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Performance, Methane Emission, Nutrient Utilization, and the Nitrate Toxicity of Ruminants with Dietary Nitrate Addition: A Meta-analysis from *In Vivo* Trials

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ABSTRACT

This study aims to evaluate the effects of dietary nitrate addition on performance, methane emission, nutrient utilization, and the nitrate toxicity of ruminants by using the meta-analysis methodology from *in vivo* trials. A total of 38 published papers and 139 studies were used. Parameters observed were feed intake, animal performance, enteric methane emission, and nitrate toxicity. Data were subjected to the mixed model methodology. Nitrate doses or forms were treated as fixed factors, while the different studies were treated as random factors. Results revealed that nitrate supplementation significantly decreased the milk protein content, milk fat content, dry matter intake, gross energy intake, the molar proportion of the propionic acid, methane production, and the metabolism of vitamin A in a linear pattern ($p<0.05$). Moreover, nitrate addition significantly increased nitrate intake, the molar proportion of the acetic acid, the ratio of acetic acid to propionic acid, hydrogen molecule production, microbial protein synthesis, and nitrite blood levels ($p<0.05$). However, treatments did not affect the milk yield, final body weight, nitrate retention, and blood methemoglobin. There was a significant interaction among the animal types and the nitrate (forms and doses) on the milk protein content, dry matter intake, rumen pH value, total volatile fatty acids, the molar proportion of propionic acid, NH_3 concentration, H_2 molecule, microbial protein synthesis, metabolism of vitamin A, and the blood methemoglobin. This concludes that nitrate supplementation is an alternative feed additive for mitigating the enteric methane in ruminants without any adverse effects on animals' health or performance despite its impact on the feed consumption rate.

***Keywords:** meta-analysis; methane; nitrate; nitrate toxicity; ruminant performance*

INTRODUCTION

Over the last decades, much attention has been directed toward methane emission as an anthropogenic greenhouse gas (Abdelbagi *et al.,* 2021). In addition, methane as a greenhouse gas was reported to have the second highest contribution to atmospheric concerns after carbon dioxide (Asanuma *et al.,* 2014). However, livestock, among the other agricultural sectors, was found to contribute to up to 40% of the total global methane emissions produced by the agricultural sector (Zhao *et al.,* 2018). The enteric methane in ruminants is the major form of methane emitted by the ruminants (Storm *et al.,* 2012). It was reported that the enteric methane emission costs the ruminants animals around 12 percent of the gross energy losses (Mamvura *et al.,* 2014). The energy losses could subsequently affect negatively on feed utilization and animal performance (Granja-Salcedo *et al.,* 2019). Therefore, several feeds, feed additives, and feed management were used to mitigate the enteric methane emission in the ruminants to improve feed utilization and animal productivity (Alemu *et al.,* 2019).

Nitrate was reported as a promising approach for mitigating the enteric methane emission in the ruminants due to its ability to act as an electron acceptor competing with the methanogens on the hydrogen ion (Guo *et al.,* 2009; Granja-Salcedo *et al.,* 2019). Moreover, it was hypothesized that nitrate is a toxic compound for many rumen microbes, such as cellulolytic bacteria, methanogenic bacteria, and protozoa (Lin *et al.,* 2013). Despite the effectiveness of the dietary nitrate supplementation on the enteric methane emission as a hydrogen sink, the inclusion of nitrate into the ruminants' diet is still limited due to its toxicity in the ruminants (Patra & Yu, 2013). Nitrate toxicity occurs when the highest concentration of nitrate is supplemented into the ration with low-energy content (Alemu *et al.,* 2019). It was proposed that nitrate metabolism be followed by nitrite formation and then converted to ammonia (van Zijderveld *et al.,* 2010). However, the initial reduction of nitrate to nitrite is significantly faster than the subsequent reduction to ammonia. Therefore, the potential risk of nitrate toxicity occurs when high levels of nitrite are built-up, causing oxygen transportation incapability (Methemoglobinemia) due to the oxidation effects of ferrous ion (Fe^{2+}) to (Fe^{3+}) ferric state.

Several *in vivo* and *in vitro* trials have reported the significance of nitrate as an alternative methane mitigation method (Lin *et al.,* 2011; Abdelbagi *et al.,* 2021). Further, nitrate was used effectively to replace the urea without adverse effects on animal health. The effects of nitrate on animal growth performance were previously investigated by Lee & Beauchemin (2014). The results have obviously demonstrated nitrate's ability to influence animal performance. To date, few meta-analysis studies have been conducted regarding the effects of nitrate on animal performance, methane emission, and nitrate toxicity. Therefore, this study aims to evaluate the effect of nitrate addition on performance, methane emission, utilization, and the nitrate toxicity of the ruminants by integrating several previously published papers using the meta-analysis methodology from *in vivo* trials.

MATERIALS AND METHODS

Database Building

In this study, the database was constructed by using previously published articles. The literature was obtained from different sources such as Google, Google Scholar, and Scopus. Data were developed concerning dietary nitrate supplementation and its effects on animal performance, enteric methane emission, and nitrate toxicity. Literature was obtained by using the keywords "nitrate supplementation", "methane emission", "animal performance", and "nitrate toxicity". The searching process was proceeded in four main steps, which were identification, screening, and the eligible papers were included in the database building (Figure 1). All the journals used for building the database were accepted if "the article is in the English language, contains control experiment, and contains nitrate supplementation as a feed additive for methane mitigation or its effects on the animal performance as well as its toxicity. The papers excluded from the database were review articles, *in vitro* experiments, or articles that use other nitro-compounds such as 3-nitrooxypropanol as a feed additive for mitigating methane emission, improving animal performance, or studying nitrate toxicity.

