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Effects of *Indigofera zollingeriana* and ammonium sulfate-Ca(OH)$_2$ as protein and non-protein nitrogen supplement on *in vitro* ruminal fermentation

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ABSTRACT

This study aimed to evaluate the effects of a combined protein–non-protein nitrogen (NPN) supplement based on *Indigofera zollingeriana* and ammonium sulfate-Ca(OH)$_2$ on *in vitro* rumen fermentation characteristics. A completely randomized design was applied with five treatments, N0 (100% ammonium sulfate), ID0 (100% *Indigofera*), NID19 (NPN: *Indigofera* = 1:9), NID11 (1:1), and NID91 (9:1), with six replications. Parameters measured included ammonia (NH_3) concentration, total volatile fatty acids (TVFA), acetate, propionate, butyrate, microbial protein synthesis (MPS), dry matter degradation, total gas production, and methane production (CH_4). The NID19 treatment associated with significantly higher ($P<0.05$) total VFA (380.99 mM), acetate (350.87 mM), microbial protein (17.79 mg/dL), and gas production (97.67 mL), while resulting in lower NH_3 than N0. The N0 treatment exhibited the highest NH_3 (117.48 mM) but the lowest gas and methane production. The inclusion of Ca(OH)$_2$ in the protein–NPN supplement reduced NH_3 concentration, gas production, gas production rate, and methane output. Meanwhile, *Indigofera* inclusion contributed to higher microbial protein synthesis, VFA concentrations, and dry matter degradation in the rumen.

Introduction

Ruminant diets typically consist of forage, concentrates, or complete feed. When feed fails to fulfill the animals' maintenance and production requirements, productivity declines as nutrients are utilized solely for survival. Protein is a critical component of ruminant nutrition and can be differentiated into rumen-degradable protein (RDP) and rumen-undegradable protein (RUP) (Rashmi *et al*., 2024). Optimal protein utilization occurs when the nutritional requirements of rumen microbes are aligned with the metabolic demands of the host animal. Rumen microbes utilize ammonia nitrogen (NH_3-N) (Ma *et al*., 2025), including that derived from non-protein nitrogen (NPN) sources such as ammonium sulfate (ZA). Although ZA is an effective source of NPN, its rapid hydrolysis may lead to the accumulation of excess NH_3 accumulation and toxicity. To mitigate this, slow-release strategies using binding agents such as limestone have been explored. As reported by Harahap *et al*. (2018), calcium compounds have been shown to lower ammonia release rates and improve nitrogen utilization efficiency.

Indigofera zollingeriana, a protein-rich legume containing secondary metabolites, offers two benefits owing to its high protein content and partial resistance to ruminal degradation (Puastuti *et al*., 2024). When combined with slow-release NPN sources, such as ZA-Ca(OH)$_2$, *Indigofera* may enhance microbial protein synthesis and sustain post-ruminal metabolism. Additionally, an energy source such as corn is essential for synchronizing nitrogen release with microbial energy demand, thus optimizing microbial growth (Hanlon *et al*., 2023). This study aimed to evaluate the effects of combining *Indigofera* and ZA-Ca(OH)$_2$ as a protein-NPN supplement on ruminal hydrolysis products in ruminants. It is hypothesized that increasing the proportion of nitrogen derived from *Indigofera* while reducing the contribution of ZA-Ca(OH)$_2$ to the supplement improves the efficiency of ruminal hydrolysis, thereby supporting microbial activity and enhancing nutrient availability for ruminant productivity.

Materials and methods

Ethical clearance approval

All procedures complied with the ethical standards established by the Commission of Ethical Clearance for Animal Husbandry and Application Research BRIN, No 112/KE.02/SK/05/2024.

Protein-NPN supplement preparation

The slow-release nitrogen supplement was prepared by combining ZA and Ca(OH)$_2$ using a 1.5:1 (b/v) mixing rasio in water, with vanilla powder (10% of the ZA–Ca(OH)$_2$ mixture) added as a flavoring agent. The mixture was then added to corn (Table 1). The protein-NPN supplement was prepared based on the ratios of N-NPN to N from *Indigofera*, which were 1:9, 1:1, and 9:1, with crude protein content of 30, 35, and 40%, respectively. The formulations of the materials and nutrient content used in this study are presented in Table 2.

