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Dynamics of *in vitro* rumen methane production after nitrate addition

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ABSTRACT

The present study aimed to assess the dynamics of rumen methane (CH_4) production following the addition of $NaNO_3$. This was done using an *in vitro* rumen fermentation system that ensures continuous gas and methane assessments. Four different levels of $NaNO_3$ were used to get the final nitrate concentrations of 0.5, 1.0, 1.5, and 2.0 mg/ml of rumen fluid. For each dose, corresponding controls contained sodium chloride and urea were realised to ensure comparable levels of sodium and nitrogen. The addition of nitrates had slight effect on the intensity of fermentation because the total gas produced minus CH_4 (total methane-free gas) only went down at the highest dose (2.0 mg/ml), and the final concentrations of SCFA were the same at all doses. The most evident effect was a modification of the SCFA profile (low concentrations of propionate and valerate, progressive increments of acetate, and decreases of butyrate) and a reduction in overall CH_4 production. The CH_4 yield for the 0.5 mg/ml dose was not different from control in the entire fermentation. Yield of the 1.0 mg/ml dose was significantly lower than the control group ($p < 0.05$) only within the initial 24-h period, and higher dosages (1.5 and 2.0 mg/ml) were lower during the entire fermentation ($p < 0.01$). Methane yields were well fitted with the Gompertz model, but only the highest level of nitrate inclusion had a significant impact on the majority of model parameters ($p < 0.01$). The linear regressions between CH_4 yields (y) and the amounts of nitrates (x) at progressive fermentation durations (e.g. 6, 12, 24, and 48 h) produced equations with increasing absolute slopes (from −0.069 to −0.517 ml/mg of nitrate). Therefore, nitrate reduced rumen CH_4 yield in a dose-dependent manner: the impact of low doses was primarily observed at the initial stages of fermentation, whereas high doses exhibited effectiveness throughout the entire fermentation process. In conclusion, in batch fermentation systems, the dose effect of nitrates on methane yield was time dependent.

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Methane; sodium nitrate; rumen; *in vitro* fermentation; methane dynamics

1. Introduction

Nitrates (NO_3^-) added to ruminant diets have anti-methanogenic properties as they represent an alternative electron acceptor, diverting the rumen hydrogen flow away from carbon dioxide reduction (Olijhoek et al. 2016; Yang et al. 2016). Thermodynamically, the NO_3^- reduction to NH_3 is the preferable pathway when it was compared to methanogenesis due to

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the lower Gibbs free energy (ΔG^0, −599.6 *vs* −136 kJ/mol, respectively; Latham et al. 2016). However, the NO_3^- supplementation needs to be carefully evaluated due to possible side effects on animals. In fact, during the conversion to NH_3, the toxic intermediate nitrite (NO_2^-) can accumulate and be absorbed, interacting with the haemoglobin and altering its capacity to carry oxygen (Lee and Beauchemin 2014). As emerged from the meta-analysis conducted by Feng et al. (2020), who evaluated the possible negative effect of NO_3^- supplementation *in vivo*, 1% to 2% of nitrate in the dietary DM was identified as an acceptable dosage, reducing methane production by about 13–14%.

However, there are still important challenges in future research on this topic, mainly connected with the utilisation of the most suitable type of chemical as NO_3^- source (Almeida et al. 2022) and the possible attenuation of NO_3^- toxicity using encapsulation to slow down the release in the rumen (Feng et al. 2020). An additional aspect that warrants investigation in future studies is the impact of NO_3^- as a hydrogen acceptor on rumen fermentation stoichiometry. Studies by Janssen (2010), Wang et al. (2014), and Wenner et al. (2020) emphasised the significance of NO_3^- in the regulation of hydrogen (H_2) balance, consequently affecting methane (CH_4) production. Consequently, the utilisation of dietary NO_3^- could be a useful model for investigating the intricate dynamics of fermentation stoichiometry within the rumen.

