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Research article

Effects of slow-release ammonium sulfate as non-protein nitrogen sources on rumen fermentation characteristics: An *in vitro* study

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Abstract

This experiment aimed to evaluate the effects hydrated lime ($Ca(OH)_2$) as slow-release agent for ammonium sulfate in non-protein nitrogen supplementation on ruminal fermentation characteristics under *in vitro* method. A completely randomized design (CRD) was employed with four treatments and six replications: ZA (100% ammonium sulfate); ZA-Corn (29.26% ammonium sulfate and 70.74% corn meal); ZA+Lime-Corn (a mixture of 60% ammonium sulfate and calcium hydroxide, and 40% corn meal); and Pangola grass as standard. The variables observed included total gas production, methane (CH_4) production, ammonia (NH_3) concentration, protozoa population, microbial protein synthesis (MPS), and *in vitro* dry matter (IVDMD) and organic matter (IVOMD) degradability. Results presented that the inclusion of $Ca(OH)_2$ as a binding agent for slow-release ammonium sulfate significantly reduced ($P<0.05$) total gas production compared to ammonium sulfate and corn meal combination, which showed the highest gas production. There were no differences ($P>0.05$) in fermenter pH across treatments. However, the inclusion of $Ca(OH)_2$ decreased cumulative gas production, NH_3 concentration, MPS, IVDMD, IVOMD and protozoa populations ($P < 0.05$). Conversely, the ZA-Corn treatment significantly increased ($P<0.05$) cumulative gas production, MPS, CH_4 production, IVDMD, and IVOMD. The ZA treatment increased ($P < 0.05$) NH_3, IVDMD, and IVOMD. These findings suggest that the inclusion of $Ca(OH)_2$ slows the ruminal nitrogen release and prevents excessive NH_3 accumulation, though it can slightly suppress microbial synthesis and digestibility.

Keywords: Ammonium sulfate, Calcium hydroxide, Fermentation, Rumen, Slow-release.

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INTRODUCTION

Protein is one of the most expensive components in the ruminant diets, playing a crucial role as both a nitrogen source for the rumen microbes and a provider of amino acids for the host animal (Rashmi et al., 2024). Non-protein nitrogen (NPN) can partially replace true protein in diets, serving as an alternative source of rumen-degradable protein to enhance microbial protein synthesis (MPS) (Norrapoke et al., 2018; Rashmi et al., 2024; Vargas., 2024). Within the rumen, NPN sources such urea and other nitrogenous compounds released ammonia (NH_3) through enzymatic degradation. This reaction, largely driven by rumen bacteria enzymes, convert NPN into NH_3. The released ammonia is subsequently used by rumen microorganisms to synthesize microbial protein (Stefański et al., 2020). Beside urea, NPN sources such as ammonium sulfate are frequently used as cost-effective substitutes for feed protein in ruminant diets (Crookshank et al., 1973; da Silva et al., 2014; de Mendonça Lopes et al., 2023). Ammonium sulfate (ZA) is a potential NPN source due to its high nitrogen content, which supports microbial activity and fermentation in the rumen. However, its rapid hydrolisis into ammonia can lead to inefficient nitrogen utilization, resulting in an imbalance between nitrogen availability and microbial energy requirements. This imbalance may cause excessive ammonia accumulation, reducing nitrogen retention efficiency and posing a risk of ammonia toxicity (Pacheco et al., 2021; Zheng et al., 2024).

Microbial protein synhesis in the rumen is a highly coordinated process that depends on the simultaneous availability of energy and nitrogen. This concept refers to the temporal alignment between the supply of fermentable energy and nitrogen sources within the rumen environment (Fitriyah et al., 2024). To enhance the efficiency of MPS, an adequate energy source must be available to synchronize the rate of ammonia release (Cherdthong and Wanapat, 2010; Harahap et al., 2019). Corn, as a readily fermentable carbohydrate source, provides the necessary energy for rumen microbial proliferation. The combination of nitrogen from ammonium sulfate and energy from corn can enhance rumen fermentation efficiency.

Several slow-release nitrogen products have been developed and evaluated in previous studies including biuret, starea, urea-formaldehyde, uromol, urea coated with flaxseed oil and talc, lactosylurea, polyvinyl alcohol urea, urea-lignocellulosic complexes, uromalt, isobutyraldehyde monourea, epogen, microcapsules, and calcium chloride-bonded urea (Niazifar et al., 2024). Additionally, Harahap et al. (2018) reported that the inclusion of limestone ($CaCO_3$) as a binding agent can effectively reduce ammonia concentrations in the rumen. Calcium forms chelates with urea through hydrogen bonding, thereby slowing ammonia release and improving nitrogen utilization efficiency.