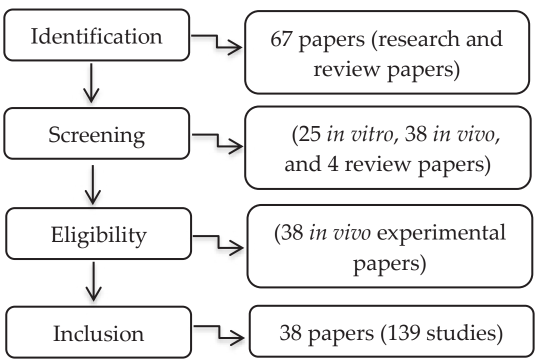

Figure 1. The steps and procedures of structuring the meta-analysis database

After investigation, 67 published papers were identified, 60 articles passed the screening process, while 38 papers containing 139 studies were eligible to be involved in the database. This is a study using different ruminant types, e.g., cows and cattle (21 papers), goats (4 papers), and sheep (13 papers). The animal sex and state are presented in Table 1. Papers were identified based on the topic titles, whereas the screening process was done deeply by reading the abstracts and then by fully scanning the details of the full paper of each paper. Subsequently, an excel spreadsheet was created. In Excel's sheet, parameters were classified into two different main groups, which are independent parameters (Author name, year, study, experiments, number of study, nitrate dose as well as nitrate form) and the dependent parameters or the response (feed consumption (feed intake, nitrate intake [NI], and the gross energy intake), nutrient digestibility (dry matter digestibility (DMD), neutral detergent fiber digestibility (NDFD), acid detergent fiber digestibility (ADFD), ether extract digestibility (EED), crude protein digestibility (CPD), and starch digestibility (starch D)), methane production (daily methane production, methane production/dry matter intake (DMI), methane production/gross energy intake (GEI), methane production/metabolic body weight (BW)….etc), animal performance (final body weight, average daily gain (ADG), feed conversion ratio (FCR) …etc), and nitrate toxicity (total hemoglobin (total-HB), methemoglobin…etc).

Nitrate forms were categorized into different groups based on the nitrate forms. These forms are control treatment (zero percent nitrate), nitrate addition (NO_3) (unencapsulated nitrate and uncoated nitrate, encapsulated nitrate, or the coated nitrate), nitrate in a salt-form (unencapsulated and uncoated nitrate salt, encapsulated nitrate salt, and the coated nitrate salt). Nitrate salt involves sodium nitrate, potassium nitrate, calcium nitrate, and ammonium nitrate. However, the information about the authors, years, experiments, and studies is presented in Table 1. In this study, some literature has used urea as a control diet. This urea treatment was not used as a control treatment in the database unless the nitrate content of the urea used as a control treatment was known and mentioned in the study.

Statistical Analysis

Datasets were subjected to the mixed model methodology as performed by Abdelbagi *et al*. (2021). Nitrate doses or forms were treated as fixed factors, while the studies were considered to be random effects. P-values were used as statistical models for determining the significant effects of the treatments. The results were accepted if the p-value was less than 0.05. The interaction effect among animal types, nitrate forms, and nitrate dose was accepted to be significant if the p-value was

Table 1. The studies that were used for developing the meta-analysis database

No	Authors	Year	Studies	Dose (g/kg)	Animal	Treatment
1	Alemu *et al*. (2019)	2019	7	0-17.85	Male beef	(Encapsulated ammonium nitrate and microencapsulated blend essential oils (EO))
2	de Raphélis-soissan *et al*., (2017)	2017	6	5.09-9.2	Sheep	(Uncoated nitrate; coated with palm oil or coated with paraffin wax)
3	Alaboudit & Receivedg (1985)	1985	9	0-19.1	Female sheep	(Potassium nitrate)
4	El-Zaiat *et al*. (2014)	2014	2	0-27.43	Male lamb	(Urea and calcium nitrate)
5	Guyader *et al*. (2015)	2015	3	0-22.5	Dairy cow	(Urea and encapsulated nitrate product)
6	Nguyen *et al*. (2016a)	2016	1	0.00 -22.5	Female lambs	Calcium nitrate
7	Nolan *et al*. (2016)	2016	1	0-30.6	Female lambs	(Potassium nitrate)
8	Özdemir *et al*. (2014)	2014	11	0-10.9	Male goat	(Sodium nitrate and Sodium sulfate)
9	Pal *et al*. (2015)	2015	2	0-1.53	Male sheep	(Potassium nitrate)
10	Rebelo *et al*. (2019)	2019	2	0.14.3	Male beef	(Encapsulated nitrate)
11	Sar *et al*. (2002)	2002	1	0-0.88	Sheep	Sodium nitrate
12	Sar *et al*. (2005)	2005	1	0-0.88		Sodium nitrate
13	Silivong *et al*. (2011)	2011	1	0.2.87	Female goat	Calcium nitrate
14	Silveira *et al*. (2019)	2019	1	0-18.9	Male goat	Calcium nitrate
15	Sun *et al*. (2017)	2017	3	0-10	Male beef	Sodium nitrate
16	Takahashi & Young (1991)	1991	1	0-0.88	Sheep	Sodium nitrate
17	Takahashi *et al*. (1998)	1998	3	0.6-0.97	Sheep	(Sulphur, L-cysteine, and Sodium nitrate)
18	van Zijderveld *et al*. (2010)	2010	1	0-19.5	Male lamb	(Calcium nitrate and sulphate)
19	Weicttenthal *et al*. (1940)	1940	6	0-8.02	Male beef	(Vitamin A and sodium nitrate)
20	Granja-Salcedo *et al*. (2019)	2019	1	0- 47	Male beef	(Urea and encapsulated calcium nitrate)
21	Guyader *et al*. (2018)	2018	1	0-17.25	Non-lactating cow	(Tea saponin and nitrate)
22	Paengkoum *et al*. (2021)	2021	11	19.46-29.46	Male meat goat	(Potassium nitrate)
23	van Zijderveld *et al*., (2011)	2011	4	0-6.6	Cow	(Urea and calcium nitrate)
24	van Wyngaard *et al*. (2018)	2018	3	0-17.25	Dairy cow	(Calcium nitrate)
25	Velazco *et al*. (2014)	2014	1	0-2.57	Male cow	(Urea and calcium nitrate)
26	Wang *et al*. 2018)	2018	1	0-14.6	Lactating cow	(Urea and Sodium nitrate)
27	Farra & Satter (1971)	1970	23	0-20	Non-lactating cow	(Sodium and potassium nitrate)
28	Klop *et al*. (2016)	2016	1	0-15.75	Lactating cow	(Urea and nitrate)
29	Lee *et al*. (2017)	2017	2	0-17.85	Male beef	Encapsulated (Calcium ammonium and urea)
30	Li *et al*. (2012)	2012	5	0-22.9	Female sheep	(Urea and calcium nitrate)
31	Li *et al*. (2013)	2013	3	0-18.8	Male sheep	(Calcium ammonium nitrate)
32	Meller *et al*. (2019)	2019	4	0-3.41	Lactating cow	(Calcium ammonium nitrate)
33	Powers *et al*. (2014)	2014	3	0-18	Male cow	(Calcium ammonium nitrate)
34	Olijhoek *et al*. (2016)	2016	2	0-15.82	Lactating cow	(Calcium ammonium nitrate)
35	Veneman *et al*. (2015)	2015	2	0-15	Dairy cow	(Linseed oil and nitrate)
36	Tomkins *et al*. (2016)	2016	2	0-5.973	Male beef	Nitrate
37	Hulshof *et al*. (2012)	2012	2	0-18	Male beef	Nitrate
38	Sinclair & Jones (1964)	1964	4	3.06	Sheep	Potassium nitrate
	TOTAL		139			

