015; Guyader et al., 2016). In contrast, Klop et al. (2016) discovered no differences in milk yield and MUN in nitrate diet fed cows; however, milk yield was higher in nitrate plus fat supplement fed cows compared with the other groups.

It is noteworthy that to the knowledge of authors, effect of nitrate supplementation on milk vitamins has been reported only once. In the present study, vitamins A and E in milk were decreased by dietary nitrate. Similar to our results, Aytekin et al. reported the negative effect of nitrate intake on vitamins A and E in the blood (Aytekin and Aypak, 2011). Vitamins A and E are involved in regulation of meth-hemoglobin levels by eliminating the causative agents like the nitrate (Atyabi et al., 2012). Moreover, it is expected that with increasing levels of met-hemoglobin the level of blood vitamins would decrease.

To our knowledge, the effect of dietary nitrate on milk FAs composition has only been reported once. Our observations were comparable to the findings of Klop et al. (2016) reporting higher milk fat C4:0 and isomers of C18:1 in nitrate supplemented diet fed dairy cows. Unlike the other saturated short-chain FAs, C4:0 in milk fat does not need acetate for its production as it can be produced directly from blood derived BHBA. Moreover, in our study the concentration of blood derived BHBA was higher in nitrate supplemented groups. Similar to our results Klop et al. (2016) reported lower *trans-10* C18:1 in nitrate supplemented group. Contrary to our findings, in the study of Klop et al. (2016) nitrate addition had no effect on C14:0, UFA and n-6/n-3. In this regard, Duthie et al. (2018) described that trans-10 FAs as a ruminal biohydrogenation intermediate, decrease upon nitrate feeding and absorption. While, absence of the trans-10 FAs can stimulate de novo synthesis of FA in the mammary gland. Nitrate supplementation had no effect on C18:0, which is indicative of bio-hydrogenation in the rumen. Finally, AI was decreased which is a sign of enhanced immune response in humans (Klop et al., 2016; Duthie et al., 2018).

Blood metabolite and *in-vivo* rumen parameters

In recent years, analyses of blood metabolites for diagnosing nutritional imbalances have become increasingly important due to their simplicity and reproducibility. In this study, plasma concentrations of glucose, BHBA, BUN, and met-Hb in all treatments as presented in Table 4 were significantly different ($P<0.05$). The BHBA level is considered as an indicator of energy balance. Increasing BHBA content by nitrate supplementation may indicate partial utilization of feed energy for conversion of nitrate into harmless compounds, thus resulting in its lower availability to the animal (Wankhade et al., 2017; Duthie et al., 2018). Studies (Veneman et al., 2015; Klop et al., 2016) have reported the higher level of met-Hb upon nitrate consumption in lactating dairy cows. Concentrations of 30% and higher would lead to subclinical methemoglobinemia (Duthie et al., 2018).

Ruminal NH_3-N, nitrite, acetate, and A+B/P ratio were greater in nitrate supplemented diet fed animals compared with the control group. Ruminal NH_3-N in the present study is in agreement with those reported by Olijhoek et al. (2016) and Guyader et al. (2016) for lactating dairy cows. In those studies, the nitrate was not replaced by another nitrogen source in the control diet, explaining the greater NH_3-N amounts in the nitrate supplemented diets. Nevertheless, we expected this difference since our experimental diets were iso-nitrogenous and feed intake was similar for all the groups. The inhibitory effect of nitrate on ruminal methane emission can be due to reduction of nitrate and nitrite to NH_3-N competing for H_2 with CO_2 (Zhou et al., 2017). In our study, the increased ruminal NH_3-N concentration in response to the addition of nitrate supports this theory. Likewise, our results are comparable with the observations of Veneman et al. (2015) where the proportion of acetic acid and A+B/P ratio increased, and propionic acid decreased in response to nitrate. In contrary, Zhao et al. (2015) and Olijhoek et al. (2016) found that nitrate addition had no effect on acetate, propionate, and A+B/P ratio. Moreover, our results agreed with those of Veneman et al. (2015), Hulshof et al. (2012) and Olijhoek et al. (2016) reporting the insignificant effect of nitrate (F40N and F60N) on VFA concentration. This finding might be due to the numerically reduced feed intake. In contrast, Zhao et al. (2015) found a greater total VFA concentration in nitrate supplemented diet fed animals compared with the other experimental groups.

The results of this study revealed that increasing forage level intensifies the effect of nitrate on ruminal nitrite and NH_3-N. Increasing the concentrate-to-forage ratio improves ruminant resistance to large amounts of nitrate or NH_3-N (van Zijderveld et al., 2011). Thus, high forage due to decreasing the rapid-digesting carbohydrates will illustrate the negative effects of nitrate on the fermentation process more than low forage level. In this regard, Lee et al. (2017) showed that increasing the fast-digesting carbohydrates of the diet in heifers reduces the ruminal NH_3-N and BUN concentrations. Reducing the amount of fast-digesting carbohydrates when consuming nitrate causes an increase in urinary-N to make the animal more tolerant to nitrate or excess NH_3-N in the rumen (Lee and Beauchemin, 2014). Sharifi et al. (2019) reported that by providing rapid-digesting carbohydrates and its simultaneous release with nitrate, not only the microbial mass would increase, but also the concentration of ruminal NH_3-N, nitrite, and BUN would be significantly reduced. Consistent with the above results, this study revealed that high forage intensifies the effect of nitrate on reducing propionate and increasing NH_3-N, nitrite, and acetate in the rumen. Therefore, the use of nitrate as a dietary supplement to reduce methane emission requires the provision of fast-digesting carbohydrates and its simultaneous release with the accumulation of nitrate in the rumen.