Despite the growing interest in slow-release nitrogen studies, most existing approaches have focused exclusively on urea-based compound, with limited exploration of ammonium sulfate in similar formulations. Moreover, while calcium-based binders like limestone have shown potential in reducing ammonia release, the use of hydrated lime ($Ca(OH)_2$) as a binding agent to control ammonia release from ammonium sulfate remains largely unexplored. This study addresses this gap by investigating the potential of hydrated lime to function as slow release binder for ammonium sulfate. We hypothesize that the inclusion of $Ca(OH)_2$ will enhance nitrogen stability and modulate ammonia release in a way that aligns more closely with microbial energy requirements. Such synchronization could reduce nitrogen losses, mitigate ammonia toxicity risks, and promote microbial protein synthesis. Therefore, the objective of this study is to evaluate the effectiveness of $Ca(OH)_2$ as slow-release binding agent for ammonium sulfate in optimizing rumen fermentation and nitrogen utilization efficiency in ruminants.

MATERIALS AND METHODS

Ethical clearance approval

All experimental procedures followed the ethical guidelines established by the Animal Care and Use Ethics Committee of the National Research and Innovation Agency. Ethical clearance was obtained under approval reference number: 112/KE.02/SK/05/2024.

Non-Protein Nitrogen Supplement Preparation

The nutrient composition (%) and formulation proportions (%) of the NPN supplements are presented in Table 1. The experimental groups included: (1) ZA, consisting of 100% $(NH_4)_2SO_4$ (ammonium sulfate); (2) ZA-Corn, a mixture of 29.26% ammonium sulfate and 70.74% corn meal; (3) ZA+Lime-Corn, composed of a mixture containing ammonium sulfate and $Ca(OH)_2$ combined with 40% corn and (4) PNG, pangola grass. The preparation of the ZA+Lime was arranged according to Harahap et al. (2018) method with some modifications. The ZA+Lime was prepared by mixing ZA and $Ca(OH)_2$ at 1.5:1 (w/w) ratio in water, with 10% vanilla powder added as a flavoring agent.

All experimental groups received ammonium sulfate (ZA) as the primary NPN source, except the PNG group, which served as the standard control and did not received any NPN supplementation. The amounts and specific proportions of each component in the dietary treatments are detailed in Tables 1 and 2.

Table 1 Formula of slow-release non-protein nitrogen

Item	Ingredients		
	ZA	$Ca(OH)_2$	Total
Proportion, %	45.5	54.5	100
Nutrient content, %			
N	9.30	-	9.30
Ca	-	53.85	53.85

N = nitrogen; Ca = Calcium; ZA = ammonium sulfate

Table 2 Formula of supplement non-protein nitrogen

Item	Ingredients			
	ZA	ZA-$Ca(OH)_2$	Corn	Total
Proportion, %				
ZA	100	-	-	100
ZA-Corn	29.26	-	70.74	100
ZA+Lime-Corn	-	60	40	100

ZA = ammonium sulfate; ZA-Corn = ammonium sulfate with corn; ZA+Lime-Corn = slow-release ammonium sulfate with corn

Fourier Transform Infrared Spectroscopy (FTIR)

The constituent materials of each treatment were analyzed in 60 s using an Alpha II (Bruker) FTIR spectrometer. Spectra were recorded over a wavelenght range of 4000 – 600 cm^{-1}, transmittance positions were identified accordingly.

In Vitro Assay

Rumen liquor was collected from a fistulated cow maintained at the Animal Husbandry Farm, Faculty of Animal Science, Universitas Gadjah Mada. The rumen fluid was immediately transferred into a prewarmed thermos to maintain temperature and filtered through a fabric until fully refined under anaerobic conditions.

The *in vitro* gas production technique was performed following the method described by Theodorou et al. (1994). Half a gram of each sample was placed into labeled serum bottles, to which 50 mL of a rumen fluid and buffer mixture (1:2 ratio) was added. Carbon dioxide (CO_2) was flushed through the bottles to maintain anaerobic conditions before sealing. The bottles were then incubated at 39°C for 72 hours in water bath (Memmert WNB 45, Germany). After 72 h of incubation, the pH of the rumen fluid was immediately measured using a calibrated pH meter (Eutech PC 700 Thermo Scientific) standardized with pH 4 and pH 7 buffer solutions. Subsequently, rumen fluid and residue samples were collected for the analysis rumen fermentation profiles.

Dry matter degradabilty (IVDMD) was determined by filtering the incubation residue through a Gooch crucible, followed by drying at 105°C. Organic matter degradability (IVOMD) was determined by ashing the dried residue at 600°C. Dry matter and organic matter analyses were conducted according to AOAC (2005).

Ammonia (NH_3) concentration was analyzed using the Conway microdiffusion method (Conway and Byrne, 1933). The sample supernatant was reacted with sodium carbonate (Na_2CO_3) and boric acid with a phenolphthalein indicator, incubated for 24 hours and titrated with 0.005 N sufuric acid (H_2SO_4). Ammonia concentration (mM/L) was calculated using the following formula: NH_3 concentration (mM/L) = ml H_2SO_4 (titration result) × N H_2SO_4 × 1000.

Protozoa populations were counted using the Ogimoto and Imai (1981) method. A methyl green formalin solution (MFS) was used as a stain, and protozoa were observed under a microscope (Olympus CKX53, Olympus, China) at 40× magnification using a Fuchs-Rosenthal counting chamber. The protozoa population was calculated using the formula: N × 104 × dilution factor.

