Research Article

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Effects of encapsulation and combining probiotics with different nitrate forms on methane emission and *in vitro* rumen fermentation characteristics

https://doi.org/10.1515/opag-2022-0377
received December 20, 2023; accepted October 7, 2024

Abstract: This study aimed to evaluate the effects of encapsulation and combining probiotics with different nitrate forms on methane emission and the *in vitro* fermentation process of ruminants. Sodium nitrate ($NaNO_3$) and nitric acid (HNO_3) were used as nitrate forms, while lactic acid bacteria *Lactiplantibacillus plantarum* TSD-10 was used as a probiotic source. Twelve different treatments with four replicates were allocated in the factorial block design (2 × 2 × 3). During each replicate, the test was conducted individually in a different week so that each block could be considered separately. Data analysis followed the analysis of variance (ANOVA) and then continued with the Duncan multiple range test. After encapsulation, significant increases (p < 0.05) in gas production, gas kinetics, total volatile fatty acids (TVFAs), and production of propionic acid were observed. In addition, encapsulation significantly decreased (p < 0.05) the pH, ammonia concentration (NH_3), nutrient digestibility, and the ratio of acetic to propionic acid (p < 0.05). The addition of combined encapsulated probiotics and encapsulated nitrate significantly increased (p < 0.05) gas production, maximum gas production, TVFAs, and the molar portion of propionic acid, and significantly decreased (p < 0.05) enteric methane emission, acetic acid, ammonia concentration, pH, and nutrient digestibility. The addition of sodium nitrate significantly increased (p < 0.05) the concentration of TVFAs and acetic acid, while nitric acid significantly increased (p < 0.05) the gas production rate. However, there was no significant effect due to combining unencapsulated probiotics with unencapsulated nitrate forms on the rumen fermentation process. There was a significant interaction (p < 0.05) between encapsulation probiotics and nitrate on ammonia concentration. In conclusion, combining encapsulated probiotics with encapsulated nitrate is an alternative method for enhancing the fermentation process and mitigating enteric methane emission in ruminants.

Keywords: encapsulation, nitrate, probiotics, methane emission, fermentation process

1 Introduction

Methane (CH_4) is normally produced as a result of microbial activity, especially archaeal methanogens, during the fermentation process in ruminants [1]. In addition, methane is the second greatest greenhouse gas (GHG) after carbon dioxide [1]. Methane gained its importance among the other greenhouse gases due to its potential contribution to climate change and global warming phenomena [2–4]. On the other hand, the livestock sector has an important contribution to methane emission, which reaches about 14.5% of the total emitted methane in the globe [5]. In addition, 12% of gross energy (GE) losses in ruminants are lost due to the enteric methane emission. Thus, the issue of enteric methane emission is in relation to feed utilization, productivity, and global warming [6]. Therefore, the dietary options that are used for mitigating enteric methane emissions in ruminants are

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effective in elevating environmental concerns, improving utilization, and improving animal productivity [7]. Among the dietary options for mitigating such enteric methane emissions, the use of feed additives has been considered a promising option [8].

Both nitrate and probiotics have been suggested as feed additives to inhibit methane emission in ruminant animals [9–12]. Nitrate has the potential to inhibit enteric methane in ruminants due to its ability to act as an electron acceptor [13,14]. Moreover, nitrate is toxic for both methanogens [15] and ruminants themselves due to its relation with methemoglobinemia that occurs as a result of consuming high nitrate diets [16–18]. Despite the effectiveness of nitrate as a methane inhibitor, nitrate is still widely unused in ruminant nutrition. On the other hand, probiotics were proposed to mitigate enteric methane emission in ruminants [19] through two different mechanisms: first, probiotics have the ability to stimulate the growth of lactic acid utilizing bacteria, resulting in high production of propionic acid and subsequently decrease hydrogen molecules for forming methane during the fermentation process in ruminant animals [20]. Second, probiotics contribute to providing some nutrients for bacterial growth. The nutrients include some metabolic intermediates and vitamins that are essentially used for bacterial growth, and therefore, this may negatively affect methanogen growth [21].

Many experiments have been investigated to determine the effects of combining nitrate with other inhibitors on reducing enteric methane emission, improving utilization, and improving animal productivity. For instance, nitrate and nitrate-reducing bacteria have been used to enhance nitrate reduction [22]. Also, combining nitrate with saponin was previously examined. The authors did not observe significant effects due to combining nitrate with saponin on rumen microorganisms [23]. Additionally, the effects of combining nitrate with garlic oils were examined by using different raw materials. The results showed a significant effect of combining methane with garlic oils on methanogens [24].

Encapsulation is a process that is used to prevent nutrients from undesirable conditions over time by improving their stability and bioavailability and controlling their release rate at specific times and places [25]. Encapsulated nitrate was reported to reduce methane production without negatively affecting the performance of animals [26]. To date, there has been no research on the effects of combining probiotics with nitrate forms. Therefore, we hypothesized that encapsulation and combining probiotics with nitrate forms would influence the rumen fermentation process and decrease enteric methane emissions in ruminants. Therefore, in this study, we aimed to evaluate the effects of encapsulation and combine probiotics with different nitrate forms on enteric methane emission and *in vitro* fermentation characteristics.

2 Materials and methods

All research procedures in the present study were performed at the Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong, Indonesia, and the Department of Nutrition and Feed Technology, IPB University, Bogor, Indonesia.