In vitro fermentation

Rumen liquor was obtained from a fistulated cow maintained by the Department of the Faculty of Animal Husbandry at Gadjah Mada University. The liquor was transferred into thermos pre-conditioned with hot water to maintain a temperature of 39°C and filtered through the fabric until it was fully refined under anaerobic conditions. The *in vitro* gas production technique described by Theodorou *et al*. (1994) was used to assess the rumen fermentation. A sample (0.5 g) was added to 100 mL serum bottles, which were labeled according to the treatment. Fifty milliliters of rumen liquor and buffer solution (in a 1:2 ratio) were added, and CO_2 was used to sustain anaerobic conditions. The bottles were sealed and incubated at 39°C for 72 h in a water bath (Memmert WNB 45, Germany). The pH of the rumen fluid was determined using a calibrated pH meter (Eutech PC 700, Thermo Scientific) with standard buffer solutions

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at pH 4 and pH 7. Gas production was measured and collected *in vitro* at 2, 4, 8, 16, 24, and 48 h, using a gas syringe. Methane (CH_4) was analyzed in gas samples collected after 48 h of incubation by gas chromatography (Shimadzu, Japan). Ammonia (NH_3) concentrations were determined using the Conway microdiffusion method. The supernatant of the sample was reacted with Na_2CO_3 and boric acid with a phenolphthalein indicator, incubated for 24 h, and titrated with 0.005 N H_2SO_4 to ascertain the NH_4 concentration. The ammonia concentration was calculated using the formula described by Nocek *et al.* (1987): ammonia concentration (mM/l) = ml H_2SO_4 (result of titration) × N H_2SO_4 × 1000. Volatile fatty acid measurements were conducted using a GC-FID (Thermo Scientific, Trace1310 GC, USA) following the method described by Luo *et al.* (2015). Standard solutions were prepared by dissolving crotonic acid and metaphosphoric acid in ultrapure water, and acetate, propionate, and butyrate standards were diluted. The standard solution was added to metaphosphoric acid and crotonic acid, incubated at 4°C for 30 min, centrifuged, mixed with methanol (1:9), filtered through a 0.22 µm membrane, and analyzed. Microbial protein synthesis was assessed by centrifuging the supernatant and precipitating it with trichloroacetic acid (TCA) (Makkar *et al.*, 1982) and protein destruction with 0.25 N NaOH (Lowry *et al.*, 1951). Absorbance at 650 nm was determined using a spectrophotometer (Thermo Scientific Multiskan Go, Thermo Fisher Scientific, USA). Dry matter degradation was determined by filtering the incubation residue through a gooch crucible and drying at 105°C (Memmert, Germany) to measure the dry matter content.

Table 1. Chemical Composition of NPN Supplements.

Ingredients	NPN Supplement
Proportion, %	
ZA	27.3
$Ca(OH)_2$	32.7
Corn	40
Nutrient content, %	
N	6.61
CP	41.33
Carbohydrate	35.24
Mineral content, %	
Macro	
Ca	37.69
P	5.14
K	25.71
Mg	6.25
S	2.37
Micro	
Zn	0.33
Fe	1.28
Mn	0.1
Cu	0.07

N = nitrogen; ZA = ammonium sulfate; CP = crude protein; ZA-$Ca(OH)_2$ = nitrogen supplement

Table 2. Chemical Composition of Protein-NPN supplements.

Ingredients	Treatments				
	N0	ID0	NID19	NID11	NID91
Proportion,%					
Nitrogen Supplement	-	-	7.5	42.4	86.9
Indigofera	-	100	92.5	57.6	13.1
ZA	100	-	-	-	-
Nutrient, %					
N	22	4.85	4.98	4.98	6.38
Carbohydrate	-	53.22	51.87	45.6	37.6
CP	137.5	30.31	31.14	34.99	39.89
CF	-	19.16	17.85	11.75	3.97
EE	-	5.66	5.25	3.33	0.89
Ash	0.01	10.81	12.49	20.3	30.6
Mineral, %					
Macro					
Na	-	-	0.87	0.72	0.86
Ca	0.29	11.27	15.45	25.56	34.17
P	0.26	0.25	0.33	0.31	0.42
K	0.04	4.15	3.09	1.67	5.47
Mg	-	0.25	0.11	0.06	0.01
S	24.41	0.29	1.01	3.81	5.47
Micro					
Zn	-	0.02	0.01	0.01	0.01
Fe	0.03	0.15	0.23	0.11	0.04
Mn	-	0.05	0.05	0.03	0.02
Cu	-	0.01	0.01	0.03	2
N Ratio					
NPN : *Indigofera*	-	-	1:09	1:01	9:01