Microbial protein synthesis was measured by centrifuging the supernatant and precipitation with trichloroacetic acid (TCA) (Makkar et al., 1982). The precipitated protein was then hydrolyzed with 0.25 N sodium hydroxide (NaOH) (Lowry et al., 1951). Absorbance was measured at a wavelength of 650 nm using a spectrophotometer (Thermo Scientific Multiskan Go, ThermoFisher Scientific, USA).

Gas production kinetics were recorded at 2, 4, 8, 16, 24, 48, and 72 hours using a gas syringe. Methane gas (CH_4) measurement was carried out at 48 hours. The collected gas was transferred to vacutainer tubes using gas syringe. Methane (CH_4) content was analyzed using gas chromatography (GC 2014, Shimadzu, Kyoto, Japan), equippet with a Porapak N column and operated with nitrogen and hydrogen as the carrier gas, along with a flame ionization detector and electron capture detector at the laboratory of Indonesian Agricultural Environment Research Institute, Pati, Indonesia. Cumulative total gas production was fitted according to the Gompertz model (Machado et al., 2014; Anggraeni et al., 2024):

$$y = ae^{-be\ -ct}$$

Where :

y = cumulative total gas production (mL);
a = maximum gas production (mL);
b = lag period before exponential gas production (h);
c = gas production rate (mL/h) at time (h);
e = Euler's constant (2.7183).

Experimental Design and Data Analysis

This study used a completely randomized design (CRD) with four treatments and six replications: ZA (ammonium sulfate), ZA-Corn (supplement nitrogen from ZA with corn), ZA+Lime-Corn (supplement nitrogen slow-release ZA-$Ca(OH)_2$ with corn), PNG (pangola grass / standard control). The linear model used was:

$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$

Where :

Y_{ij} = observation for the i^{th} treatment in the j^{th} replication
μ = overall mean value
τ_i = effect of treatment i
ε_{ij} = experimental error for the i^{th} treatment in the j^{th} replication
i = treatment
j = replication

Data were analyzed using Analysis of Variance (ANOVA), and significant differences ($p<0.05$) were subsequently analyzed using Duncan's Multiple Range Test in R-Statistical software R 4.4.2 (R Core Team, 2024). Hierarchical cluster analysis (HCA) and heatmap visualization were performed to examine relationships among various parameters in this study. The HCA and heatmap analysis were conducted using the free accessible program MetaboAnalyst 6.0 at https://www.metaboanalyst.ca/.

RESULTS

The chemical composition of the three NPN supplement treatments is presented in Table 3. The crude protein content of ZA-Corn and ZA+Lime-Corn was formulated to be isoproteic, while the crude protein in ZA remained in its original form, which was higher than that of the other NPN supplements. Mineral analysis showed that Ca and S levels in ZA+Lime-Corn were higher than those in ZA-Corn. However, ZA contained the highest S content, exceeding 24%, and had lower micro-mineral concentrations compared to the other NPN supplement treatments.

The Fourier transform infrared (FTIR) spectra of ZA, ZA-Corn, and ZA+Lime-Corn are shown in Figure 1. A new O-H stretching peaks appeared at 3849.06 cm^{-1} in ZA-Corn, while peaks at 2053.60 cm^{-1} and 1655.93 cm^{-1} indicated amine-related changes. The ZA+Lime-Corn exhibited peaks at 3887.50 cm^{-1}, 3643.19 cm^{-1}, 2924.99 cm^{-1} and 2516.76 cm^{-1} along with shift at 1784.38 and 1685.05 suggesting futher structural modifications due to $Ca(OH)_2$ addition. The effect of $Ca(OH)_2$ inclusion on gas production kinetics is presented in Figure 2. Meanwhile, Table 4 summarizes the ruminal fermentation characteristics of the three NPN supplement treatments, with pangola grass serving as the standard control.

The inclusion of $Ca(OH)_2$ as a binding agent for slow nitrogen release reduced ($P<0.05$) total gas production compared to ZA-Corn, which resulted in the highest total gas production ($P<0.05$). However, there were no differences in fermenter pH among treatments. The inclusion of $Ca(OH)_2$ decreased cumulative gas production, NH_3, microbial protein synthesis (MPS), CH_4 production, *in vitro* dry matter degradability (IVDMD), and *in vitro* organic matter degradability (IVOMD) ($P<0.05$), yet lowered protozoa populations ($P<0.05$).

Conversely, the ZA-Corn treatment significantly increased ($P<0.05$) cumulative gas production, microbial protein synthesis (MPS), CH_4 production, *in vitro* dry matter degradability (IVDMD), and *in vitro* organic matter degradability (IVOMD) ($P<0.05$). The ZA treatment increased ($P<0.05$) NH_3, IVDMD, and IVOMD compared to the other treatments. However, the addition of $Ca(OH)_2$ reduced NH_3 concentrations, IVDMD, and IVOMD.