In vitro rumen batch fermentation systems are largely used to test a wide number of additives to reduce CH_4 yield, including NO_3^- (Yáñez-Ruiz et al. 2016). Several *in vitro* batch studies (Patra and Yu 2014; Wu et al. 2019) were focused on a single incubation time and specific NO_3^- dose, preventing the investigation of possible interaction between the fermentation phase and supplementation level. Recently, we have tested (Braidot et al. 2022) a new *in vitro* fermentation system that allows continuous gas and CH_4 assessment, and thus main aim of the present research was to evaluate the dynamics of CH_4 production for different NO_3^- dosages over time. Our tentative was to analyse the temporal changes in gas and CH_4 yield to better understand the dynamics of CH_4 formation.

2. Material and methods

2.1. Experimental design

The experiment consisted of four fermentation runs conducted over consecutive periods (weeks), utilising $NaNO_3$ (Sigma Aldrich, Milan, Italy) as the source of nitrate ions.

The $NaNO_3$ was added to the substrate to achieve a final NO_3^- concentration of 0.5, 1.0, 1.5, and 2.0 mg/ml of incubated rumen fluid. For each dosage, a respective control was prepared with the addition of sodium chloride (Sigma Aldrich, Milan, Italy) and urea (Carlo Erba, Milan, Italy) to guarantee a comparable amount as mg of sodium and nitrogen (ratio 1:1,95) (Table 1).

2.2. In vitro *experiment*

The *in vitro* apparatus is composed of eight fermentation glass bottles with a total available capacity of 750 ml. The gas originated during the fermentation process flows from the fermenters to the gas counter and then to the infrared gas analyser sensor,

Table 1. Dosages of the chemicals used in the experiment.

	Chemical additions [mg/ml rumen fluid]							
	NaCl+Urea				$NaNO_3$			
Dose[a]	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Chemicals, [mg]								
Urea (CH_4 N_2 O)	39	79	118	158	–	–	–	–
Sodium Chloride (NaCl)	76	152	228	305	–	–	–	–
Sodium Nitrate ($NaNO_3$)	–	–	–	–	112	224	336	448
Nitrate (NO_3^-)[b]	–	–	–	–	82	165	247	330

[a]The doses are expressed in mg of NO_3^- for each ml of rumen fluid. In control bottles, the doses correspond to amounts of NO_3^- added to the corresponding treated bottles.
[b]Nitrate amounts calculated from sodium nitrate addition.

which continuously detects and registers gas volume and methane concentration (Braidot et al. 2022).

The fermenters were filled with 500 ml of buffered rumen fluid (rumen fluid: buffer, 1:2, v/v) following the methodology proposed by Menke et al. (1979). The rumen fluid was collected in the same slaughterhouse from culled dairy productive cows and was delivered within half an hour after collection to the laboratory in airtight bottles refluxed with CO_2 and maintained at 39°C.

A total mixed ration for ruminants based on corn silage (crude protein: 14% DM; neutral detergent fibre: 33% DM) was used as substrate (3300 mg of DM/fermentation glass bottle) in all experiments. The substrate was grounded at 0.5 mm length, weighted, and introduced in each bottle as dry material. The fermenters were hermetically sealed and incubated in a water bath at 39°C for 48 h.

2.3. Sampling of fermentation fluid and analysis

At the end of the incubation, pH was directly measured (GLP 22, Crison Instruments, S. A. Barcelona, Spain), while samples of fermentation fluid were collected for NH_3 (10 ml) and short-chain volatile fatty acid (SCFA, 5 ml of rumen fluid added with 5 ml of H_2SO_4 0.01 N) determinations and were stored at −20°C until being analysed. Rumen fluid for protozoa count (5 ml) was also collected at the end of incubation and added with an 18.5% formaldehyde solution (1:1, v/v). Protozoa were quantified using a microscope as described by Dehority (2003). The NH_3 concentration was determined using an Ammonia Gas Sensing Combination Electrode (Hach Company, Colorado, USA). The SCFA samples were centrifuged at 20,000 g for 20 min at 4°C, and the supernatant was filtered using a polypore filter (RC 0.45 µm, 25 mm, DTO Servizi Srl, Venice, Italy). The filtrate was transferred into autosampler vials, and 20 µl were injected into HPLC (Shimadzu Corporation, Kyoto, Japan). The instrument was composed of an LC-20AT pump, a vacuum degasser, a Prominence SPD-M20A photodiode-array detector, a Prominence SIL-20AC HT autosampler, and a Prominence CTO-20AC column oven set at 40°C. The HPLC separations were obtained using an Aminex HPX-87 H column (300 mm x 7.8 mm) with a pre-column (Bio-Rad, Hercules, California, USA). Sulphuric acid 0.008 N was used as the mobile phase at a flow rate of 0.6 ml/min. Full spectra were recorded in the range of 190–400 nm, and the optimum wavelength detection for all SCFA was found to be 220 nm. Peaks of analyses were compared with the retention times