less than 0.05. All the statistical analysis process was performed using SAS software version 9.4.

RESULTS

The effects of nitrate dose on animal performance, methane emission, utilization, and nitrate toxicity are presented in Table 2. Treatments did not affect animal weight, feed conversion, milk yield, milk protein content, or milk fat content. At the same time, there was a significant decrease in the daily milk protein content, daily milk fat content, dry matter intake (DMI), DMI/metabolic body weight (BW), gross energy intake (GEI), and the GEI/metabolic BW due to the effect of nitrate dose. In contrast, the dietary nitrate addition has significantly increased the nitrate intake compared with the control diet (Table 2). In addition, the addition of nitrate has significantly influenced the rumen fermentation process. As the nitrate dose increases the acetic acid molar proportion, the ratio of acetic to propionic acid molar proportion, hydrogen molecules, and the rumen microbial protein synthesis increase significantly, while

Table 2. Regression equation on the methane production, performance, nitrate toxicity, and ruminal fermentation profile influenced by nitrate addition in ruminant diet

Item	No	Unit	Model	Parameter estimates				Trend	Model estimates	
				Intercept	SE-intercept	Slope	SE-slope		AIC	p-value
Feed intake	13	kg/day	L	8.661	0.881	-0.025	0.028	-	61.2	0.540
DMI	136	kg/day	L	7.593	0.847	-0.013	0.007	-	569.1	0.037
DM_ metabolic weight	128	kg/kg MBW	L	0.102	0.005	0.000	0.000	-	-662.2	0.007
GEI	12	MJ/day	L	102.94	63.780	-0.223	0.084	-	104.8	0.045
GEI-metabolic weight	10	MJ/kg MBW	L	1.699	0.283	-0.005	0.001	-	6.3	0.011
NI	23	g N/day	L	51.775	24.696	8.667	1.091	+	242.6	<.0001
TVFAs	116	mM	L	97.824	2.778	-0.171	0.110	-	984.2	0.124
C_2	116	%	L	64.614	0.919	0.319	0.048	+	757.9	<.0001
C_3	116	%	L	22.639	1.016	-0.246	0.065	-	799.4	0.000
C_4	116	%	L	10.388	0.398	-0.075	0.021	-	568.6	0.001
Iso-C_4	22	%	L	2.259	1.086	0.002	0.002	+	27.2	0.449
C_5	32	%	L	1.987	0.431	-0.009	0.014	-	98.5	0.515
Iso-C_5	22	%	L	1.410	0.471	-0.018	0.009	-	50.9	0.059
C_2/C_3	51	%	L	3.794	0.259	0.018	0.007	+	129.1	0.012
pH	72		L	6.472	0.095	0.009	0.009	+	141.3	0.273
NH_3	100	mM	L	19.243	5.616	-0.013	0.092	-	884.1	0.892
H_2-Day	14	mM	L	0.703	0.626	0.081	0.027	+	45.4	0.015
CO_2	12	mM	L	9.941	5.077	0.018	0.014	+	51.5	0.243
NO_3-rumen	21	μg/ml	L	3.267	2.395	0.189	0.149	+	138.2	0.231
NO_2-rumen	29	μg/ml	L	3.236	1.532	-0.007	0.107	-	182.6	0.950
Methanogens	10	Log	L	7.172	1.717	-0.025	0.011	-	31.2	0.064
Total-bacteria	10	Log	L	10.759	0.533	-0.01	0.005	-	17.4	0.140
Total-protozoa	28	log	L	4.204	0.89	-0.009	0.005	-	79.9	0.581
Vitamin-A	22	μg/dL	L	29.046	3.194	-0.85	0.308	-	160.4	0.020
β- carotene	12	μg/dL	L	2.992	0.333	-0.135	0.045	-	34.7	0.030
CH_4	80	g/day	L	172.78	21.823	-1.969	0.345	-	862.7	<.0001
CH_4/DMI	78	g/kg	L	18.871	0.654	-0.199	0.025	-	387.3	<.0001
CH_4/BW	14	g/kg	L	0.417	0.107	-0.007	0.005	-	8.3	0.220
CH_4/GEI	34	% GEI	L	6.303	0.244	-0.075	0.013	-	91.2	<.0001
CH_4/milk prod	11	g/kg	L	15.081	2.452	-0.052	0.093	-	57.6	0.602
Initial-BW	162	kg	L	228.92	27.018	-0.005	0.046	-	1545.2	0.923
Final-BW	40	kg	L	232.79	54.409	0.077	0.165	+	388.3	0.644
LWG	18	kg	L	0.665	0.195	0.004	0.008	+	32.0	0.650
ADG	52	kg/day	L	0.656	0.125	-0.004	0.003	-	43.6	0.109
FCR	41	kg/kg	L	5.346	2.903	0.172	0.154	-	308.6	0.277
Feed efficiency	10		L	0.143	0.012	-0.006	0.008	-	-22.4	0.534
DMD	40	%	L	60.717	3.654	0.055	0.033	+	248.3	0.107
NDFD	56	%	L	54.555	3.415	0.024	0.045	+	361.1	0.588
ADFD	23	%	L	52.663	2.733	0.114	0.095	+	144.3	0.251
CPD	30	%	L	65.116	3.273	-0.028	0.152	-	211.9	0.857
EED	16	%	L	64.177	2.024	-0.039	0.249	-	100.1	0.880
Starch D	12	%	L	97.906	0.360	0.020	0.025	+	34.4	0.463
Nitrate-retention	15	g/day	L	11.190	3.095	0.029	0.100	+	96.4	0.779
Urea	18	mg/dL	L	58.254	7.934	-0.151	0.149	-	136.1	0.338
Microbial protein	19	g/day	L	9.094	1.977	0.083	0.023	+	86.5	0.004
NO_2-Plasma	35	μg/ml	L	1.153	0.56	0.096	0.048	+	161.6	0.060
NO3-plasma	27	μg/ml	L	0.373	0.181	0.052	0.014	+	61.7	0.003
Total HB	28	mg/dL	L	9.329	1.469	-0.009	0.018	-	115.1	0.650
Methemoglobin	55	%	L	3.588	1.020	-0.043	0.116	-	351.3	0.714
Methemoglobin min	18	%	L	2.611	1.517	-0.033	0.145	-	111.9	0.826
Methemoglobin max	12	%	L	6.414	3.368	0.344	0.525	-	83.6	0.541
Milk-yield	31	kg/day	L	21.368	1.534	-0.052	0.031	-	153.4	0.112
Milk fat (%)	29	%	L	3.960	0.315	0.004	0.006	+	56.0	0.521
Milk fat (kg/day)	15	kg/day	L	1.040	0.006	-0.006	0.001	-	-20.7	0.002
Milk-protein	25	%	L	3.401	0.094	0.000	0.006	-	22.7	0.993
Milk-protein	11	kg/day	L	0.785	0.071	-0.003	0.002	-	-13.7	0.047
Milk-lactose	23	%	L	4.564	0.065	0.004	0.007	+	6.7	0.542