Additionally, a heatmap illustrating the relationship between treatments and *in vitro* rumen fermentation characteristics of the NPN supplements is provided in

Figure 3. The ZA+Lime-Corn treatment exhibits a close association with Pangola, indicating similarities in their effects, while both treatments also share a connection with ZA-Corn. However, ZA forms a distinct cluster, reflecting a fermentation profile that differs from the other treatments.

Table 3 Chemical content of supplement non-protein nitrogen (100%DM)

Item	Treatments			
	ZA	ZA-Corn	ZA+Lime-Corn	PNG
Nutrient content, %				
N	22.03	7.04	6.18	1.11
CP	137.50	41.35	41.33	6.95
Carbohydrate	-	62.33	35.24	54.73
Mineral content, %				
Ca	0.29	8.06	37.69	0.78
P	0.26	7.69	5.14	0.05
K	0.04	38.46	25.71	1.94
Mg	-	8.98	6.25	-
S	24.41	9.15	16.65	-
Zn	-	0.50	0.33	-
Fe	0.03	1.88	1.28	-
Mn	-	0.15	0.10	-
Cu	-	0.11	0.07	-

N = nitrogen; CP = crude protein; ZA = ammonium sulfate; ZA-Corn = ammonium sulfate with corn; ZA+Lime-Corn = slow-release ammonium sulfate with corn; PNG = Pangola

Table 4 *In vitro* ruminal fermentation characteristics of supplement non-protein nitrogen

Variable	Treatment				SEM	*P* value
	ZA	ZA-Corn	ZA+Lime-Corn	PNG		
Gas production kinetic parameters, 500 mg DM						
a (mL/g)	5.09^{d}	112.72^{a}	62.22^{c}	99.66^{b}	2.65	<0.001
b (h)	1.26^{c}	3.32^{a}	3.50^{a}	2.30^{b}	0.12	<0.001
c (mL/h)	1.52^{a}	0.12^{b}	0.09^{b}	0.07^{b}	0.24	0.002
Cumulative gas 72 h (mL)	4.40^{d}	116.42^{a}	64.01^{c}	100.82^{b}	1.05	<0.001
NH_3 (mM)	117.48^{a}	47.40^{b}	16.42^{c}	20.02^{c}	1.30	<0.001
MPS (mg/dL)	11.37^{b}	15.76^{a}	11.04^{b}	15.28^{a}	0.33	<0.001
Protozoa (log/mL)	3.00^{b}	3.30^{ab}	2.81^{b}	6.10^{a}	3.66	0.050
CH_4 (%)	0.14^{d}	6.75^{a}	3.51^{c}	4.98^{b}	0.10	<0.001
pH	6.86	6.80	6.84	6.82	0.02	0.745
IVDMD (%)	80.49^{a}	88.99^{a}	45.97^{b}	52.40^{b}	1.37	<0.001
IVOMD (%)	87.94^{a}	89.68^{a}	54.77^{b}	50.24^{b}	1.44	<0.001

Different superscripts within the same row in the treatment average indicate significant differences ($P < 0.05$)
a = the maximum gas production (mL/g); b = the lag phase before exponential gas production (h); c = gas production rate (mL/h) at time t (h); MPS = Microbial protein synthesis; IVDMD = *in vitro* dry matter digestibility; IVOMD = *in vitro* organic matter digestibility; ZA = ammonium sulfate; ZA-Corn = ammonium sulfate with corn; ZA+Lime-Corn = slow-release ammonium sulfate with corn; PNG = Pangola; SEM = standard error of the mean