2.1 Materials

A commercial concentrate containing soya bean meal, rice bran, corn meal, corn gluten feed (CGF), distiller dried grains with solubles (DDGS), and others was purchased from the Indofeed Mini Feed mill, Bogor, West Java. In this study, elephant grass (*Pennisetum purpureum*) was used as a source of forage. Forage was collected from the surrounding area of the research farm of KST Soekarno-BRIN, Cibinong, West Java, Indonesia. *Lactiplantibacillus plantarum (*10 log CFU/ml (TSD-10)) was used as a probiotic source. Probiotics were prepared by culturing *L. plantarum* in a facultative fermentation medium at 30°C in deMan Rogosa Sharpe (MRS) broth medium (Merck, Darmstadt, Germany). Preparation was done in the Genomic and Environmental Laboratory of the National Research and Innovation Agency (BRIN), Cibinong. In this study, maltodextrin was used as a coating material for encapsulation. Maltodextrin was in a powder readily used form. Sodium nitrate ($NaNO_3$), 99% purity, and nitric acid (HNO_3), 70% purity, were used as sources of nitrate. Sodium nitrate was supplied by Merck (Darmstadt, Germany), while nitric acid was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India).

2.2 Encapsulation process

Encapsulation was done by using a freeze dryer, according to Chen Man et al. [27]. Briefly, 10 mM of $NaNO_3$ and HNO_3 were dissolved in 10 ml of distilled water. Then, 10 ml of probiotics containing 10 (log CFU/ml) was mixed with 10 g of maltodextrin. The mixture was prepared from different nitrate forms (sodium nitrate, nitric acid), probiotics, and maltodextrin (1:1:1) to obtain 10 mM nitrate. A total of 10 g

of maltodextrin, 10 ml of sodium nitrate and nitric acid, and 10 ml of probiotics *L. plantarum* TSD-10 were mixed. The mixture was immediately kept at 20°C in the freezer for 15 min to homogenize. Subsequently, samples were placed overnight in an 80°C deep freezer (CHRIST Alpha 1-4 LD plus) until they became completely dry. After that, samples were ground by using a mortar and pestle to be used in the next steps.

2.3 *In vitro* experimental procedure

Feed materials (concentrates and the forage) were ground to pass a 1 mm screen size. Then, feed samples (concentrate and forage materials) were analyzed before adding nitrate forms or probiotics using the method described by Ridwan et al. [28] (Table 1). A feed ratio of 60% concentrates and 40% forages was used. Further, diets were designed in a 2 × 3 × 3 factorial design with 12 different treatments. Treatments were prepared by adding 0.5 g of nitrate forms and 0.5 ml of *L. plantarum* TSD-10. Treatments included T1(encapsulated $NaNO_3$ without probiotics), T2 (encapsulated $NaNO_3$ with probiotics), T3 (encapsulated $NaNO_3$ with encapsulated probiotics), T4 (non-encapsulated $NaNO_3$ probiotics without probiotics), T5 (non-encapsulated $NaNO_3$ probiotics with probiotics), T6 (non-encapsulated $NaNO_3$ probiotics with encapsulated probiotics), T7 (encapsulated HNO_3 without probiotics), T8 (encapsulated HNO_3 with probiotics), T9 (encapsulated HNO_3 with encapsulated probiotics), T10 (non-encapsulated HNO_3 probiotics without probiotics), T11 (non-encapsulated HNO_3 with probiotics), and T12 (non-encapsulated HNO_3 probiotics with encapsulated probiotics). Treatments were quadruplicated according to the number of *in vitro* incubation runs. Each of the replicates was run individually in different weeks. Each week was considered a block by itself.

Table 1: Chemical composition of fistulated cattle basal diet and *in vitro* substrate (% dry matter)

Item	Basal diet		*In vitro* substrate
	Forage	Concentrate	
Ash	2.3	3.0	3.2
CP	8.25	16.0	16.6
EE	1.92	5.30	4.83
CF	35.8	15.8	21.1
NDF	61.1	44.1	44.1
ADF	40.7	33.0	28.1

CP, crude protein; EE, ether extract; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber.

The buffer medium was prepared anaerobically following the method of McDougall [29]. Rumen fluids were collected from two rumen fistulated Ongole crossbred males with an average body weight of 550 ± 30 kg. Steers were handled and maintained in accordance with the protocols of animal welfare of the Animal Care and Use Committee of the Indonesian Institute of Sciences 2015. Animals were fed two times a day (morning and afternoon). The feed substrate consisted of 40% forages and 60% concentrates. Water was freely accessible by animals. The rumen fluid collection was done before the morning feeding, around 7:00 a.m. Rumen solutions were sieved through a four-layer cheesecloth. A total of 500 ml of rumen fluid from each animal was collected and kept separately in pre-warm bottles. After collection, solutions were brought immediately to the laboratory and kept in a water bath at 39°C. Each of the collected fluids was separately transferred to the conical flask, sealed with an aluminum foil. After that, the rumen pH was determined and recorded. The pH was measured by using a TRAI BP3001 pH meter, e.g., the average pH of the samples collected from steer No. 1 was 6.93, and that of the sample from steer No. 2 was 6.91. The rumen buffered solution was mixed at 1:2 of rumen fluid/buffered solution. Subsequently, a rumen buffer solution was placed in a conical flask, which was sealed with an aluminum foil. Each of the rumen buffer solutions was continuously purged with CO_2 to maintain the pH value and the anaerobic conditions. The pH value of the mixture was also recorded (pH 7.2 and 7.1). Incubation was done in accordance with a modified protocol of Theodorou et al. [30]. Forty-eight vials (100 ml) were filled with 50 ml of rumen buffer fluid containing 500 mg of the experimental substrate. All bottles were sealed with butyl rubber stoppers and aluminum crimps before placing into a 39°C water bath. Then, all bottles were incubated for 72 h using a 39°C water bath. However, the bottles were frequently shaken every 1 h. Each of the treatments had two blank bottles. Incubation was run four times during four different weeks. Each week was considered as a replicate by itself.