of a standard mixture, and quantification was based on the external standard method. SCFA standards for acetic acid, propionic acid, butyric acid, iso-butyric acid, iso-valeric acid, and valeric acid were obtained from Merck (Darmstadt, Germany). From the SCFA profile composition (in mM), the net hydrogen produced was estimated as reported by Wang et al. (2014):

$$H_2 \text{ mM} = 2[\text{acetate} + \text{n-butyrate} + \text{iso-butyrate}] - [\text{propionate} + \text{iso-valerate} + \text{valerate}].$$

2.4. Calculations and statistical analysis

Cumulative methane production was recorded continuously for 48 h and measurements at each hour were fitted using the Gompertz equation:

$$Y_t = B\left(1 - exp^{\left(-Cexp^{-At}\right)}\right)$$

where Y_t is the CH_4 produced (ml) at a specific time (t), B is the asymptotic methane volume (ml), C is the specific CH_4 production rate (1/h) dependent on time (t), A is a constant that describes the decline of production rate. From the equation, other parameters have been calculated: the lag phase (Lag), the maximum fermentation rate (MFR), and the time needs to reach the maximum fermentation rate (TMFR) as reported by Lavrenčič et al. (2015).

The kinetics parameters derived from the Gompertz model and fermentation data measured at the end of incubation (total gas, methane-free gas, CH_4 percentage, protozoa count, total SFCA content, SCFA composition, ammonia content, net H_2 produced) were statistically analysed with SAS software (Version 9.4, SAS Institute Inc., USA) and the following multifactorial model:

$$Y_{ijk} = \mu + a_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

where Y_{ijk} is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation run (i = 1,4); β_j is the fixed effect of the dose (j = 1,4); γ_k is the effect of the type of addition (k = 1,2; NO_3^- or urea and NaCl) and ε_{ijk} is the residual error.

The CH_4 produced at 6-h progressive times (e.g. at 6, 12, 18, 24, 30, 36, 42, and 48 h) was analysed with the previous model added with the fixed effect of time (σ_z; z = 1,8) in a factorial model with repeated measures, taking in account all possible interactions between factors. The MIXED procedure of SAS Software was used for the analysis.

The linear relationship between the amount of NO_3^- added (X) and the methane yield (Y) was tested at 4 selected times of fermentation (6, 12, 24, and 48 h) considering the effect of the separate fermentation runs. The MIXED procedure of SAS Software was used as described by St-Pierre (2001) and the model applied was the following:

$$Y_{ij} = a + bX_i + \gamma_j + \varepsilon_{ij}$$

Where Y_{ij} is the CH_4 produced for the inclusion level *i* of the *j* run, *a* represents the overall intercept, *b* is the overall regression coefficients, X_i is the level of NO_3^- inclusion expressed in total mg (*i* = 82, 165, 247, and 330 mg), γ_j is the random effect of the run (*j* = 1,4), and ε_{ij} is the residual error. The coefficient of determination (R^2) was obtained from

a single-factor regression analysis between the adjusted values derived from the mixed model and the measured values.

3. Results

Table 2 reports the main fermentative traits at the end of the fermentative process (48 h). Ammonia, total SCFA concentration, and protozoa count in the fermentation fluid were not changed by factors considered, whereas there was an increment in final pH with the increasing dosages ($p < 0.01$) regardless of the type of chemical addition. For the total gas, the total methane-free gas and the CH_4 concentration, the interaction of the model was significant ($p < 0.01$). The additions of urea and NaCl never determined significant variations, while NO_3^- doses of 1.5 and 2.0 mg/ml lowered ($p < 0.01$) total gas and the total methane-free gas compared with other doses. Only the highest NO_3^- addition determined a significant reduction in the CH_4 concentration compared with lower doses.