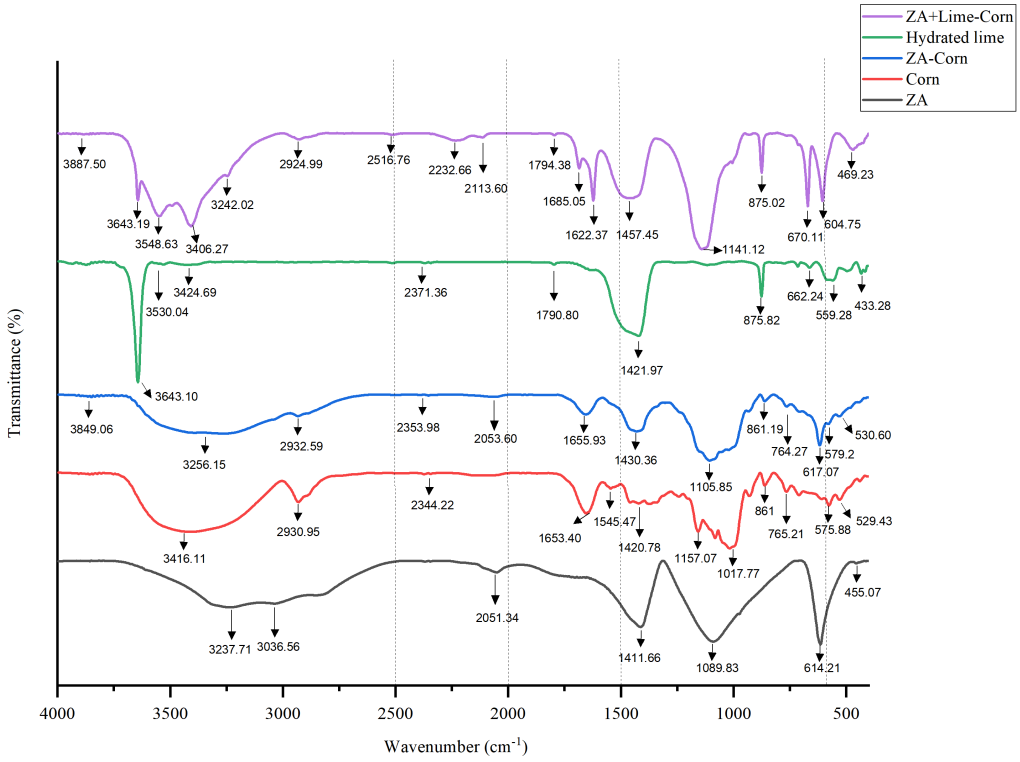

Figure 1 Fourier transform infrared spectra of ZA, ZA-Corn, and ZA+Lime-Corn

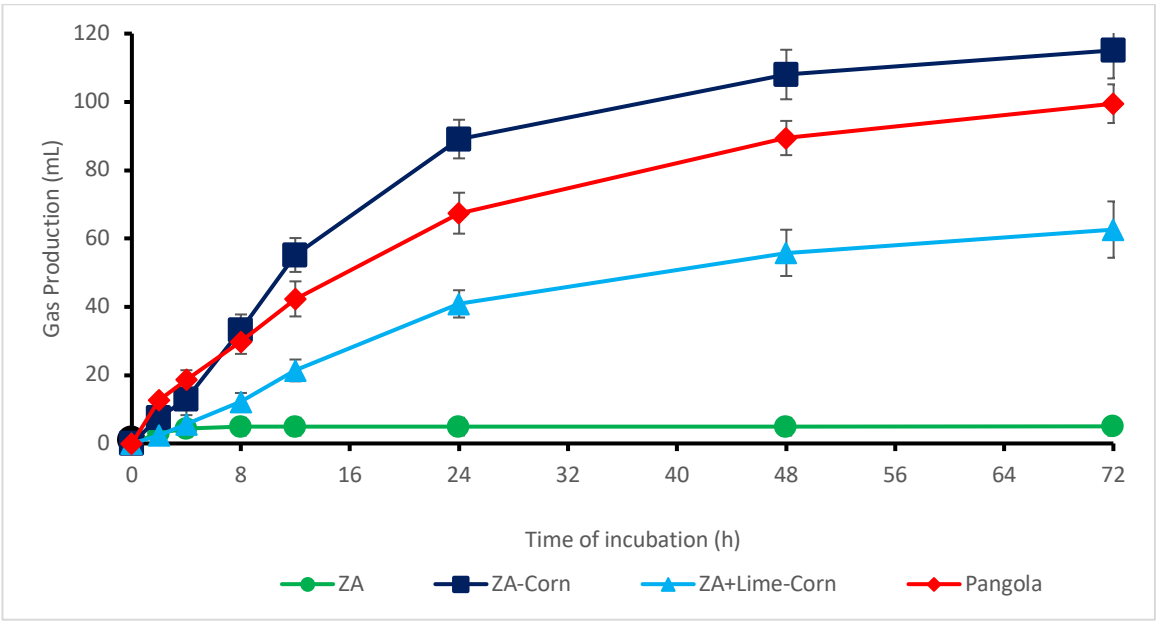

Figure 2 *In vitro* gas production kinetics of non-protein nitrogen supplement. ZA = ammonium sulfate; ZA-Corn = ammonium sulfate with corn; ZA+Lime-Corn = slow-release ammonium sulfate with corn