After 72 h of incubation, the gas production of each bottle was vented and recorded at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. Methane concentration was measured at 8, 10, 12, 24, 48, and 72 h. Total gas production was measured using a 50 ml syringe, while methane concentration was measured using a methane analyzer (RIKEN KEIKI RX415). Gas production kinetics were estimated using the Ørskov equation: $p = a + b\,(1 - e^{-c.t})$ [31]. After 72 h of incubation, the serum in each bottle was sieved carefully in the plastic corning and the pH of the residues was measured. However, calibration of the Cyberscan pH 310 Eutech equipment was

Table 2: Effects of treatments on *in vitro* gas production and methane emission

Item		Total gas (ml/g DM)	a + b (ml/g DM)	c (/h)	CH_4 (% gas)
Encapsulation	ENCAP	216 ± 32.5^{b}	216 ± 29.4^{b}	0.112 ± 0.016^{b}	7.35 ± 4.25
	Non-ENCAP	123 ± 75.6^{a}	128 ± 70.5^{a}	0.064 ± 0.043^{a}	7.05 ± 3.81
Nitrate type	N1	163 ± 77.1	162 ± 75.7^{a}	0.089 ± 0.035	7.66 ± 4.43
	N2	176 ± 72.9	181 ± 62.5^{b}	0.087 ± 0.045	6.75 ± 3.87
Probiotics	Without PRO	140 ± 71.9^{a}	150 ± 62.4^{a}	0.068 ± 0.038^{a}	9.08 ± 4.16^{b}
	With PRO	141 ± 73.8^{a}	140 ± 66.9^{a}	0.076 ± 0.035^{a}	8.35 ± 3.39^{b}
	With ENCAP-PRO	228 ± 63.7^{b}	232 ± 30.2^{b}	0.120 ± 0.025^{b}	4.18 ± 3.16^{a}
Treatment	T1	204 ± 14.5^{b}	194 ± 11.5^{c}	0.100 ± 0.018^{bc}	8.97 ± 5.05
	T2	206 ± 16.4^{b}	196 ± 14.1^{cd}	0.107 ± 0.005^{bc}	8.45 ± 4.52
	T3	226 ± 51.7^{b}	239 ± 43.2^{de}	0.127 ± 0.032^{cd}	2.20 ± 1.24
	T4	60.3 ± 12.9^{a}	63.0 ± 18.2^{a}	0.037 ± 0.005^{a}	8.03 ± 5.90
	T5	63.5 ± 4.26^{a}	63.0 ± 4.31^{a}	0.050 ± 0.008^{ab}	7.35 ± 2.67
	T6	222 ± 20.3^{b}	218 ± 13.2^{d}	0.102 ± 0.012^{bc}	5.51 ± 4.23
	T7	210 ± 22.6^{b}	205 ± 13.7^{cd}	0.110 ± 0.001^{c}	10.6 ± 3.73
	T8	215 ± 19.1^{b}	210 ± 9.54^{d}	0.112 ± 0.009^{c}	9.22 ± 4.17
	T9	238 ± 21.3^{b}	249 ± 25.0^{de}	0.117 ± 0.015^{c}	4.74 ± 3.17
	T10	84.8 ± 21.1^{a}	114 ± 22.6^{ab}	0.027 ± 0.012^{a}	8.78 ± 2.77
	T11	79.5 ± 10.5^{a}	89.5 ± 6.96^{a}	0.035 ± 0.006^{a}	8.38 ± 3.17
	T12	228 ± 22.5^{b}	221 ± 32.4^{cd}	0.135 ± 0.029^{cd}	4.26 ± 3.51
p-value	Encapsulation	<0.001	<0.001	<0.001	0.786
	Nitrate type	0.117	0.003	0.634	0.421
	Probiotics	<0.001	<0.001	<0.001	0.002
	Treatment	<0.001	0.669	0.116	0.130
	ENC*NITR	0.691	0.212	0.924	0.518
	ENC*PRO	<0.001	<0.001	<0.001	0.552
	NITR*PRO	0.949	<0.275	0.305	0.982
	ENC*NITR*PRO	0.816	0.287	0.007	0.747
	Block	<0.001	<0.001	<0.001	<0.001

a + b, potential gas production; c, gas production rate; ENCAP, encapsulation; Non-ENCAP, non-encapsulation; N1, sodium nitrate; N2, nitric acid; PRO, probiotics; ENCAP-PRO, encapsulated probiotics; ENC*NTR, the interaction between encapsulated and the nitrate type; ENCA*PRO, the interaction between encapsulated and probiotics; NTR*PRO, the interaction between the nitrate type and probiotics; ENC*NITR*PRO, the interaction among the encapsulated, nitrate type, and probiotics; T1, encapsulated sodium nitrate without probiotics; T2, encapsulated sodium nitrate with probiotics; T3, encapsulated sodium nitrate with encapsulated probiotics; T4, non-encapsulated sodium nitrate without probiotics; T5, non-encapsulated sodium nitrate with probiotics; T6, non-encapsulated sodium with encapsulated probiotics; T7, encapsulated nitric acid without probiotics; T8, encapsulated nitric acid with probiotics; T9, encapsulated nitric acid with encapsulated nitric acid; T10, non-encapsulated nitric acid without probiotics; T11, non-encapsulated nitric acid with probiotics; T12, non-encapsulated nitric acid with encapsulated probiotics; SEM, standard error of means; probability was considered significant when *p*-value <0.05; Small letter superscripts are in ascending order.