Note: DMI= dry matter intake, MBW= metabolic body weight, NI= nitrogen intake= NDF intake= neutral detergent fiber intake, ADF intake= acid detergent fiber intake, GEI= gross energy intake= DMD= dry matter digestibility, NDFD= neutral detergent fiber digestibility, ADFD= acid detergent fiber digestibility, starch D= starch digestibility, EED= ether extract digestibility, CPD= crude protein digestibility, Total-HB= total hemoglobin, methemoglobin Max= maximum methemoglobin, methemoglobin Min= minimum methemoglobin, initial-BW= initial body weight, final- BW= final body weight, LWG= live weight gain, FCR= feed conversion ratio, CH4/DMI= methane production per total dry matter intake, CH4/BW= methane production per total body weight, CH4/GEI= methane production per total gross energy intake, AIC= Akaike information criterion, N= number of data.

the increase of nitrate dose in the diet decreases significantly the propionic acid molar proportion in the rumen ($p<0.05$). Moreover, treating with nitrate has significantly decreased the enteric methane emission in the rumen (Table 2). Furthermore, nitrate addition has decreased the metabolism of vitamin A and the β-carotene. There was no significant effect of nitrate addition on urea blood levels or nitrate retention. On the other hand, the inclusion of nitrate in the diet has resulted in a significant increase in nitrate levels in the rumen, while there was no significant effect of nitrate addition on blood nitrite and nitrate levels, blood hemoglobin, or blood methemoglobin levels ($p<0.05$).

In terms of the nitrate forms, the different nitrate forms significantly influenced ($p<0.05$) animal performance, methane emission, nutrient utilization, and nitrate toxicity in different ways. As it is demonstrated in Table 3, the higher amount of the daily fat milk content, dry matter intake/BW, and molar proportion of the acetic acid in the rumen, the ratio of acetic to propionic acid, and the higher rumen hydrogen concentration were due to the inclusion of nitrate group treatment (NO_3). In contrast, the higher molar proportion of the propionic acid in the rumen, the higher microbial protein synthesis, the higher daily methane production, and the higher nitrite blood concentration were caused by the addition of the nitrate salts group (NO_3-salt) ($p<0.05$).

In Table 4, it is shown that there was a significant interaction between animal species, nitrate forms, and nitrate doses on the milk protein content, the amount of the daily milk protein content, the amount of the daily fat content, dry matter intake, the molar proportion of acetic acid, rumen pH value, NH_3 concentration, H_2 molecules, vitamin A metabolism, and the blood methemoglobin concentration.

DISCUSSION

The effects of nitrate supplementation on feed consumption and feed consumption elements are shown in Tables 2 and 3. Nitrate addition has significantly reduced dry matter intake, dry matter intake/metabolic body weight, and gross energy intake/metabolic body weight. Also, there was a numerical reduction in the feed consumption rate due to nitrate inclusion in the diet. Feed consumption was reported to be reduced by nitrate addition to the diet (Hulshof *et al.,* 2012). It is explained that nitrate salts are recognized to have poor palatability and strong bitterness, which may reduce the feed consumption rate and the rate of the other feed consumption elements (El-Zaiat *et al.,* 2014). It was suggested that the negative organoleptic effects of nitrate on the ruminant feeding behavior because of the nitrate salts bitterness could be eliminated by using encapsulation or the coating process (Alemu *et al.,* 2019), which may result in a better consumption rate. However, from the evaluation of the collected data in this study, the results showed there was no significant difference in the feed intake or the DM intake among the nitrate forms. At the same time, there was a significant interaction effect among nitrate dose, nitrate forms, and the animal types on dry matter intake. The significant interaction effects indicate that feeding nitrate to livestock should consider the dose and the form of nitrate given to the animal and the animal type to prevent depression on feed consumed by the animal.