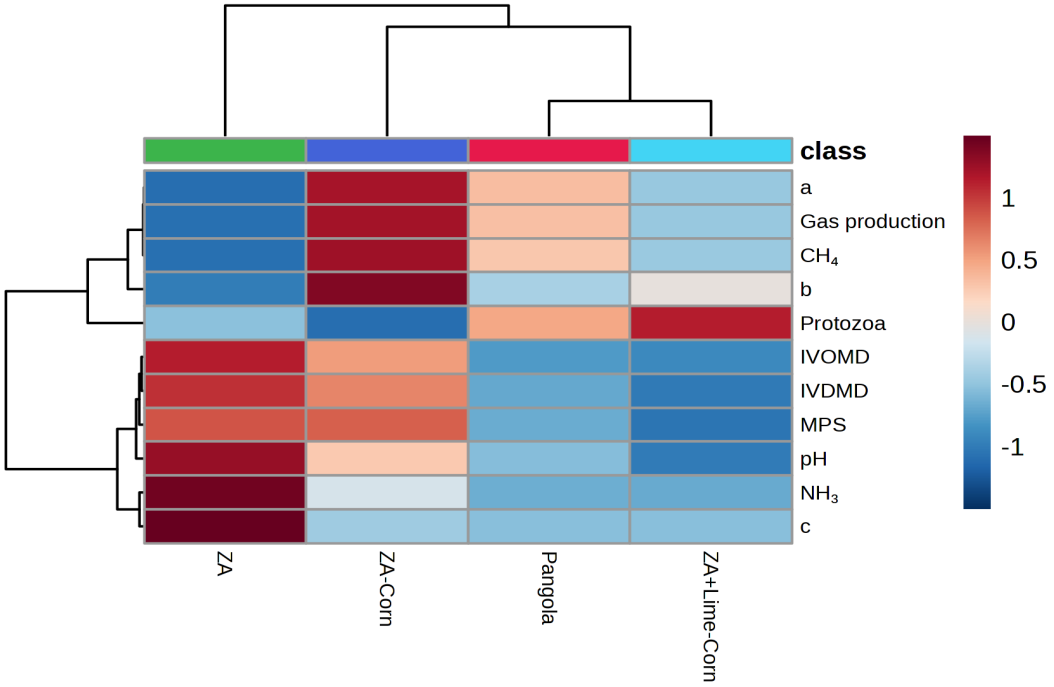

Figure 3 The heatmap of the relationship between treatments and in vitro rumen fermentation characteristics of non-protein nitrogen supplement Colours reflect a trend of treatment effects from a decreasing trend (blue) to an increasing trend (red) a = the maximum gas production (mL/g); b = the lag phase before exponential gas production (h); c = gas production rate (mL/h) at time t (h); IVDMD = *in vitro* dry matter digestibility; IVOMD = *in vitro* organic matter digestibility; ZA = ammonium sulfate; ZA-Corn = ammonium sulfate with corn; ZA+Lime-Corn = slow-release ammonium sulfate with corn

DISCUSSION

Characteristics of Non-Protein Nitrogen Supplement

Fourier transform infrared (FTIR) spectroscopy is a valuable technique for observing changes in functional groups and reflecting the transformation of organic ligands following the chelation of calcium ions with nitrogen. Figure 1 ilustrated clear differences in functional group compositions among ZA, corn, ZA-Corn, hydrated lime, and ZA+Lime-Corn. In the ZA-Corn, the appearance of new peak at 3849.06 cm^{-1} associated with O–H stretching vibrations, indicating the presence of hydroxyl groups derived from corn biomass. This supported by a absorption in corn alone at 3416.11 cm^{-1}. A similar absorption band was reported at around 3340 cm^{-1} in the FTIR analysis of sweet corn cob polysaccharide – Fe(III) complexes, which was attributed to O–H stretching vibrations (Xiu et al., 2023). Additionally, the peaks at 2053.60 cm^{-1} and 1655.93 cm^{-1} in ZA-Corn correspond to N–H bending and amide-related vibrations, suggesting possible interactions between ammonium or amine groups in ZA and organic component in corn.

In the ZA+Lime-Corn treatment, the spectrum showed futher complexity. A strong O–H stretching region appears at 3887.50 cm^{-1} and 3643.19 cm^{-1}, consistent with $Ca(OH)_2$ indicating strong hydrogen bonding. Peaks at 2924.99 cm^{-1} and 2516.76 cm^{-1} may reflect new C–H and C–O bond formations, potentially arising from interactions between Ca^{2+} ions and hydroxyl or carboxyl groups of corn components. Moreover, the appearance of absorption bands at 1794.38, 1685.05, and 1622.37 cm^{-1} suggests changes in C=O and N–H vibrations, likely due to calcium-organic complexation or chelation structures. The hydrated lime spectrum supports this interpretation, as similar peaks appear in ZA+Lime-Corn treatment

but are shifted in wavenumber and reduced intensity, suggesting the formation of new chemical bonds rather than simple physical mixtures.

These observations were consistent with the previous studies, which report that shifts in the 1600 – 3400 cm^{-1} range typically reflect the formation of hydrogen bonds, peptide-calcium coordination, or metal-ligand interactions (Chen et al., 2013; Zhao et al., 2014; Nandiyanto and Ragadhita., 2019). Futhermore, additional bands observed in the fingerpront region of ZA+Lime-Corn, such as 875.02, 670.11, and 604.75 cm^{-1}, may be attributed to sulfate and phosphate group vibrations, whose positions are sensitive to interactions with metal ions. The spectral shifts and emergence of new peaks in ZA-Corn and ZA+Lime-Corn indicate chemical interactions beyond simple mixing, particularly involving hydrogen bonding, amine modifications, and calcium-mediated chelation with organic ligands.