done using a pH 7 buffer solution. Later, each corning was centrifuged at 6,000 for 10 min at −4°C to determine the nutrient digestibility (dry matter and organic matter digestibility). The nutrient digestibility was measured as described by Tilley and Terry [32]. Residues were added to 20 ml of 0.2% pepsin HCL solution. Then, all samples were incubated for another 24 h. After incubation, the samples were dried at 130°C for 8 h and then burnt at 600°C for 3 h to obtain the nutrient digestibility (DMD and the OMD). The *in vitro* nutrient digestibility of the dry matter (IVDMD) and organic matter (IVOMD) was determined by subtracting the amount of the initial substrate from the substrates after the drying and burning processes. Total and partial volatile fatty acids were determined by using 10 ml of the supernatant, which was filtered carefully and collected in a plastic corning. The concentration of total volatile fatty acids (TVFAs, mg/L) was determined by using a spectrophotometer (495λ), as described in the study of Biswabandhu and Radhakrishnan [33]. Further, the molar portions of partial volatile fatty acids were determined using a GC machine (GC-MS-QP2010 SE) using a MEGA-WAX MS column (025-02530). Another 5 ml of the supernatant was used for determining ammonia concentration. Ammonia concentrations were quantified using a spectrophotometer (630λ) in accordance with the study of Souza et al. [34].

2.4 Statistical analysis

Data were analyzed using the general linear model procedure with a 2 × 2 × 3 factorial arrangement. The first factor included two different physical forms (encapsulated and noncapsulated). The second factor included two different chemical forms ($NaNO_3$ and HNO_3). The third factor included three different probiotic treatments (without probiotics, with probiotics, and with encapsulated probiotics). The allocation of treatments to experimental units followed a completely randomized block factorial design. Different *in vitro* operations served as blocks due to population variations and rumen microbial activity with each sampling time (each week). Data were analyzed by analysis of variance (ANOVA) based on a completely randomized factorial block design. When the ANOVA results showed $p < 0.05$ for a particular parameter, a post-hoc test, namely Duncan's multiple range test, was applied to the data. Data analysis was performed using SAS Statistics software version 9.1.4. The figures are presented using Microsoft Office Excel.

3 Results

The effects of encapsulated and combining probiotics with nitrate forms on gas production and methane production are shown in Table 2. Gas production kinetics on the effects of encapsulation and nitrate types are presented in Figures 1 and 2, respectively. The effects of encapsulated and combining probiotics with nitrate forms on rumen fermentation parameters are presented in Table 3. The effects of encapsulated and combined probiotics with nitrate forms on the partial volatile fatty acids are shown in Table 4. Treatments significantly influenced the fermentation process. After encapsulation, we observed a significant increase in the gas production, gas kinetics, TVFAs, and production of propionic acid. In addition, encapsulation significantly decreased

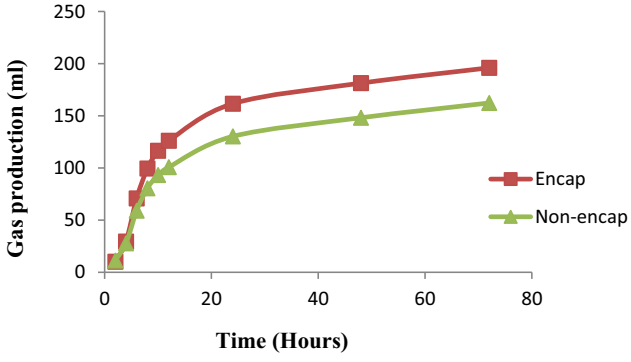

Figure 1: Effects of encapsulation on gas production kinetics.

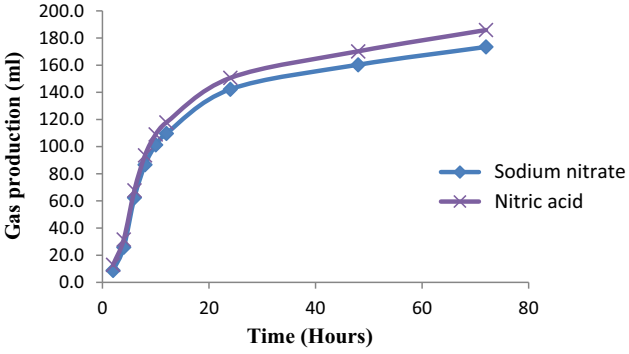

Figure 2: Effects of nitrate types on gas production kinetics.

the pH, ammonia concentration (NH_3), nutrient digestibility, and the ratio of acetic to propionic acid ($p < 0.05$). The addition of combined encapsulated probiotics and encapsulated nitrate significantly increased the gas production, maximum gas production, TVFAs, and the molar portion of propionic acid and significantly decreased enteric methane emission, acetic acid, ammonia concentration, pH, and nutrient digestibility ($p < 0.05$). The addition of sodium nitrate significantly increased the concentration of TVFAs and acetic acid, while nitric acid significantly increased the gas production rate ($p < 0.05$). However, there was no significant effect due to combining unencapsulated probiotics with unencapsulated nitrate forms on the rumen fermentation process. There was a significant interaction among encapsulation probiotics and nitrate on ammonia concentration.

4 Discussion

We observed a significant influence of encapsulation on the fermentation process. Encapsulation increased significantly the TVFAs, gas production, maximum gas production, and gas production rate. Also, we observed a significant increase in gas production and TVFAs due to combining encapsulated probiotics with encapsulated nitrate forms. Among other treatments, the highest gas production and the highest TVFAs were scored as a result of combining encapsulated probiotics with encapsulated nitrate forms before or after encapsulation. The results were consistent with those of previous studies [9,35], which reported a significant increase in the total produced gas and TVFAs due to the addition of encapsulated probiotics and encapsulated nitrate. Also, there was a significant interaction between probiotics and encapsulation in gas production.