The NO_3^- supplementation had a significant impact on the SCFA profile. The interaction between factors was significant ($p < 0.01$) for the concentration of acetate and butyrate, that showed no significant variations for the NaCl and urea additions, while they were modified by nitrate additions. The acetate showed higher concentrations than the respective controls at 1.0 (67.8 *vs* 62.4% of total SCFA concentration), 1.5 (67.9 *vs* 62.9% of total SCFA concentration), and 2.0 mg/ml (68.1 *vs* 61.9% of total SCFA concentration). The lowest nitrate dosage showed similar concentrations as the respective control (63.9 *vs* 62.6% of total SCFA concentration). On the contrary, the butyrate showed a lower concentration with respect to the controls at the NO_3^- doses of 1.0, 1.5 and 2.0 mg/ml. Furthermore, the NO_3^- addition

Table 2. Main fermentative traits at the end of the fermentative process (48 h) for the different inclusion levels of sodium nitrate ($NaNO_3$) or other chemicals (NaCl + urea)[a].

	Chemical additions [CA, mg/ml rumen fluid]											
	NaCl+Urea				$NaNO_3$							
Dose[b]	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	CA	Dose	CA* Dose	RMSE
pH	6.71	6.73	6.75	6.76	6.71	6.75	6.76	6.75	NS	**	NS	0.019
NH_3 [mg/dl]	67.6	71.1	71.6	76.7	66.9	74.3	74.9	81.4	NS	NS	NS	8.04
Total protozoa, 10[c] [cell/ml]	86	82.4	86.8	90	77.2	80.4	95.2	91.6	NS	NS	NS	12.17
Total gas, [ml]	1429	1403	1387^{X}	1380^{X}	1413^{A}	1379^{A}	1263^{YB}	1117^{YC}	**	**	**	40.72
Methane, [%]	20.4	19.1	19.6	20.1^{X}	19.9^{A}	17.7^{A}	16.8^{A}	13.2^{YB}	**	**	**	1.
Methane-free gas[c], [ml]	1137	1135	1114	1104^{X}	1137^{A}	1133^{A}	1047^{B}	968^{YC}	**	**	**	36.17
Total SCFA, [mmol/l]	79.5	86.6	89.4	91.6	92.4	94.0	86.4	94.9	NS	NS	NS	7.52
Acetate, [%] of SCFA	62.6	62.4^{Y}	62.9^{Y}	61.9^{Y}	63.9^{B}	67.8^{XA}	67.9^{XA}	68.1^{XA}	**	*	**	1.32
Propionate, [%] of SCFA	17.1	17.4	17.7	17.7	16.2	15.2	16.4	16.9	**	NS	NS	0.88
Iso-butyrate, [%] of SCFA	1.07	0.96	0.95	0.98	1.09	0.91	0.90	0.85	NS	*	NS	0.13
Butyrate, [%] of SCFA	13.9	13.8^{X}	13.3^{X}	14.0^{X}	13.2^{A}	11.3^{YA}	9.67^{YB}	8.98^{YB}	**	**	**	0.94
Iso-valerate, [%] of SCFA	4.00	4.03	3.90	4.06	4.22	3.68	3.98	3.98	NS	NS	NS	0.21
Valerate, [%] of SCFA	1.19	1.29	1.23	1.23	1.20	1.08	1.10	1.09	**	NS	NS	0.11
Acetate:Propionate	3.76	3.60^{Y}	3.57^{Y}	3.53^{Y}	3.96	4.47^{X}	4.15^{X}	4.05^{X}	**	NS	NS	0.26
Net H_2 produced [d], [mM]	105	113	117	119	124	130	116	114	NS	NS	NS	10.43
[mol/100 mol] of SCFA	133	131	131	131	135	139	135	133	**	NS	NS	2.88

[a]Means with different superscripts are statistically different (^{A,B,C,D} between dose and within chemical additions ($p < 0.01$); ^{X,Y}, between type chemical addition and within dose, $p < 0.01$).
[b]The doses refer to the final NO3 concentration in fermenters (mg/ml of rumen fluid);
[c]Total gas minus methane.
[d]in mM = 2[acetate + n-butyrate + iso-butyrate] − [propionate + iso-valerate + valerate].

resulted in lower propionate and valerate concentrations and an increase in the acetate: propionate ratio than in controls (16.2 *vs* 17.5%, 1.12 *vs* 1.24%, 4.16 *vs* 3.62, $p < 0.01$, respectively), with no effects caused by increasing dosages.