Despite the significant increase in nitrate intake due to the inclusion of nitrate into the diet, the different nitrate forms did not show any significant difference. Also, there was no interaction effect among nitrate dose, forms, or animal types on the nitrate consumption rate. Recently, Alemu *et al*. (2019) reported that the average daily nitrate consumption was higher in treating the encapsulated nitrate. However, Rebelo *et al*. (2019) stated that the animals fed encapsulated nitrate consumed less than those fed urea treatment. To date, there are few papers concerning the effects of encapsulation or the coating process on the average daily consumed nitrate. Therefore, more studies are needed to evaluate the effects of encapsulation and the coating process on the nitrate consumption rate of the ruminants.

In addition, the concentrations of both rumen nitrate and nitrite did not change after the addition of nitrate into the diet, but the concentration of blood nitrate was significantly higher as compared with the control diet without nitrate addition ($p<0.05$). The encapsulation process significantly increases plasma nitrate and nitrite concentrations, while the coating process significantly increases the rumen nitrate concentration and decreases the rumen and the plasma nitrate and nitrite concentrations ($p<0.05$). According to Alemu *et al*. (2019), the significant increases in the rumen nitrate and the nitrite concentrations are due to the slow release of the nitrate of the encapsulated nitrate in the rumen. Moreover, the inclusion of nitrate into the diet did not influence the concentration of the blood hemoglobin, the minimum blood methemoglobin, the maximum blood methemoglobin, or the total blood methemoglobin concentration ($p<0.05$). It was mentioned that the inclusion of 20g/kg of nitrate into the ruminants' diet is considered to be safe (de Raphélis-soissan *et al.,* 2017), while the inclusion of nitrate up to the level of 14.9 g/kg was observed to be toxic for sheep (Rebelo *et al.,* 2019) and this could be considered as a minimum dose of lethal nitrate. An experiment conducted on sheep observed up to 45% of the methemoglobin was due to the inclusion of 3% nitrate in the diet (de Raphélis-Soissan *et al.,* 2014). However, de Raphélis-Soissan *et al*. (2014) reported that the rumen bacteria produce about 0.3% nitrous oxide as a by-product. Various animal species, such as chickens, meadow voles, mice, pigeons, pigs, possums, rabbits, and rats, have been used to elucidate the toxicity of nitro compounds and poisoning (Anderson *et al.,* 2005). Nitrate toxicity is attributed due to the high accumulation of nitrite in the blood after the levels of nitrite exceed the lethal dose in the blood resulting in nitrate toxicity. At the high accumulation of nitrite in the blood, the absorbed nitrite oxidizes the ferrous iron (Fe^{2+}) in hemoglobin into ferric form, hampering its ability to transport the oxygen, causing blood methemoglobinemia (El-Zaiat *et al.,* 2014). This occurs when the lower energy diets are

Table 3. Comparison of the methane production, performance, nitrate toxicity, and ruminal fermentation profile influenced by nitrate forms in ruminant diet