The increase in the gas production rate and amounts of TVFAs could probably be due to the effects of the encapsulation process. In this study, maltodextrin was used as a

Table 3: Effects of treatments on the rumen fermentation characteristics

Item		pH	NH_3 (mg/ml)	IVDMD (%)	IVOMD (%)	TVFAs (mmol/g)
Encapsulation	ENCAP	5.75 ± 0.57^{a}	28.8 ± 15.6^{a}	47.7 ± 17.0^{b}	57.7 ± 15.2^{a}	111 ± 17.6^{b}
	Non-ENCAP	6.53 ± 0.33^{b}	39.1 ± 11.0^{b}	58.8 ± 4.73^{a}	68.5 ± 5.16^{b}	64.6 ± 32.6^{a}
Nitrate forms	N1	6.19 ± 0.60	34.5 ± 14.4	53.3 ± 12.8	63.5 ± 12.3	91.9 ± 32.6^{b}
	N2	6.09 ± 0.61	33.4 ± 14.8	53.2 ± 14.6	62.7 ± 12.9	83.9 ± 37.5^{a}
Probiotics	Without encapsulation	6.41 ± 0.34^{ab}	40.4 ± 8.74^{b}	59.2 ± 5.85^{b}	68.1 ± 4.49^{b}	77.7 ± 36.4^{a}
	With encapsulation	6.38 ± 0.37^{ab}	36.6 ± 12.4^{b}	59.3 ± 17.1^{b}	67.9 ± 3.40^{b}	75.5 ± 37.0^{a}
	With encapsulation	5.63 ± 0.69^{c}	24.9 ± 16.7^{a}	41.4 ± 4.50^{a}	53.3 ± 17.4^{a}	110 ± 17.8^{b}
Treatment	T1	6.20 ± 0.07^{b}	40.9 ± 5.81^{cd}	61.1 ± 5.92^{b}	69.9 ± 4.46^{b}	98.9 ± 16.0^{b}
	T2	6.13 ± 0.07^{b}	30.2 ± 1.25^{bc}	57.6 ± 4.97^{b}	66.7 ± 3.77^{b}	110 ± 20.3^{b}
	T3	5.21 ± 0.08^{a}	9.21 ± 3.63^{a}	27.8 ± 8.35^{a}	39.6 ± 6.58^{a}	97.0 ± 7.70^{b}
	T4	6.76 ± 0.04^{c}	35.0 ± 11.1^{bc}	57.2 ± 4.99^{b}	65.8 ± 4.07^{b}	43.2 ± 11.5^{a}
	T5	6.74 ± 0.03^{c}	41.9 ± 16.9^{bcde}	59.3 ± 3.65^{b}	67.8 ± 2.33^{b}	42.6 ± 12.1^{a}
	T6	6.12 ± 0.08^{b}	42.9 ± 5.47^{cd}	57.0 ± 4.38^{b}	71.2 ± 10.5^{b}	112 ± 13.8^{bcd}
	T7	5.98 ± 0.07^{b}	35.5 ± 4.76^{c}	58.6 ± 6.36^{b}	67.6 ± 4.83^{b}	121 ± 13.2^{cd}
	T8	5.94 ± 0.09^{b}	47.7 ± 6.86^{de}	58.1 ± 4.84^{b}	66.9 ± 3.64^{b}	108 ± 10.5^{bc}
	T9	5.05 ± 0.04^{c}	9.26 ± 1.74^{a}	23.3 ± 4.91^{a}	35.7 ± 4.09^{a}	131 ± 14.0^{d}
	T10	6.70 ± 0.04^{c}	50.1 ± 2.42^{de}	59.9 ± 7.72^{b}	68.9 ± 5.03^{b}	47.2 ± 11.1^{a}
	T11	6.71 ± 0.07^{c}	26.6 ± 6.12^{b}	62.1 ± 4.86^{b}	70.1 ± 3.89^{b}	41.4 ± 13.5^{a}
	T12	6.14 ± 0.40^{b}	38.0 ± 5.27^{cd}	57.4 ± 2.16^{b}	66.9 ± 1.04^{b}	102 ± 14.5
p-value	Encapsulation	<0.001	<0.001	<0.001	<0.001	<0.001
	Nitrate type	0.215	0.584	0.938	0.572	0.049
	Probiotics	<0.001	<0.001	<0.001	<0.001	<0.001
	Treatment	<0.001	<0.001	<0.001	<0.001	<0.001
	ENC*NITR	0.329	0.179	0.203	0.427	0.011
	ENC*PRO	0.187	<0.001	<0.001	<0.001	<0.001
	NITR*PRO	0.927	0.381	0.645	0.292	0.253
	ENC*NITR*PRO	0.999	<0.001	0.926	0.707	0.074
	Block	0.948	0.838	<0.001	<0.001	<0.001

pH; the rumen pH value, NH_3; ammonia concentration, IVDMD; *in vitro* dry matter digestibility, IVOMD; *in vitro* organic matter digestibility, ENCAP; encapsulation, Non-ENCAP; non-encapsulation, N1; sodium nitrate, N2; nitric acid, PRO; probiotics ENCAP-PRO; encapsulated probiotics, INTR; the interaction among the factors, ENC*NTR; the interaction between encapsulated and the nitrate type, ENCA*PRO; the interaction between encapsulated and the probiotics, NTR*PRO; the interaction between the nitrate type and probiotics, ENC*NITR*PRO; the interaction among the encapsulation, nitrate type, and probiotics,T1; encapsulated sodium nitrate without probiotics, T2; encapsulated sodium nitrate with probiotics, T3; encapsulated sodium nitrate with the encapsulated probiotics, T4; non-encapsulated sodium nitrate without probiotics, T5; non-encapsulated sodium nitrate with probiotics, T6; non-encapsulated sodium with encapsulated probiotics, T7; encapsulated nitric acid without probiotics, T8; encapsulated nitric acid with probiotics, T9; encapsulated nitric acid with encapsulated nitric acid, T10; non-encapsulated nitric acid without probiotics, T11; non-encapsulated nitric acid with probiotics, T12; non-encapsulated nitric acid with encapsulated probiotics, SEM; standard error of means, probability was considered when *p*-value <0.05. Small letter superscripts a: z are in ascending order.