The net H_2 availability expressed as mol/100 mol of SCFA, calculated by a stoichiometric model, was higher in the NO_3^- added bottles (136 *vs* 132 mol/100 mol, $p < 0.01$).

Table 3 reports the CH_4 yield at progressive incubation times (6 h intervals) and the kinetic parameters of the Gompertz model used to fit the CH_4 production. For CH_4, the interaction between fermentation time, type of chemical addition, and dose was significant ($p < 0.01$). The CH_4 yield was never influenced by the addition of NaCl and urea, whereas in the NO_3^- added fermenters the CH_4 yield was reduced in a dose-dependent manner. The lowest of NO_3^- dose did not affect CH_4 production during the fermentative process. The dose of 1.0 mg/ml resulted in lower CH_4 yields compared to the corresponding control ($p < 0.05$) only within the first 24 h, while the higher two dosages showed differences ($p < 0.01$) at all times considered. For the Gompertz parameters, the interaction between dose and chemical addition was significant for most parameters except the MFR. The NaCl and urea additions did not significantly change any parameter, while NO_3^- nitrate additions reduced (at the highest dose) the asymptotic CH_4 yield, the lag phase, and the TMFR, and there was a significant increment in the A parameter. Finally, MFR was lower for NO_3^- additions (15.6 *vs* 19.7 ml/h) and declined with increasing dosages (19.6, 17.1, 16.8, and 17.1 for 0.5, 1.0, 1.5, and 2.0 mg/ml of chemicals added).

Figure 1 describes the CH_4 reductions for the various NO_3^- inclusion levels throughout the fermentation while Figure 2 shows the results of the linear regression analysis,

Table 3. Methane yield at progressive incubation times (6 h intervals) and kinetic parameters of the fitting Gompertz model of the methane yield for the different inclusion levels of sodium nitrate ($NaNO_3$) or other chemicals (NaCl + urea)[a].

	Chemical additions [CA, mg/ml rumen fluid]											
	NaCl+Urea				$NaNO_3$						CA*	
Dose[b]	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	CA	Dose	Dose	RMSE
Methane yield[c], [ml]												
6	108	99.2	98.8	99.3^{x}	92.5	77.3	78.5	73.2^{y}				
12	173	159^{x}	161^{X}	163^{X}	158^{A}	131^{yAB}	119^{YBC}	99.4^{YC}				
18	211	195^{x}	197^{X}	201^{X}	198^{A}	167^{yAB}	148^{YB}	113^{YC}				
24	241	222^{x}	225^{X}	228^{X}	227^{A}	195^{yAB}	171^{YB}	124^{YC}				
30	263	241	244^{X}	248^{X}	251^{A}	216^{B}	188^{YB}	132^{YC}				
36	276	252	257^{X}	260^{X}	263^{A}	229^{B}	200^{YB}	138^{YC}				
42	282	257	262^{X}	265^{X}	270^{A}	234^{B}	207^{YB}	142^{YC}				
48	292	269	273^{X}	276^{X}	282^{A}	245^{B}	217^{YB}	149^{YC}				
									**	**	**	**18.2**
Methane kinetic parameters												
B, [ml]	267	245	249^{X}	253^{X}	258^{A}	225^{A}	197^{YA}	133^{YB}	**	**	**	22.5
A, [1/h]	0.21	0.21	0.21	0.21^{X}	0.19^{B}	0.18^{B}	0.21^{B}	0.31^{YA}	NS	*	*	0.04
Lag, [h]	2.89	2.87	2.91	2.91^{x}	3.17^{A}	3.40^{A}	3.06^{A}	2.09^{yB}	NS	*	*	0.44
MFR[c], [ml/h]	20.8	19.2	19.2	19.6	18.3	15.0	14.4	14.6	**	**	NS	1.32
TMFR[d], [h]	7.64	7.59	7.69	7.68^{x}	8.38^{A}	8.97^{A}	8.08^{A}	5.52^{yB}	NS	*	*	1.16

[a]Means with different superscripts are statistically different (^{A,B,C,D} between dose and within chemical additions ($p < 0.01$); ^{X,Y}($p < 0.01$) or ^{x,y}($p < 0.05$), between type of chemical addition and within dose).
[b]The doses refer to the final NO3 concentration in fermenters;
[c]The interaction dose*time and dose*time*CA are statistically significant ($p < 0.01$);
[d]Calculated Maximum Fermentation Rate;
[e]Calculated Time at Maximum Fermentation Rate.