N0 = 100% ammonium sulfate; ID0 = 100% *Indigofera*; NID19 = NPN: *Indigofera* = 1:9; NID11 = NPN: *Indigofera* = 1:1; NID91 = NPN: *Indigofera* = 9:1; N = nitrogen; CP = crude protein; CF = crude fiber; EE = extract eter.

Experimental design

A completely randomized design was applied with five treatments and six replicates : N0 (100% ammonium sulfate), ID0 (100% *Indigofera*), NID19 (NPN : *Indigofera* = 1:9), NID11 (1:1), and NID91 (9:1).

Stastitical analysis

Data were analyzed using 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).

Results

Different nitrogen-protein supplement combinations affected ruminal fermentation parameters, microbial protein synthesis, gas production, and dry matter degradation (Table 3). N0 had a significantly higher ruminal NH_3 concentration ($P<0.05$), indicating excessive ammonia. Treatments ID0, NID19, NID11, and NID91 had significantly lower NH_3 concentrations than N0 ($P<0.05$). A significantly higher TVFA concentration ($P<0.05$) was observed in NID19, whereas N0 had the lowest concentration. Acetate concentration followed a similar trend, with NID19 being significantly higher ($P<0.05$) than that of the other treatments. Propionate concentrations were elevated in treatments with *Indigofera*, including ID0, NID11, and NID19 ($P>0.05$). Butyrate concentration was significantly higher ($P<0.05$) in ID0, while the lowest was observed in N0 ($P<0.05$). Microbial protein synthesis was enhanced in NID19 ($P<0.05$), while N0 was the lowest ($P<0.05$). Total gas production after 48 h was highest in ID0 ($P<0.05$), but was not different from that in NID19 ($P>0.05$), while methane (CH_4) production was reduced in NID91 and N0 compared to that in ID0 ($P<0.05$). N0 showed the highest IVDMD ($P<0.05$) and was not statistically different from that of NID19 ($P>0.05$), whereas *Indigofera* with higher ZA-$Ca(OH)_2$ (NID91) showed the lowest IVDMD ($P<0.05$).

Discussion

The combination of protein-NPN supplements with varying ratios of nitrogen from *Indigofera zollingeriana* and slow-release NPN (ZA-$Ca(OH)_2$) influenced rumen fermentation. Total gas production after 48 h of incubation was highest in the ID0 and NID19 treatments, and the lowest in N0. Gas production is influenced by the chemical components of the substrate, particularly carbohydrates. ID0, which contained *Indigofera* with 53.22% carbohydrates and 19.16% crude fiber, showed the highest gas production. NID19, which combined 92.5% *Indigofera* and 7.5% NPN supplementation, contained 51.87% total carbohydrates. Wang *et al*. (2025) reported gas production correlation with substrate carbohydrates. N0 showed low gas production, as it contained only 22% N and minerals, without a nutrient substrate. The reduced gas production was caused by slow nitrogen release, which limited hydrolysis and substrate degradation. Approximately 40% of the total gas in *in vitro* systems is produced by substrate fermentation (Spanghero *et al*., 2018). Nitrogen compound fermentation reduces gas production due to ammonia production (Heidari *et al*., 2022).

Methane production was lowest in N0, and NID91 may have been inhibited due to limited hydrogen availability caused by the low amount of fermentable organic matter in these treatments, which restricted the substrate supply for methanogenic archaea. This can also be seen in Table 3. This indicates lower gas production in N0 and NID91, which correlates with a decrease in methane gas production. High methane gas production in ID0 due to 100% *Indigofera* contained more fiber than the other treatments (Table 2). A higher fiber component increases methane gas production because it affects the production of H2 as a substrate for methanogenesis. Lan and Yang (2019) reported that a high CH_4 production is typically associated with the fermentation of structural carbohydrates, which generate H_2 and CO_2 as key substrates for methanogenesis by rumen methanogenic archaea.