matrix for coating probiotics and nitrate during the encapsulation process. Maltodextrin is a readily fermented carbohydrate. Therefore, it could easily be attacked by rumen microorganisms, resulting in higher TVFAs and higher gas production [36]. However, several coating materials have been previously investigated [37,38]. For instance, maltodextrin was reported to increase the gas production rate and the amount of TVFAs produced, while a lower gas production rate was observed when sodium alginate was used as a matrix for encapsulation. Therefore, an increase in gas production and TVFAs is correlated with the types of coating materials. For example, carbohydrate materials are known to have a greater gas production rate as compared with other ingredients [39]. Generally, an increase in gas production does not indicate a deficiency of feed utilization; however, it indicates a higher fermentation rate of substrate degraded by rumen microorganisms [19]. According to Rahman et al. [40], changes in the gas production rates of different substrates are correlated with a significant shift in portions of TVFAs. Total gas naturally includes CO_2, CH_4, and small amounts of H_2, N_2, and O_2 as a result of degrading nutrient substrates [41]. To avoid bias due to encapsulation, the amount of gas produced in this study as a result of degrading maltodextrin (about 20 ml) of the blank substrate was subtracted from the total gas production. On the other hand, there was no significant difference in gas production

Table 4: Effects of treatments on the partial volatile fatty acids

Item		Acetate (%)	Propionate (%)	Isobutyrate (%)	Butyrate (%)	Isovalerate (%)	Valerate (%)	Acetate: Propionate (%)
Encapsulation	Encap	49.1 ± 4.02^{a}	40.9 ± 6.93^{b}	0.75 ± 0.32^{b}	5.97 ± 1.87^{b}	0.34 ± 0.34^{a}	1.46 ± 0.0.43^{b}	1.26 ± 0.34^{a}
	Non-encap	60.3 ± 10.2^{b}	30.9 ± 10.8^{a}	0.43 ± 0.69^{a}	4.14 ± 2.0^{a}	0.60 ± 0.37^{b}	1.24 ± 0.51^{a}	2.28 ± 1.01^{b}
Nitrate type	N1	55.9 ± 9.77^{b}	36.7 ± 10.5	0.60 ± 0.54	5.30 ± 2.28	0.52 ± 0.37	1.45 ± 0.43	1.85 ± 0.96
	N2	53.5 ± 9.31^{a}	34.9 ± 10.3	0.57 ± 0.59	4.81 ± 1.99	0.51 ± 0.36	1.24 ± 0.52	1.67 ± 0.87
Probiotics	Without	57.4 ± 9.23^{b}	33.6 ± 9.78^{a}	0.72 ± 0.62^{b}	6.12 ± 1.86^{b}	0.63 ± 0.34^{b}	1.20 ± 0.32^{a}	1.95 ± 0.94^{b}
	With	58.3 ± 10.9^{b}	32.6 ± 11.4^{a}	0.68 ± 0.61^{b}	5.87 ± 1.97^{a}	0.58 ± 0.31^{b}	1.15 ± 0.44^{a}	2.12 ± 1.05^{b}
	With-ENCAP	48.5 ± 4.20^{a}	41.4 ± 7.67^{b}	0.38 ± 0.37^{a}	4.47 ± 2.25^{a}	0.32 ± 0.37^{a}	1.69 ± 0.49^{b}	1.23 ± 0.37^{a}
Treatment	T1	50.9 ± 3.65^{b}	38.0 ± 5.45^{b}	6.62 ± 0.26^{bcd}	6.62 ± 2.51^{cd}	0.67 ± 0.42^{bcde}	1.40 ± 0.24^{bc}	1.37 ± 0.27^{a}
	T2	50.2 ± 5.44^{b}	39.5 ± 10.4^{b}	6.35 ± 0.45^{bcd}	6.34 ± 2.48^{cd}	0.67 ± 0.45^{bcde}	1.34 ± 0.51^{abc}	1.37 ± 0.50^{a}
	T3	51.0 ± 6.11^{b}	38.8 ± 10.2^{b}	6.49 ± 0.37^{bcd}	6.49 ± 1.80^{cd}	0.39 ± 0.38^{abc}	1.23 ± 0.56^{abc}	1.42 ± 0.58^{a}
	T4	66.8 ± 1.32^{a}	25.5 ± 6.03^{a}	2.92 ± 0.85^{a}	2.92 ± 0.74^{a}	0.71 ± 0.46^{bcd}	0.90 ± 0.20^{ab}	2.78 ± 0.90^{b}
	T5	70.0 ± 2.36^{a}	22.8 ± 4.32^{a}	2.68 ± 0.74^{a}	2.68 ± 0.32^{a}	0.51 ± 0.13^{bc}	0.88 ± 0.20^{ab}	3.16 ± 0.65^{b}
	T6	46.8 ± 1.87^{b}	45.4 ± 4.58^{b}	3.81 ± 0.28^{ab}	3.81 ± 0.48^{ab}	0.16 ± 0.06^{a}	1.68 ± 0.31^{c}	1.06 ± 0.12^{a}
	T7	46.8 ± 1.34^{b}	45.0 ± 4.33^{b}	4.36 ± 0.24^{ab}	4.36 ± 0.58^{bc}	0.25 ± 0.49^{ab}	1.47 ± 0.25^{bc}	1.05 ± 0.12^{a}
	T8	46.6 ± 3.48^{b}	43.8 ± 6.01^{b}	5.09 ± 0.36^{bc}	5.89 ± 1.91^{c}	0.47 ± 0.23^{bc}	1.40 ± 0.61^{bc}	1.08 ± 0.22^{a}
	T9	49.4 ± 1.84^{b}	40.1 ± 4.22^{b}	6.94 ± 0.24^{c}	6.94 ± 0.61^{d}	0.21 ± 0.08^{a}	1.90 ± 0.12^{c}	1.24 ± 0.16^{a}
	T10	65.1 ± 2.37^{a}	26.0 ± 5.57^{a}	3.97 ± 0.89^{ab}	3.97 ± 0.36^{ab}	0.69 ± 0.07^{c}	1.02 ± 0.23^{ab}	2.61 ± 0.67^{b}
	T11	66.4 ± 2.03^{a}	24.2 ± 5.17^{a}	4.23 ± 0.84^{ab}	4.23 ± 3.41^{bc}	0.87 ± 0.30^{cde}	0.98 ± 0.21^{ab}	2.85 ± 0.69^{b}
	T12	46.7 ± 3.48^{b}	41.3 ± 10.9^{b}	7.25 ± 0.57^{de}	7.25 ± 2.13^{df}	0.63 ± 0.54^{abcde}	1.94 ± 0.58^{cd}	1.22 ± 0.44^{a}
p-value	ENCAP	<0.001	<0.001	0.0574	0.0005	0.0757	0.0496	<0.0001
	NITR	0.021	0.384	0.7862	0.3104	0.8926	0.0621	0.229
	PRO	<0.001	0.002	0.1870	0.0129	0.0218	0.0014	0.0001
	Treatment	<0.001	<0.001	0.5742	0.0012	0.0334	0.000	<0.0001
	ENC*NITR	0.494	0.222	0.4325	0.0031	0.0041	0.6279	0.583
	ENC*PRO	<0.001	<0.001	0.6273	0.6282	0.8568	0.0163	0.0001
	NITR*PRO	0.507	0.531	0.8628	0.0985	0.2957	0.2674	0.6888
	ENC*TR*PRO	0.890	0.931	0.8708	0.9768	0.7481	0.6228	0.866
	Block	0.187	0.220	<0.0001	0.4337	0.3773	0.0033	<0.0001