Gas production, *in-vitro* fermentation and H_2 balance

Results from the batch culture experiment confirmed the methane-inhibiting activity of nitrate, although the inhibition attribute of the compound was more prominent in F40N group compared with other treatments. Similar

results on gas and methane production were reported by Lin et al. (2011), Maccarana et al. (2016) and Sharifi et al. (2019). Methane in the rumen is mainly produced by methanogens using CO_2 and H_2. Since H_2 is the main limiter of methane production by methanogens, its consumption by other compounds can prevent the formation of methane. Therefore, conversion of nitrate to ammonium by consuming H_2 leads to lower availability of dissolved molecular hydrogen for methane production (Zhou et al., 2017). Another inhibition mechanism of nitrate on methane production could be nitrite's (the first intermediate of nitrate reduction) toxicity on methanogens (Zhou et al., 2015). In addition, Guo et al. (2009) demonstrated that nitrate supplementation can decrease NH_3-N in ruminal batch culture. In this regard, Lin et al. (2011) described that rapid hydrolysis of nitrogen from urea apparently accounted for a greater NH_3-N concentration than that from nitrate, which can cause higher accumulation of NH_3-N in the batch culture. In agreement with our results, Liu et al. (2017) reported that nitrate supplemented diets can increase NH_3-N in ruminal culture. Under *in-vitro* experimental setting, the nitrate inclusion resulted in higher acetate and lower propionate, irrespective of forage levels. Anderson et al. (2010) found that nitrate supplementation had no effect on acetate, propionate, butyrate and short chain fatty acids. The unchanged VFA production in our study could be due to the moderate nitrate supplementation, which overlaps with the H_2 sink and methanogens toxicity. In H_2 sink, nitrate promotes the H_2 formation and acetate production as a result of facilitation of H_2 consumption. But for methanogens toxicity, nitrate impaired healthy microbial system of H_2 utilization, thus hindering H_2 formation (Wang et al., 2014). In the present study, both expressed states can be confirmed by increasing acetate and H_2. The H_2 balance could be estimated through the flow of reducing equivalences according to the stoichiometry of main anaerobic pathways of H_2-producing and H_2-using reactions. In this regard, it was found that nitrate supplementation increases the H_2-generated, while the percentage of recovery decreased due to the reduction of less H_2-consumed (Wang et al., 2016). Furthermore, Anderson et al. (2010) showed that H_2-consumed and recovery percent were decreased significantly; while hexose fermented and H_2-generated were increased with nitrate supplementation. Nitrate supplementation lowered H_2 recoveries, which could be attributed to VFA production, especially the low acetate production in comparison with the control group. In agreement with our result, Anderson et al. (2010) demonstrated that H_2 gas was significantly higher in nitrate containing batch culture. It is generally assumed that the H_2 gas accumulation will cause an increase in intracellular NADH/NAD ratios which would decrease the overall fermentation efficiency and hexose fermented by limiting availability of required cofactors for glycolysis. Anderson et al. (2010) despite our observations showed that the H_2 gas increased at 12 and 24 h incubation by nitrate. Our stoichiometric H_2 balance estimates indicated that the amounts of fermented hexose and fermentation efficiencies were not compromised within these cultures.

Conclusions

Overall, the results of this study showed that nitrate would increase milk production, milk protein content, cis-9 C18: 1 concentration, unsaturated factors and n-6 /n-3, yet would also increase MUN and impose negative effects on vitamins A and E. In addition, the negative effects of nitrate increased significantly with increasing forage level. Therefore, reducing forage levels is a good way to reduce nitrate toxicity. Also, the outcomes exhibited that nitrate supplementation in diets had no effects on DMI, milk yield, milk fat, protein yields and ECM/ DMI. However, high forage feeding improved C14:1, trans-10 C18: 1, unsaturated FAs and monounsaturated FAs. Despite the increase in acetate concentration and A+B/P ratio, nitrate caused adverse changes in ruminal parameters like increased ruminal nitrite and NH_3-N concentrations, decreased propionate, decreased VFA, and increased met-Hb and BUN in the blood. By reducing the level of forage consumption and increasing the concentration of concentrate, the toxicity of nitrate in the rumen and blood can be effectively reduced. Furthermore, the results indicated that nitrate supplementation has no effect on VFA and its profile in batch culture. Therefore, nitrate supplementation, unlike forage level, cannot be effective in transferring energy from methane to fermented products such as acetate and propionate. Notwithstanding the reduction in methane emission by nitrate supplementation at both forage levels, the results showed that the nitrate addition and high forage increased the gas volume due to the reduction in H_2 consumption and recycling percentage.

Implications

This study showed that nitrate can improve rumen fermentation and milk production in dairy cows while decreasing *in-vitro* methane emission. Despite the decline in methane emission, this study showed that the transfer of hydrogen from CO_2 to other compounds did not lead to the production of volatile fatty acids and increased hydrogen gas in the rumen. These results suggest that methane could feasibly be mitigated by nitrate without undesirable changes in livestock performance.

Conflicts of interest

The authors have no conflicts of interest to declare.

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