The highest ruminal ammonia concentration was observed in the N0 group, which used 100% ammonium sulfate as a rapidly degradable NPN source. This elevated NH_3 level reflects the asynchrony between nitrogen release and microbial uptake, potentially leading to inefficient utilization and toxicity (Khattab *et al*., 2020). In contrast, the NID19, NID11, and NID91 treatments had significantly lower ammonia concentrations, which may be attributed to the lower nitrogen content and slower hydrolysis of NPN when bound to $Ca(OH)_2$. Harahap *et al.* (2018) reported that Ca^{2+} can form stable complexes with amino groups, forming chelates that reduce nitrogen solubility, thus limiting the rapid release of ammonia in the rumen. Interestingly, although NID11 and NID91 contained higher crude protein levels (35 and 40%, respectively) than NID19 (30%), their ammonia concentrations were lower. This confirms that NH_3 accumulation is not only determined by protein quantity, but is also more influenced by the rate of nitrogen release and its synchronization with microbial demand (Chen *et al*., 2022).

The highest total volatile fatty acid (TVFA) concentration was in NID19 treatment, showing enhanced microbial fermentation and acetate concentration, indicating effective fiber degradation. VFA concentration from the protein-NPN supplement treatment ranged from 92.62 - 380.99 mM. Chen *et al.* (2022) reported VFA concentrations of 72.7 - 88.3 mM after 6 hours and 119 - 140 mM after 48 hours incubation. The high VFA concentration resulted from a long incubation time, as evident from the gas production at 48 h, showing continued fermentation (Figure 1). VFA production correlates with gas production volume, as gas forms during feed fermentation into acetic and butyric acids (Tunkala *et al*., 2023). McCarthy *et al.* (2023) reported VFA concentrations of 53.97 - 293.87 mM in forage-fed cattle. Despite high VFA concentration, pH values remained

Table 3. *In vitro* ruminal fermentation and gas production of protein-NPN supplement.

Parameter	Treatments					
	N0	ID0	NID19	NID11	NID91	SEM
Total gas production 48 h (mL)	13.68^{d}	98.17^{a}	97.67^{a}	86.58^{b}	69.00^{c}	32.66
CH_4 (mL)	2.04^{e}	6.04^{a}	5.18^{b}	4.28^{c}	2.75^{d}	2.18
NH_3 (mM)	117.48^{a}	20.52^{c}	34.72^{b}	22.84^{c}	20.42^{c}	7.73
TVFA (mM)	92.32^{c}	159.29^{bc}	380.99^{a}	239.45^{b}	154.67^{bc}	31.23
Acetate	75.67^{b}	128.97^{b}	350.87^{a}	209.80^{b}	132.68^{b}	30.3
Propionate	9.83^{c}	17.45^{a}	16.58^{a}	17.37^{a}	13.30^{b}	0.82
Butyrate	4.81^{c}	7.67^{a}	6.57^{b}	7.10^{ab}	5.45^{c}	0.31
Microbial protein synthesis (mg/dL)	11.37^{c}	15.08^{b}	17.79^{a}	14.00^{bc}	13.35^{bc}	0.56
Dry matter degradation (%)	80.49^{a}	69.90^{b}	73.20^{ab}	62.87^{c}	56.45^{c}	1.88

N0 = 100% ammonium sulfate; ID0 = 100% *Indigofera*; NID19 = NPN : *Indigofera* = 1:9; NID11 = NPN : *Indigofera* = 1:1; NID91 = NPN : *Indigofera* = 9:1; TVFA = total volatile fatty acid; SEM = standard error of mean.

normal (6.76 - 6.86). Zhang *et al.* (2023) stated that normal rumen pH ranges from 5.5 - 7.0.