ENCAP; encapsulation, Non-ENCAP; non-encapsulation, N1; sodium nitrate, N2; nitric acid, PRO; probiotics ENCAP-PRO; encapsulated probiotics, INTR; the interaction among the factors, ENC*NTR; the interaction between encapsulated and the nitrate type, ENCA*PRO; the interaction between encapsulated and the probiotics, NTR*PRO; the interaction between the nitrate type and probiotics, ENC*NITR*PRO; the interaction among the encapsulation, nitrate type, and probiotics,T1; encapsulated sodium nitrate without probiotics, T2; encapsulated sodium nitrate with probiotics, T3; encapsulated sodium nitrate with the encapsulated probiotics; T4; non-encapsulated sodium nitrate without probiotics, T5; non-encapsulated sodium nitrate with probiotics, T6; non-encapsulated sodium with encapsulated probiotics, T7; encapsulated nitric acid without probiotics, T8; encapsulated nitric acid with probiotics, T9; encapsulated nitric acid with encapsulated nitric acid; T10; non-encapsulated nitric acid without probiotics, T11; non-encapsulated nitric acid with probiotics, T12; non-encapsulated nitric acid with encapsulated probiotics, SEM; standard error of means, probability was considered when *p*-value < 0.05. Small letter superscripts a: z are in ascending order.

and TVFAs after adding unencapsulated probiotics to the diet before or after encapsulation. The results were consistent with those of previous studies [19,42]. This indicates the ability of probiotics to improve the fermentation process and maintain improved rumen conditions. Among nitrate forms, nitric acid has been shown to increase the gas production. Nitric acid is an acidic ion. It could reduce the pH values in rumen, resulting negatively in pathogen population and improving the fermentation rate by increasing both the gas production rate and amount of TFVAs.

After encapsulation, we observed a significant decrease in the pH value. Also, there was a significant decrease in the rumen pH value due to the addition of encapsulated probiotics and encapsulated nitrate in the diet. Among treatments, the lowest pH value was recorded due to combining encapsulated probiotics with encapsulated acid. There was a numerical reduction in the pH value due to combining encapsulated probiotics with encapsulated nitric acid as compared with combining encapsulated probiotics with encapsulated sodium nitrate. Among nitrate types, the pH value of nitric acid was numerically lower than sodium nitrate. Gawad and Fellner [35] found a significant decrease in the pH value due to encapsulation. In addition, Jiao et al. [42] observed no significant differences in the pH and TVFAs among encapsulated and non-encapsulated yeasts. Generally, the reduction of rumen pH is attributed to the rapid accumulation of TVFAs in the rumen [9]. The rapid commutation of organic acids is due to the significant increase of TFVAs in rumen as a result of improving the fermentation rate after encapsulation. This could correlate significantly with a significant reduction in the pH value in the rumen.

Also, the acid properties of nitric acid increase the reduction in pH. This indicates that both the encapsulation and acid properties of nitric acid would contribute to significantly reducing the rumen pH. Therefore, the lowest pH value among treatments was due to the addition of encapsulated probiotics and encapsulated nitric acid and can be attributed to the effects of both encapsulation and acid properties of nitric acid. After adding unencapsulated probiotics, the rumen pH value tends to be closer to the pH of treatments before encapsulation. We did not observe a significant difference between the addition of unencapsulated probiotics and combining unencapsulated with nitrate forms before and after encapsulation (T1 and T2, T4 and T5, T7 and T8, and T10 and T11). Thus, this indicates the ability of probiotics to sustain rumen conditions in the normal range. It is suggested that probiotics have the ability to sustain normal pH values [43]. According to Sari et al. [44], the normal pH value in the rumen is between 6.4 and 6.7.