Item	No	Unit	Nitrate forms						p-value
			Control	NO_3	Encap-NO_3	Coat- NO_3	NO_3-salt	Encap NO_3-salt	
Feed intake	13	kg/day	8.739	-	-	-	8.176	-	0.369
DMI	136	kg/day	7.602	7.672			7.316	7.295	0.055
DMI/MBW	128	kg/kg MBW	0.102	0.102	-	-	0.098	-	0.015
GEI	12	MJ/day	102.73	-	-	-	100.380	95.333	0.332
GEI/MBW	10	MJ/kg MBW	1.705	-	-	-	1.617	-	0.000
NI	23	g	208.14	211.65	-	-	203.94	205.420	0.721
TVFAs	116	mM	99.433	93.891	-	88.715	93.943	107.570	0.051
C_2	116	%	63.826	72.020	-	71.969	69.930	57.832	<.0001
C_3	116	%	23.835	17.164	-	16.886	18.164	20.112	0.000
C_4	116	%	10.435	8.064	-	7.999	9.326	11.120	0.058
Iso-C_4	22	%	2.268	-	-	-	2.241	2.379	0.085
C_5	32	%	1.862	-	-	-	2.025	1.789	0.736
Iso-C_5	22	%	1.418	-	-	-	1.402	0.409	0.162
C_2/C_3	51	%	3.736	4.498	-	-	4.126	3.568	0.011
pH	72		6.553	6.655	-	-	6.497	6.689	0.950
NH_3	100	mM	12.688	17.537	19.307	-	14.102	9.124	0.159
H_2-Day	14	mM	0.566	2.714	-	-	1.809	0.821	0.029
CO_2	12	mM	10.010	10.129	-	-	9.755	11.148	0.072
NO_3-rumen	21	µg/mL	0.874	24.242	-	24.240	1.108	-	<.0001
NO_2-rumen	29	µg/mL	1.082	17.020	-	10.810	3.202	-	0.015
Methanogens	10	Log	7.167	_	-	-	6.844	-	0.130
Total-bacteria	10	Log	10.762	_	-	-	10.618	-	0.110
Total-protozoa	28	Log	4.204	-	-	-	4.168	4.138	0.880
Vitamin-A	22	µg/dL	28.692	_	-	-	21.465	-	0.046
β- carotene	12	µg/dL	2.990	-	-	-	1.590	-	0.033
CH4	80	g/day	178.99	139.98	-	-	141.100	158.980	<.0001
CH4/DMI	78	g/kg	19.281	16.179	-	-	15.641	16.766	<.0001
CH4/BW	14	g/kg	0.487	-	-	-	0.283	0.315	0.064
CH4/GEI	34	% GEI	6.438	5.380	-	-	4.891	6.223	<.0001
CH4/milk prod	11	g/kg	15.640	13.603	-	-	15.788	-	0.098
Initial-BW	162	kg	228.96	227.800	-	227.79	229.85	224.340	0.028
Final-BW	40	kg	231.83	-	-	-	234.59	234.15	0.640
LWG	15	kg	0.687	0.959	-	-	0.610	-	0.408
ADG	52	kg/day	0.631	0.567	-	-	0.603	0.569	0.794
FCR	41	kg/kg	3.785	6.268	-	-	11.870	1.429	0.613
Feed efficiency	10	kg	0.145	-	-	-	0.131	-	0.421
DMD	40	%	60.600	64.714	-	-	61.418	62.323	0.192
NDFD	56	%	54.774	55.592	-	-	54.416	59.057	0.325
ADFD	23	%	53.854	_	-	-	53.858	60.705	0.272
CPD	30	%	62.398	_	-	-	65.715	-	0.185
EED	16	%	65.237	64.231	-	-	61.419	-	0.400
Starch D	12	%	97.883	98.135	-	-	98.080	-	0.722
Nitrate-retention	15	g/day	11.291	12.002	-	-	12.157	8.807	0.907
Urea	18	mg/dL	57.817	_	-	-	59.030	53.187	0.391
Microbial protein	19	g/day	9.095	8.696	-	-	10.923	10.630	0.053
NO_2-plasma	35	µg/mL	0.462	0.944	-	0.360	2.382	3.668	0.047
NO_3-plasma	27	µg/mL	0.498	0.016	-	0.010	0.499	1.680	0.011
Total HB	28	mg/dL	9.467	8.157	-	-	9.606	9.595	0.014
Methemoglobin	55	%	1.064	4.302	-	2.665	6.742	0.965	0.026
Methemoglobin min	18	%	0.266	0.650	-	-	9.630	0.200	0.032
Methemoglobin max	12	%	0.808	14.175	-	-	27.550	3.400	0.008
Milk-yield	31	kg/day	21.180	22.168	-	-	20.390	-	0.190
Milk fat (%)	29	%	4.008	3.811	-	-	4.039	-	0.487
Milk fat (kg/day)	15	kg/day	1.036	1.028	-	-	0.948	-	0.022
Milk protein	25	%	3.436	3.127			3.508	-	0.088
Milk protein	11	kg/day	0.781	0.765	-	-	0.751	-	0.590
Milk-lactose	23	%	4.567	4.538	-	-	4.638	-	0.660

Note: NO3 means uncoated encapsulated nitrate, Coat- NO3= coated nitrate, Encap-NO3= encapsulated, NO3-salt= uncoated and un-encapsulated NO3-salt, nitrate salt Coat- NO3-salt, coated nitrate-salt, Encap-NO3-salt, encapsulated nitrate-salt DMI= dry matter intake, MBW= metabolic body weight, NI= nitrogen intake= NDF intake= neutral detergent fiber intake, ADF intake= acid detergent fiber intake, GEI= gross energy intake= DMD= dry matter digestibility, NDFD= neutral detergent fiber digestibility, ADFD= acid detergent fiber digestibility, starch D= starch digestibility, EED= ether extract digestibility, CPD= crude protein digestibility, Total-HB= total hemoglobin, methemoglobin Max= maximum methemoglobin, methemoglobin Min= minimum methemoglobin, initial-BW= initial body weight, final- BW= final body weight, LWG= life weight gain, FCR= feed conversion ratio, CH4/DMI= methane production per total dry matter intake, CH4/BW= methane production per total body weight, CH4/GEI= methane production per total gross energy intake, N= number of data.

Table 4. The interaction effects between animal types, nitrate doses, and nitrate forms on the methane emission, animal performance, and nitrate toxicity in the ruminants' animals

Item	Fixed effect			Interaction effects			
	A	N	D	A*N	A*D	N*D	A*N*D
Feed intake	*	NS	NS	-	-	-	-
DMI	**	NS	*	**	*	NS	*
DMI/MBW	**	*	*	**	*	NS	NS
GEI	*	NS	*	NS	-	-	-
GEI/MBW	NS	*	*	*	NS	NS	NS
NI	NS	*	**	NS	-	-	-
TVFAs	*	NS	NS	*	NS	NS	NS
C_2	NS	**	**	**	*	*	*
C_3	*	*	*	**	NS	NS	NS
C_4	NS	*	*	*	*	*	*
Iso-C_4	NS	NS	NS	NS	NS	NS	NS
C_5	*	NS	NS	NS	NS	NS	NS
Iso-C_5	*	NS	NS	**	-	-	-
C_2/C_3	NS	*	*	NS	NS	NS	NS
pH	NS	NS	NS	NS	**	**	**
NH_3	*	NS	NS	*	**	**	**
H_2-Day	-	*	*	*	*	*	*
CO_2	NS	NS	NS	NS	-	-	-
NO_3-rumen	NS	NS	**	**	**	*	**
NO_2-rumen	**	NS	*	**	**	**	**
Methanogens	NS	NS	NS	NS	NS	NS	NS
Total-bacteria	NS	NS	NS	NS	NS	*	*
Total-protozoa	*	NS	NS	*	NS	*	*
Vitamin-A	NS	*	*	*	*	*	*
β- carotene	*	*	*	*	*	*	*
CH_4	NS	*	*	NS	NS	NS	NS
CH_4/DMI	-	NS	NS	NS	*	*	*
CH_4/BW	NS	*	NS	NS	NS	NS	NS
CH_4/GEI	*	**	**	**	*	*	*
CH_4/milk prod	-	NS	NS	NS	*	*	*
Initial-BW	**	NS	NS	**	**	NS	**
Final-BW	**	NS	NS	**	NS	NS	NS
LWG	NS	NS	NS	NS	**	**	NS
ADG	NS	NS	NS	NS	NS	NS	NS
FCR	**	NS	NS	*	*	*	*
Feed efficiency	NS	NS	NS	NS	NS	NS	NS
DMD	NS	NS	NS	NS	NS	NS	NS
NDFD	NS	NS	NS	NS	NS	NS	NS
ADFD	NS	NS	NS	NS	NS	NS	NS
CPD	NS	NS	NS	NS	NS	NS	NS
EED	NS	NS	NS	NS	NS	NS	NS
Starch D	-	NS	NS	NS	NS	NS	NS
Feed efficiency	NS	NS	NS	NS	NS	NS	NS
Nitrate-retention	NS	NS	NS	NS	-	-	-
Urea	**	NS	NS	*	**	-	**
Microbial protein	*	NS	NS	*	NS	NS	NS
NO_2-plasma	*	NS	*	*	NS	*	**
NO_3-plasma	NS	*	NS	NS	*	NS	*
Total HB	NS	NS	*	*	NS	NS	NS
Methemoglobin	*	NS	NS	*	*	*	**
Methemoglobin min	NS	NS	*	NS	*	*	*
Methemoglobin max	*	NS	NS	*	*	*	*
Milk-yield	NS	NS	*	*	*	*	*
Milk fat (%)	NS	NS	-	NS	NS	NS	NS
Milk fat (kg/day)	*	*	*	*	*	*	*
Milk protein (%)	NS	NS	-	NS	*	NS	*
Milk protein (kg/day)	*	NS	-	NS	*	*	*
Milk-lactose	-	NS	*	NS	*	*	*