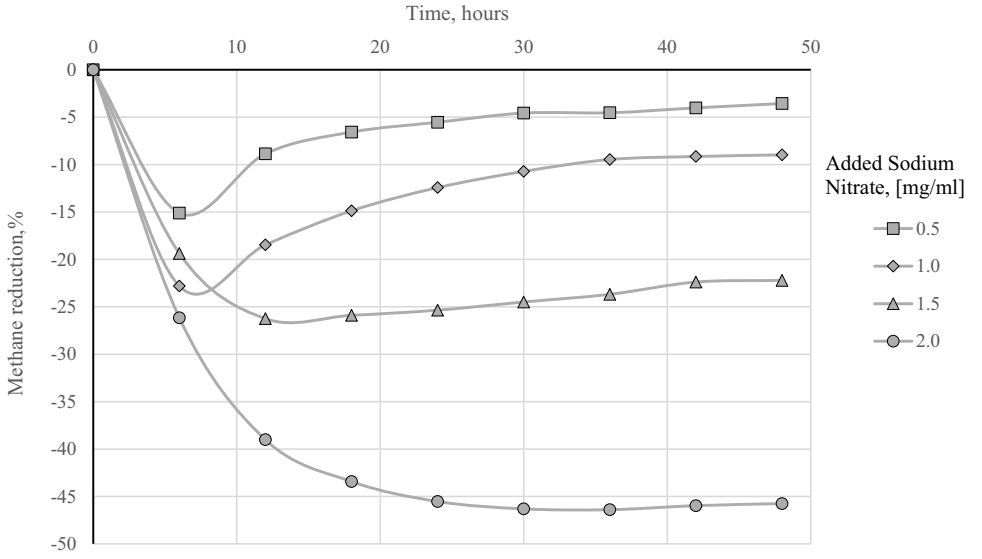

Figure 1. Methane reduction as a percentage of the relative controls for the different additions of sodium nitrate obtained during fermentation.

calculated within the fermentation runs at selected incubation times (6, 12, 24, and 48 h) between the CH_4 production and of NO_3^- dosages. Regressions were significant for all the fermentation length (R^2 ranging from 0.49 to 0.86) with slopes between −0.069 and −0.517 ml of CH_4 per mg of NO_3^- added.

4. Discussion

4.1. Total gas and fermentative parameters

In control fermenters, the progressive addition of chemicals had no relevant effects on fermentation and only caused small increases in the final pH of the fermentation fluid due to the buffering effect of ammonia generated by urea.

In fermenters treated with progressive doses of NO_3^-, in addition to an increase in pH in the fermentation fluid, there were quantitative and qualitative impacts on fermentation. The most evident effect was a progressive decrease in overall CH_4 yield. However, under our conditions, the intensity of fermentation measured by the total gas produced minus the CH_4 (e.g. total methane-free gas) decreased only at the highest NO_3^- dose (2.0 mg/ml). Also, the final SCFA concentrations were similar between doses. Overall, these results did not support a substantial modification in fermentability caused by NO_3^- addition, while previous articles (Sakthivel et al. 2012; Patra and Yu 2013; Yang et al. 2016) described a decrease in fermentation activity due to the detrimental effect of NO_3^- and its redox intermediate NO_2^- on the rumen microbiota. Finally, NO_3^- addition did not appear to depress protozoa populations, despite contradictory data in the literature. Protozoa counts were reduced in goats (Asanuma et al. 2015), but not in dairy cows (van Zijderveld et al. 2010) or *in vitro* rumen continuous fermenters (Wenner et al. 2020). The maintenance of