In Vitro Ruminal Characteristics

The relationship between all treatments and *in vitro* rumen fermentation characteristics is illustrated in Figure 3**.** The ZA+Lime-Corn treatment reduces NH_3 concentration, IVDMD, IVODM, microbial protein synthesis, and CH_4 production while increasing protozoa populations. On the other hand, ZA-Corn reduces protozoa populations while enhancing microbial protein synthesis, IVDMD, and IVOMD. Meanwhile, ZA treatment significantly increased NH_3 concentrations due to its rapid nitrogen release, leading to higher digestibility but also increase the risk of nitrogen losses and disrupt microbial fermentation, as indicated by the lowest total gas production. The reduction in NH_3 and CH_4 production observed in ZA+Lime-Corn indicated a positive shift toward environmentally sustainable ruminal fermentation. However, this benefit must be carefully evaluated alongside the concurrent decline in MPS and digestibility parameters. Therefore, achieving an optimal dietary balance requires minimizing environmental emissions while improving nutrient utilization efficiency. The potential to reduce the use of $Ca(OH)_2$ may be explored through by formulating a mixture $Ca(OH)_2$ with ammonium sulfate. This approach may allow to determine the precise ratio that balances emission mitigation while simultaneously supporting MPS and nutrient digestibility.

pH serves a key indicator of rumen fermentation, with optimal pH conditions promoting microbial growth. In this study, the fermenter pH ranged between 6.80 and 6.86, remaining within the normal range (Table 4). Previous research on polymer-coated urea reported no significant differences in pH compared to the control group (Fan et al., 2024). However, studies examining diets in which soybean protein was partially replaced with 0.35% slow-release urea (SRU) found a slightly lower fermenter pH than the control (Guo et al., 2022). These findings suggest that the inclusion of slow-release nitrogen supplements containing 54.5% $Ca(OH)_2$ as a binding agent does not affect ruminal fermentation stability.

Gas production serves as an indicator of substrate fermentation and microbial activity in the rumen. The effects of NPN supplementation on *in vitro* gas production kinetics are presented in Figure 2. The ZA-Corn treatment demonstrated a high kinetic parameter a (mL/g), indicating the presence of readily degradable substrates in this NPN supplement. The energy and N-NH_3 derived from ZA-Corn are rapidly absorbed by microbes, thereby promoting optimal fermentation. A previous study by Sanchez-Meraz et al. (2014) presented that the inclusion of urea or a combination of urea with SRU significantly enhances gas production and forage substrate degradation. Conversely, the cumulative 72-hour gas production for the ZA+Lime-Corn treatment (64.01 mL) was lower than that of ZA-Corn (116.42 mL), indicating a reduction in fermentation activity. Futhermore, as shown in Figure 2, the ZA treatment did not show any increase in gas production throughout the observation period. This may indicate the absense of fermentation activity by rumen microbes and suggest a potential toxic effect of the ZA treatment on microbial activity. This interpretation is supported by the high ammonia concentration observed in the ZA treatment (Table 4.), which may have exceeded

the tolerance threshold for microbial fermentation. In addition, the absence of carbohydrate source in the ZA treatment likely contributed to the lack of gas production during the fermentation process.

The inclusion of $Ca(OH)_2$ reduces fermentation activity, as indicated by decreased gas production and digestibility, due to the slower availability of NH_3 compared to the ZA-Corn treatment. This is reflected in the lower kinetic a fraction from ZA+Lime-Corn, indicating a slower early fermentation process due to the delayed nitrogen release despite the provision of readily available energy. These results contrast with those reported by Cherdthong and Wanapat (2011), who found that slow-release urea products combined with cassava chips both enhanced the rate and extent of fermentation by supplying readily available energy source. The reduction in gas production in the ZA+Lime-Corn treatment is probably due to most of fermented nitrogen compounds being utilized for ammonia production, which neutralizes acids and consequently decreases gas production from the buffer system (Spanghero et al., 2018).

The slow-release nitrogen from ZA+Lime-Corn demonstrated a slower nitrogen release compared to ZA-Corn and ZA, as evidenced by its lower NH_3 concentrations (16.42 mM/L vs. 47.4 mM/L vs. 117.48 mM/L) (Table 4). These results were supported by FTIR analysis of the ZA+Lime-Corn (Figure 1), which revealed the formation of chelate bonds involving interactions between Ca ions and N or O groups with sulfate groups. The formation of theese coordination complexes likely reduce the solubility of ammonium sulfate and delays its release by rumen microbes. This slower release enhances nitrogen utilization efficiency by preventing excessive NH_3 accumulation, which could otherwise be toxic to ruminal microbes. However, excessively low nitrogen availability can limit microbial protein synthesis, as observed in the ZA+Lime-Corn treatment. Microbial protein synthesis was also lower in ZA+Lime-Corn treatment (11.04 mg/dL) compared to ZA-Corn (15.76 mg/dL), indicating that the slow-release nitrogen formulation may not sufficiently support microbial activity. Despite mitigating the risk of ammonia toxicity, the slower nitrogen release restricts microbial growth. Moreover, Fan et al. (2024) reported that the use of on polymer-coated urea and gelatinized starch-urea as slow-release nitrogen sources resulted in reduced N-NH_3 production. This suggests that slow-release urea may inhibit ruminal microbial protein synthesis. Therefore, enhancing the synchronization between NH_3-N release and carbohydrate availability can lead to increase microbial protein synthesis.