We observed a significant decrease in nutrient digestibility and ammonia concentration after encapsulation. Moreover, the lowest digestion rate and the lowest ammonia concentration were observed due to the addition of combining encapsulated probiotics and encapsulated nitrate forms. Moreover, there was a significant interaction among encapsulation, probiotics, and nitrate forms on the ammonia concentration. On the other hand, by adding unencapsulated probiotics, we observed no significant effects on the digestion rate or ammonia concentration. Previously, Lund et al. [45] have reported a numerous reduction in nutrient digestion after encapsulation. Also, Lee et al. [46] found that ammonia concentration decreased linearly by adding encapsulated nitrate. Similarly, Makled et al. [38] reported lower concentrations of ammonia after encapsulation. The reduction in nutrient digestion follows the drastic reduction of pH value, which is caused by the rapid accumulation of TVFAs in the rumen because of encapsulation [47]. Faniyi et al. [48] reported that ammonia is produced mainly as a result of microbial activity on protein sources [49]. Therefore, ammonia production is usually affected positively or negatively by the digestion rate. The reduction in pH is usually linked to a reduction in nutrient digestibility (IVDMD and IVOMD), followed by a reduction in ammonia concentration. Sari et al. [44] stated that a normal range of pH indicates normal and appropriate conditions in the rumen. In addition, Vet [50] and Sari et al. [44] observed a significant reduction in digestibility and ammonia concentration due to a gastrointestinal shift at low pH. To improve the rate of digestion during encapsulation, a suitable matrix is suggested to be used based on the resistance of rumen microorganisms and degradability during the fermentation process. It is well known that rumen microorganisms are good and sophisticated in degrading a wide range of raw materials during the fermentation process. Therefore, resistant starch or any resistance material is recommended to be used as a suitable matrix for encapsulation in rumens. Probiotics are known to improve the fermentation process by increasing the digestion rates and ammonia concentration. By adding probiotics, we did not observe any significant differences in nutrient digestibility and ammonia concentration between the treatments before encapsulation and the treatment after adding unencapsulated probiotics to the diet. Similar results were obtained by Sheikh et al. [51]. This indicates the ability of probiotics to improve digestion and improve ammonia concentration in ruminants.

Despite the significant reduction in the molar portion of acetic acid and the significant decrease in the ratio of acetic to propionic acid, there was a significant increase in propionic acid after encapsulation. Also, we observed that by combining encapsulated probiotics with encapsulated nitrate, there was a significant decrease in enteric methane emission, thereby decreasing the concentration of acetic acid, decreasing the ratio of acetic to propionic acid, and

increasing propionic acid. Similar results were observed in previous studies [9,35]. It is known that both nitrate and probiotics reduce enteric methane emissions [20,52,53]. Therefore, the reduction in methane concentration is attributed to the shallow release of probiotics and nitrate after encapsulation. This could positively affect the availability of both probiotics and nitrate to scavenge hydrogen, thereby reducing enteric methane concentration. Methane production is associated with acetic acid production due to the high production of hydrogen ion, which is used by methanogenic for the formation of methane during the process of methanogenesis [54]. The reduction in the concentration of the molar portion of acetic acid and an increase in the concentration of propionic inhibit methane formation due to a decrease of hydrogen molecules. The reduction in enteric methane emissions could also occur due to the reduction of the ratio of acetic to propionic acid. We observed in this study that there was a significant reduction in acetic acid and the ratio of acetic to propionic acid, and there was a significant increase in propionic acid after encapsulation. This indicates the effects of the encapsulation process on reducing enteric methane emissions. In addition, a significant decrease in methane concentration was observed by encapsulating nitrate in the long term [55].

5 Conclusion

Despite the limitation on digestibility, encapsulation is an effective method for enhancing the rumen fermentation process by increasing the total gas production and TVFAs. Nevertheless, encapsulation indicates effectiveness on enteric methane emission, thereby reducing the molar portion of acetic acid and the ratio of acetic to propionic acid and increasing the molar portion of propionic acid. Moreover, combining encapsulated probiotics with encapsulated nitrate forms is an effective method for improving the fermentation process in the rumen and reducing enteric methane emission by reducing the molar portion of acetic acid and the ratio of acetic to propionic acid and increasing the molar portion of propionic acid. Probiotics are effective in improving the fermentation process, thereby stabilizing and maintaining normal conditions in the rumen. Therefore, *in vivo*, long-term practices of combining probiotics with nitrate are recommended to improve the effectiveness of encapsulation on enteric methane emission.

Acknowledgment: The authors are grateful to Prof. Dr. Yantyati Widyastuti for providing the probiotic isolates used in this study.

Funding information: This research was supported by RIIM-LPDP-BRIN (Nos B-803/II.7.5/FR/6/2022 and B-1373/III.5/PR.03.08/6/2022) of the Research Center for Applied Zoology-BRIN. The authors are also grateful to the Directorate General of Higher Education, Research and Technology, Ministry of Education, Culture, Research and Technology, Republic of Indonesia, for the financial support through the "Hibah Penelitian Fundamental" scheme, the year 2024 grant number 102/E5/PG.02.00.PL/2024.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and consented to its submission to the journal, reviewed all the results and approved the final version of the manuscript. RR, NN, and AJ designed the experiments, and MA and RF carried them out. MA and SN prepared the manuscript with contributions from all co-authors.

Conflict of interest: Authors state is no conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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