Note: A= animal, N= nitrate form, D= Dose of nitrate, A*N = interaction between animal type and nitrate form, A*D = interaction between animal type and nitrate dose, N*D = interaction between nitrate form and dose, A*D*N= interaction among animal type, nitrate form, and nitrate does, NS= not significant, "-" = not calculated, DMI= dry matter intake, MBW= metabolic body weight, NI= nitrogen intake= NDF intake= neutral detergent fiber intake, ADF intake= acid detergent fiber intake, GEI= gross energy intake= DMD= dry matter digestibility, NDFD= neutral detergent fiber digestibility, ADFD= acid detergent fiber digestibility, starch D= starch digestibility, EED= ether extract digestibility, CPD= crude protein digestibility, Total-HB= total hemoglobin, methemoglobin Max= maximum methemoglobin, methemoglobin min= minimum methemoglobin, initial-BW= initial body weight, final- BW= final body weight, LWG= life weight gain, FCR= feed conversion ratio, ADG= average daily gain weight, CH4/DMI= methane production per total dry matter intake, CH4/BW= methane production per total body weight, CH4/GEI= methane production per total gross energy intake.

fed (Alemu *et al.,* 2019). Also, the methemoglobin levels showed to have a significant interaction among nitrate doses, forms, and the animal types.

On the other hand, it was found that sheep tolerate the double amount of nitrate administration in the cattle. A study on nitrite poisoning in goats and sheep showed that sheep tolerate up 50% of the nitrate conversion, while death occurs when the nitrate conversion rate exceeds 80% (Simões *et al.,* 2018). Sheep were reported to have more resistance to nitrate toxicity among the ruminants, and their tolerance to the dietary nitrate can be enhanced by acclimation (Alaboudit & Receivedg, 1985). Sinclair & Jones (1964) concluded that the rumen microorganisms could adapt to the high nitrate addition, utilizing a considerable amount of nitrate. The toxicity occurs under specific farming conditions, such as the sudden introduction of the readily consumed high nitrate diet or under specific feed composition conditions.

Nitrate addition reduced the metabolism of vitamin A as well as the metabolism of the β-carotene. Özdemir *et al*. (2014) proposed that the inclusion of nitrate reduces the carotenes and limits the conversion of the carotenes to vitamin A and subsequently, this reduces the storage of vitamin A in the liver. The β-carotene and vitamin A have significant interaction effects among nitrate dose, forms, and animal types. According to Özdemir *et al*. (2014), the level of vitamin A in the blood was not affected by the inclusion of nitrate into the diet. The author concluded that the dose included in the diet did not cause any significant reduction of vitamin A in the plasma. To date, few papers have investigated the effects of nitrate on the metabolism of vitamin A. Therefore, many studies are suggested to be carried out to fully understand why nitrate causes a significant reduction of vitamin A levels in the blood of the ruminants. However, the addition of up to 1% sodium nitrate did not affect the vitamin A levels in the plasma (Weichenthal *et al.,* 2020). The result is still limited due to the lack of studies; therefore, more studies are recommended to determine the exact amount of nitrate which causes vitamin A reduction.

There was no significant effect of nitrate additions on the rumen bacterial population. This indicates the normal conditions in the rumen (the pH value is still between 6.4 and 6.7). The result was similar to the result that was observed by van Zijderveld *et al*. (2010). The rumen pH value did not change after the nitrate inclusion (Nolan *et al.,* 2016). According to Abdelbagi *et al*. (2021), the rumen pH value is still in the normal range, which is between 6.4 and 6.7. The inclusion of nitrate into the diet resulted in a lower methanogenic population (Klop *et al.,* 2016). Similarly, Villar *et al*. (2020) found a significant reduction in the protozoal log when nitrate was included in the diet. Also, Wang *et al*. (2018) found a significant reduction in the total bacterial count due to nitrate addition to the diet.