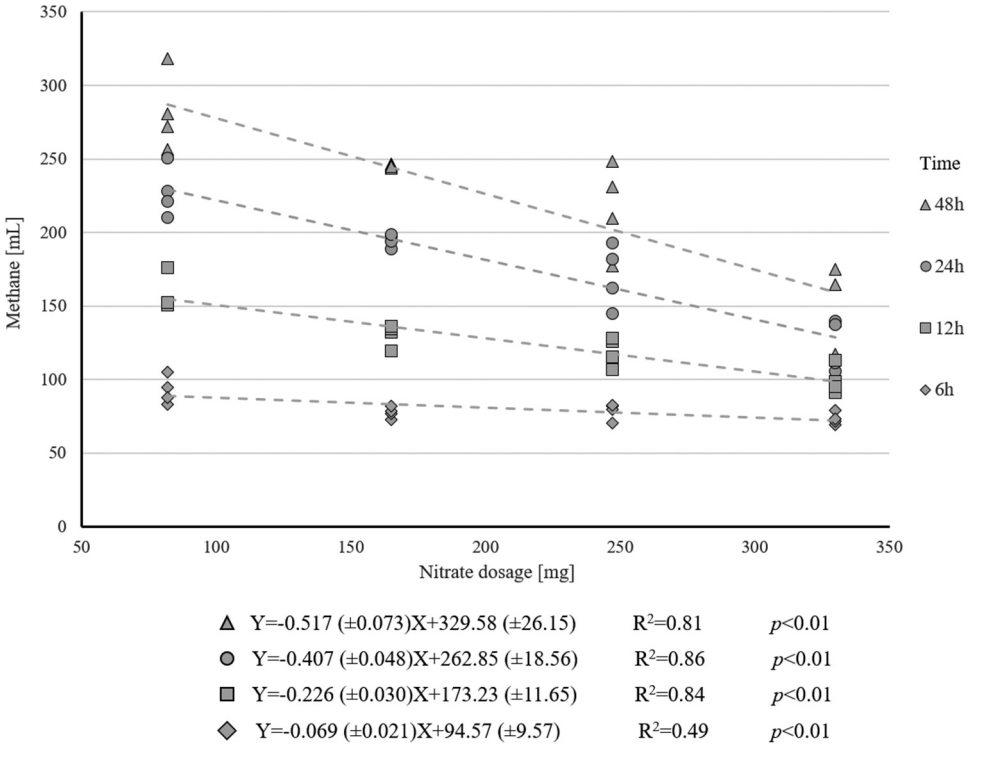

Figure 2. Linear regressions (within fermentation run) at different fermentation times (6,12, 24 and 48 hours) between methane yield (ml) and level of nitrate inclusion (mg).

the rumen protozoa population is important for nitrate metabolism because it appears to speed the conversion of NO_3^- to NO_2^- (Yang et al. 2016).

Typically, the process of NO_3^- reduction generate an increase in NH_3 concentration in fermentation fluid (Božic et al. 2009; Patra and Yu 2015; Nguyen et al. 2016). In the literature, it has been suggested that some factors, such as the incomplete conversion of NO_3^- into NH_3 with the accumulation of the intermediate NO_2^- (Yang et al. 2016) or the presence of nitro-reducing bacteria (Latham et al. 2016) could reduce the expected increment in NH_3. In our conditions, a numerical increment in NH_3 concentration was measured from the lowest to the highest addition of NO_3^-(from 66.9 to 81.4 mg/dl), but this increase did not reach statistical significance. The expected increment of NH_3-N due to the maximum addition of NO_3^- (54 mg of N corresponding to 448 mg of $NaNO_3$) is comparable to the observed increment of ammoniacal nitrogen (47 mg of NH_3-N).

The SCFA concentration in the fermentation fluid was not affected by NO_3^- additions but there were relevant modifications of the SCFA profile. We obtained a decrement in propionate and valerate caused by the type of chemical addition without any variation due to the dose, while there was an increment in acetate and a decrement in butyrate reached in response to the progressive NO_3^- additions. These results are consistent with previous findings both from *in vitro* and *in vivo* studies (Nolan et al. 2010; Lin et al. 2011; Li et al. 2012). The NO_3^- conversion to NH_3 is favoured over propionogenesis because it

is thermodynamically more favourable than the reduction of fumaric acid to succinic acid (van Zijderveld et al. 2010; Yang et al. 2016). Moreover, the increment in acetate production generates a further decrease in the H_2 available for propionate production (Nolan et al. 2010). A similar effect was also observed for butyrate concentration. The changes in individual SCFA content can be summarised in terms of net H_2 production, according to the stoichiometric model described by Wang et al. (2014). An increase in acetate, reductions in propionate and valerate as well as a change in butyrate were reflected in a significant increment of H_2 net production with the NO_3^- addition. These results indicate that the inclusion of NO_3^- favours fermentation pathways that produce more H_2.