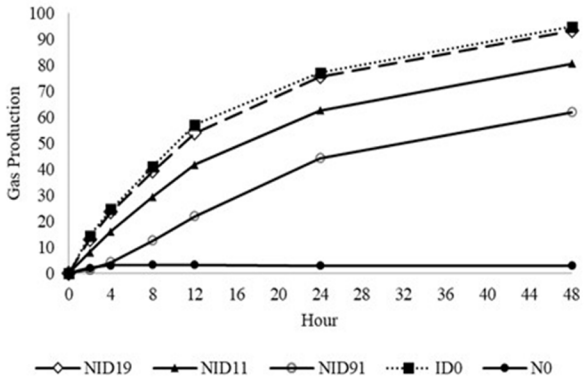

Fig 1. *In vitro* total gas production kinetics of Protein-NPN supplement.
N0 = 100% ammonium sulfate; ID0 = 100% *Indigofera*; NID19 = NPN : *Indigofera* = 1:9; NID11 = NPN : *Indigofera* = 1:1; NID91 = NPN : *Indigofera* = 9:1

The NID19 treatment showed the highest acetate concentration compared to the other treatments (N0, ID0, NID11, and NID91), likely due to structural carbohydrates in its 92.5% *Indigofera zollingeriana* formulation. Structural carbohydrates are precursors for acetate production through cellulolytic bacterial fermentation. High-fiber feed ingredients increase acetate and decrease propionate concentration in the rumen (Li *et al*., 2019). Wanapat *et al*. (2014) reported that structural carbohydrates promote acetate formation, and Gunun *et al*. (2022) observed that *Indigofera* byproducts increased the acetate concentration. Although ID0 contained 100% *Indigofera*, its lower acetate concentration than NID19 suggests that production depends on both fiber content and microbial activity, supported by higher microbial protein synthesis in NID19, indicating more active fermentation.

Propionate concentrations were the highest in ID0, followed by NID19 and NID11, which contained *Indigofera*, contributing 53.22% carbohydrates, aiding fermentation. Propionate is produced through non-structural and structural carbohydrate fermentation. Wang *et al.* (2020) noted forage polysaccharides ferment to propionate. Fiber-degrading bacteria such as *Ruminococcus albus* mainly produce acetate, while *R. flavefaciens* and *Fibrobacter succinogenes* produce succinate, which is converted to propionate (Wang *et al*., 2016). Protein-NPN supplementation affected butyrate production, with the highest levels in ID0 and comparable levels in NID11 and NID19. The N0 treatment resulted in the lowest butyrate concentrations. The high crude fiber content of *Indigofera* enhances butyrate production. Miguel *et al.* (2019) found high-forage diets stimulate butyrate-producing bacteria, while Wang *et al.* (2022) noted butyrate forms from fibrous carbohydrates and slowly fermentable starch in leguminous forages.

The NID19 treatment showed a higher microbial protein concentration due to the dominance of *Indigofera*, which provides amino acids supporting microbial proliferation. The limitation of protein sources decreases amino acid availability to rumen microbes and inhibits microbial protein synthesis (Edmunds *et al*., 2013). *Indigofera* contains amino acids for rumen microbe proliferation, including phenylalanine, isoleucine, tryptophan, proline, valine, asparagine, and tyrosyltyrosine. Rumen microbes use NH_3 as a nitrogen source for MPS and their growth (Hanlon *et al*., 2023). Lower NH_3 concentrations typically indicate an increase in MPS (Chen *et al*., 2022). However, the linear decrease in NH_3 concentration from $Ca(OH)_2$ addition contradicted the MPS results. Microbial synthesis depends on carbohydrate and nitrogen availability, with easily degradable carbohydrates and NPN increasing the rumen bacterial growth (Norrapoke *et al.* 2022).

Dry matter degradation was the highest in the N0 treatment, likely due to the high solubility of ammonium sulfate. However, this was not associated with efficient fermentation, as indicated by low VFA and microbial protein concentrations. In contrast, NID19 promoted greater nutrient conversion to microbial proteins and volatile fatty acids. Cherdthong *et al*. (2011) found that slow-release urea supplementation increases rumen digestibility and fermentation. Slower NH_3 release can be used by the rumen microbes to increase substrate degradation. The lowest degradability occurred in NID91, which may be explained by its higher ash content that reduces the proportion of fermentable organic matter in the substrate, thereby limiting microbial activity (Hao *et al*., 2016).

Conclusion

The inclusion of $Ca(OH)_2$ in the protein-NPN supplement can reduce NH_3 concentration, total gas production, gas production rate, and methane gas production. Meanwhile, the addition of *Indigofera* to the protein-NPN supplement can increase rumen microbial protein synthesis, VFA concentration, and dry matter degradation.

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Conflict of interest

The authors declare no conflicts of interest related to this research.

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