The effect of nitrate addition on the concentration of the volatile fatty acids in the rumen was previously investigated. For example, Lund *et al*. (2014) and Rebelo *et al*. (2019) have reported that the inclusion of nitrate has increased the molar proportion of acetic acid and reduced the molar proportion of the propionic acid, while the total volatile fatty acids were not affected by the nitrate inclusion. This was similar to the result observed in this research data collection. Also, van Zijderveld *et al*. (2010) and Sun *et al*. (2017) have reported that the inclusion of nitrate into the diet did not affect the concentration of the total volatile fatty acids. In addition, from the evaluation of the collected data in this study, we found a significant increase in the ratio of acetic to propionic acid. The ratios that were recorded by the unencapsulated and the uncoated and the unencapsulated and the uncoated nitrate were greater than the control treatment and the encapsulated sodium nitrate. However, the concentration of hydrogen in the rumen was greater after the inclusion of nitrate in the diet ($p<0.05$).

However, it is stated that nitrate could influence the methanogenesis process, whether by directly reducing the methanogens number or by competing with the available hydrogen in the rumen (Guyader *et al.,* 2016). Nitrate is a toxic component that could inhibit many microbes in the rumen involving the methanogen. After evaluating the collected data from this study, we observed a significant increase in the concentration of the hydrogen molecule in the rumen, while the number of methanogens was not affected by the nitrate inclusion. Based on this finding, nitrate acts as a hydrogen sink without affecting the methanogens number. So, the explanation is that nitrate is reduced in the rumen to ammonia throughout accepting hydrogen more favorable than carbon dioxide. Both nitrate and methane formations follow the same thermodynamic reaction (Olijhoek *et al.,* 2016). So, the current findings explain that the inclusion of nitrate into the diet could reduce the emitted methane by competing with the available hydrogen in the rumen, resulting in a lower methane production due to the low conversion of the CO_2 and the H_2 molecules into methane molecules. However, nitrate was proposed to affect the gas production trend (Klop *et al.,* 2016). This also indicates the effectiveness of nitrate as a hydrogen iron sink in the rumen.

However, the ammonia concentration was not affected by the nitrate addition. This could be because of the low conversion rate of nitrate molecules into ammonia. The evaluation of the collected data in this study agrees with the previous findings of van Zijderveld *et al*. (2010). On the other hand, the trend of microbial protein synthesis was increased significantly ($p<0.05$) by increasing the nitrate dose in the diet (Table 2). The same result was reported by Nguyen *et al*. (2016). Despite the increase of the microbial protein synthesis due to the increase of the nitrate dose in the diet, nitrate retention, urea production, feed efficiency, average daily gain, or the final body weight was not improved significantly by nitrate addition ($p<0.05$). This could be due to the low nutrient digestion because of lower feed consumption or lower dry matter intake because of the strong bitterness of nitrate salts (Arif *et al.,* 2016). In terms of nitrate forms, the microbial protein synthesis was numerically lower at the nitrate treatment (NO_3) as compared with the groups of unencapsulated and uncoated nitrate salt (NO_3-salt) and the encapsulated nitrate salt (NO_3-salt). No significant interaction was observed among nitrate forms, nitrate doses, or animal types on microbial protein synthesis.

In contrast, Pal *et al*. (2015) found no significant effect of nitrate on the apparent digestibility, crude fat, NDF, and starch digestibility. Silivong *et al*. (2011) reported that the inclusion of nitrate into the diet did not affect the nitrate retention of the ruminants. A similar result was reported by Guyader *et al*. (2015). Based on these results, the inclusion of nitrate at low levels in the diet is recommended to maximize the beneficial effects of nitrate inclusion in the diet. Villar *et al*. (2020) mentioned that the greater urinary nitrogen excretion of animal fed nitrate or urea suggests an inadequate amount of fermentable carbohydrates. It is stated that nitrite utilization could be accelerated by maintaining the energy supply (Nolan *et al*., 2016). In addition, the decrease in methane production might not influence performance production because hydrogen production could be another source of energy losses in the ruminants (Lee & Beauchemin, 2014). Therefore, there was no significant improvement in the meat production performance.

Based on the evaluation of the collected data, it showed that there was no significant effect of nitrate addition on animal performance or the milk yield, while there was a significant decrease in milk protein and milk fat contents. Meller *et al*. (2019) have observed numerous decreases in the final body weight due to nitrate administration. Li *et al*. (2013) have investigated the effects of nitrate and sulfur addition on the Marino lambs' growth. The authors suggested that the ruminant may benefit from the low nitrate addition. The results are in agreement with the result that was reported by Li *et al*. (2013). The same result was reported by Klop *et al*. (2016) and Meller *et al*. (2019). This result is because the dietary nitrate did not significantly affect nutrient digestibility (dry matter, organic matter, crude fat, NDF, or the starch) and subsequently did not significantly affect the feed conversion ratio, average daily gain, final body gain, and milk yield. However, Meller *et al*. (2019) have stated that the decrease of the DMI would decrease the nutrient to support the milk protein synthesis, resulting in lower milk protein content. At the same time, it was found that nitrate has decreased the fat milk content in ruminant animals (Veneman *et al*., 2015). In contrast, Olijhoek *et al*. (2016) found a numerical decrease in milk fat content due to nitrate addition to the diet. Therefore, this result suggests that the reduction of the daily amount of milk protein and fat milk content could be attributed to the low energy supply, which reduces the capacity of microbial nitrite utilization.

CONCLUSION

Nitrate toxicity could be reduced by the gradual inclusion of nitrate into the diet. Sheep appear to be more tolerant to nitrate toxicity than other ruminant animals. The encapsulation process increases the concentration of both plasma nitrate and nitrite. The coating process increases the rumen nitrate but decreases the concentrations of the plasma nitrate and nitrite. The nitrate addition is considered an effective method for mitigating the enteric methane emission in the ruminants by acting as a hydrogen sink. To improve nitrate utilization, the diet must be provided with readily fermented carbohydrates or by optimizing the energy levels of the diet and encapsulation strategy to improve the effectiveness of the nitrate addition on animal performance.

CONFLICT OF INTEREST

Nahrowi and A. Jayanegara serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors also declare there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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