The ZA-Corn treatment demonstrated the highest digestibility among all treatments, with IVDMD at 88.99% and IVOMD at 89.68%, indicating optimal fermentation efficiency (Table 4). As stated by Baffa et al. (2023), the inclusion of starch sources in a diet can increase DM digestibility. However, it may also negatively affect ruminal fermentation by increasing N-NH_3 concentrations. The N-NH_3 concentration is inversely related to carbohydrate availability. Nevertheless, fiber carbohydrate fermenting bacteria rely exclusively on ammonia as a nitrogen source. Conversely, ZA+Lime-Corn treatment showed a decrease in IVDMD and IVOMD, likely due to the slower nitrogen release, which may have led to asynchrony between nitrogen availability and the energy provided by corn. This limitation in microbial proliferation subsequently in reduced substrate degradation. Interestingly, although the ZA treatment showed no detectable fermentation activity, as indicated by the absence of gas production in Figure 2, both IVDMD and IVOMD values remained high (Table 4). This may attributed to the hygroscopic nature of ZA particles, which dissolve easily in liquid media and may have led to an overestimation of digestibility.

Alipour et al. (2020) reported that the addition of SRU at varying levels (0%, 0.5%, 1%, and 1.75%) to finishing cattle concentrate did not significantly affect dry matter, organic matter, or crude protein digestibility. However, SRU supplementation influenced neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility, with 1% SRU showing higher results compared to the control. The SRU coated with low-trans vegetable lipids did not significantly impact dry

matter and organic matter digestibility, but improved crude protein digestibility and increased MPS (de Lecuna et al., 2024). Slow-release urea demonstrates greater potential for improving synchronization with dietary carbohydrate availability compared to conventional urea, thus optimizing ruminal fermentation. However, the inclusion of slow-release nitrogen with $Ca(OH)_2$ results in reduced MPS, which consequently affects substrate digestibility. This condition may arise from delayed nitrogen release, leading to suboptimal microbial proliferation despite sufficient fermentable energy. Inadequate nitrogen utilization can decrease carbohydrate digestibility, and in the absence of sufficient carbohydrates, degraded protein may be released as ammonia. Therefore, optimizing the synchronization of nitrogen and energy release is crucial for optimizing rumen fermentation.

Table 4. presents protozoal populations were lower in the ZA+Lime-Corn treatment (2.81 log/mL) compared to ZA-Corn, which correlated with lower methane emissions (3.51%). The reduction in protozoa led to decreased fermentation activity involving protozoa and methanogens, contributing to lower microbial protein synthesis. The simultaneous decrease in protozoa and methane levels may be atrributed to interrelated shifts in rumen fermentation dynamics, particularly involving MPS and NH_3 concentrations. Protozoa are known to harbor methanogens symbiotically. Thus, a reduction in protozoal population likely disrupted this association, leading to lower methane production. The decreased methane production in ZA+Lime-Corn treatment can be linked to the reduced protozoa fermentation activity (Table 4). Ruminal methanogens use dihydrogen and carbon dioxide produced during ruminal fermentation. Therefore, methanogens maintain a symbiotic relationship involving interspecies hydrogen transfer with other ruminal microorganisms, including ruminal protozoa (Balch et al., 1979; Dai et al., 2022). Additionally, decreased NH_3 levels may have limited nitrogen availability for microbial growth, indirectly affecting protozoal proliferation and overall methanogenic activity. Protozoa employ nitrogen from bacteria as a source for growth (Majewska et al., 2020). However, in the ZA-Corn treatment, the higher protozoal population supported fermentation efficiency by facilitating energy availability from starch fermentation, thereby enhancing microbial growth and protein synthesis. Nonetheless, this also leads to a slight increase in methane production due to the symbiotic relationship between protozoa and methanogens (Morgavi et al., 2012).

CONCLUSION

The inclusion of hydrated lime ($Ca(OH)_2$) shows potential in reducing nitrogen losses by decreasing nitrogen release and limiting excessive NH_3 accumulation. Although some reductions in microbial protein synthesis and digestibility were observed, these findings highlight the importance of synchronizing slow-release nitrogen source with microbial energy and nitrogen requirements to optimize fermentation efficiency.

AUTHOR CONTRIBUTIONS

All authors were involved in all research and in writing this scientific article. Mira Ndaru Pertiwi: designed the study, collected data, analyzed data, drafted the original manuscript. Muhammad Ainsyar Harahap: conceptualized of the study, designed the study, supervised, collected data, analyzed data, drafted the original manuscript, and finalized the manuscript. Joelal Achmadi: designed the study, supervised, manuscript drafting and finalized the manuscript. Hendra Herdian: collected data, analyzed data, and manuscript drafting. Awistaros Angger Sakti: collected data, contributed to manuscript drafting and critical review. Ahmad Sofyan: analyzed data and critical review. Gunawan Gunawan: manuscript drafting

and critical review. Wulandari Wulandari: contributed to manuscript drafting. Randi Mulianda: collected data and contributed to manuscript drafting.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this research.

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