4.2. Methane dynamics

Different trends in CH_4 production were observed for the various dosages applied (Table 3). For the lowest NO_3^- dose, the CH_4 did not change significantly in the entire fermentation when compared to the respective control while reductions occurred for 1.0 mg/ml dose only within the first 24 h. For the higher doses, the differences with respective controls progressively increased up to 30 h and then were stable. Figure 1 shows the CH_4 reductions expressed as a percentage of the relative controls observed for the various NO_3^- inclusion levels at different fermentation lengths. Overall, these variations could be explained taking into account the balance between the H_2 generated during fermentation and the available NO_3^-: a dose of 1.0 mg/ml consumes all the H_2 available in 24 h while doses of 1.5 and 2.0 mg/ml require longer time. It means that at short times the limiting factor is the H_2 availability but as fermentation proceeds, the limitation becomes the NO_3^- shortage. However, the fermentation time at which the limitation shifts from H_2 to NO_3^- is dose-dependent: it is included within about 12 h of fermentation for the two lowest dosages, while it requires 24 h for the 1.5 mg/ml dose and 30 h for the inclusion level of 2.0 mg/ml.

The Gompertz model was previously employed for the kinetic study of data from *in situ* degradability or gas production (Susmel et al. 1999; Sarnataro et al. 2020) and produced satisfactory results also in fitting CH_4 data in the current study ($R^2 > 0.95$). However, the predominant effect of the NO_3^- addition to the kinetic parameters was a significant reduction in the asymptotic values and the MFR. For the other parameters, the type of chemical addition was never significant. Despite the goodness of fit obtained with the Gompertz equation, the kinetics parameters did not accurately describe the evolution of CH_4 production during the fermentative process. Even if the kinetics approach allowed for the investigation of specific CH_4 production metrics such as the MFR and TMFR, this solution did not guarantee an accurate differentiation of NO_3^- effects for the various dosages used.

Often, the use of various doses in experimental work can be useful to generate equations (by regression approach) to predict the effects of untested dosages (e.g. "dose-response" experiments). Considering this, the relationship between the four NO_3^- additions and the CH_4 production was assessed at four different durations of fermentation (i.e. 6, 12, 24, and 48 h, as shown in Figure 2). The predictive equations obtained were

different, showing an increment in the dose effect according to the fermentation time considered. From a stoichiometric calculation, 1 mg of NO_3^- completely transformed NH_3 allows a reduction in CH_4 yield of 0.409 ml (Beauchemin et al. 2020). Considering the presented equations, the slope was much lower than that expected for the 6-h fermentation (−0.069 ml/mg). The slope increased to −0.226 ml/mg at 12 h, and at 24 h it was close to what was expected (−0.407 ml/mg). A further increase in fermentation time overestimated the CH_4 reduction (−0.517 ml/mg).

As a result, in batch rumen systems, the fermentation length affects the predictive equations derived from different doses and this was consistent with our prior observations on the time dependency between NO_3^- additions and CH_4 production. In order to analyse this phenomenon, it has to be considered that in the initial stages of fermentation when larger doses are administered, the limiting component is H_2, and therefore the available NO_3^- cannot express its full potential, requiring long fermentation times to cause an overall methane drop. On the contrary, for low doses, the limit is represented by NO_3^- availability and the antimethanogenic potential is consumed in relatively short times of fermentation. This aspect causes a variation in the regression-line slope, which results time dependent.

5. Conclusion

The amounts of nitrogen and sodium equivalents to $NaNO_3$ additions did not influence rumen fermentation in batch *in vitro* systems. The experiment confirmed the potential of $NaNO_3$ as NO_3^- source to mitigate rumen methane yield and modify patterns of fermentation. However, the dose-effect depends also on the fermentation time. In batch fermentation systems, the impact of low doses was appreciable only at the beginning of the fermentation, while high doses demonstrated a complete effect only at the end of the fermentation process. In conclusion in batch fermentation systems the dose effect of nitrates on methane yield resulted time dependent.

Disclosure statement

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