Please, read the manuscript draft thoroughly and check the title, abstract, text, equations, and tables (and their corresponding values and units).

Ruminants
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

Additive effects of feed-grade urea and slow-release urea on chemical composition, *in vitro* gas kinetics and fermentation profile of beef diets varying fiber: starch ratios

Manuel **Gonzalez-Ronquillo**[1]*, Emmanuel **Medina**[2], Navid **Ghavipanje**[3,4], Babak **Darabighane**[5], Alondra Cristel **Narvaez-Lopez**[1], Reynaldo **Zaragoza**[1], Jorge **Osorio-Avalos**[1], Lizbeth Esmeralda **Robles-Jimenez**[6]

[1] Universidad Autónoma del Estado de México, Facultad de Medicina Veterinaria y Zootecnia, Toluca, Estado de México, México.
[2] Universidad Autónoma del Estado de México, Facultad de Ciencias Agricolas, El Cerrillo, El Cerrillo Piedras Blancas, Toluca de Lerdo, México.
[3] University of Birjand, Faculty of Agriculture, Department of Animal Science, Birjand, Iran.
[4] South Khorasan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization, Birjand, Iran.
[5] Semnan University, Faculty of Veterinary Medicine, Department of Animal Science, Semnan, Iran.
[6] Universidad Nacional Autónoma de México, Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Morelia, Michoacán, México.

***Corresponding author:**
mrg@uaemex.mx

Received: June 26, 2025
Accepted: December 31, 2025

How to cite: Gonzalez-Ronquillo, M.; Medina, E.; Ghavipanje, N.; Darabighane, B.; Narvaez-Lopez, A. C.; Zaragoza, R.; Osorio-Avalos, J. and Robles-Jimenez,L. E. 20xx. Additive effects of feed-grade urea and slow-release urea on chemical composition, *in vitro* gas kinetics and fermentation profile of beef diets varying fiber: starch ratios. Revista Brasileira de Zootecnia xx:xxxx.

Copyright: This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

For review only

ABSTRACT - This study determined the feasibility different of nitrogen (N) sources by evaluating *in vitro* rumen fermentation parameters, gas production, and ammonia-N (NH_3-N) concentration in beef cattle diets. The five N sources evaluated were: soybean meal (SBM), feed grade urea (FGU), and three slow-release urea (SRU) sources (Biuret, Optigen, and Nitrosure). Three fiber: starch substrates including corn stover (CS), rolled corn (RC) and 50/50 (CS/RC) were evaluated at three levels of N inclusion (0, 2, and 4% as DM) were also examined. Experiment followed a 5 × 3 × 3 factorial arrangement. The results showed that the different N sources did not affect (P>0.05) the chemical composition, gas production parameters (A, B, and C), dry matter digestibility, microbial crude protein (MCP), and metabolizable energy (ME). The SRU source (Biuret) resulted in lower *in vitro* NH_3-N at 4 h (P = 0.026), 6h (P = 0.004), and 8 h (P = 0.009). In addition, increasing the inclusion level of the N sources (i.e. 0, 2, and 4) linearly increased (P<0.001) NH_3-N concentration at all sampling times. Slow-release urea was the most effective non-protein nitrogen (NPN) source for partially replacing SBM. However, further *in vitro* and *in vivo* studies are warranted to evaluate the feasibility of incorporating NPN sources into practical ruminant diet formulations.

Keywords: ammonia nitrogen, non-protein nitrogen, rumen, ruminant, sustainability

1. Introduction

In ruminant nutrition, dietary proteins play a crucial role in supplying amino acids (AA) and non-protein nitrogen (NPN) as sources of nitrogen for the synthesis of microbial crude protein (MCP) in the rumen (Calsamiglia et al., 2010). MCP serves as the primary source of crude protein (CP) that flowing

into the small intestine, and when combined with dietary rumen undegradable protein (RUP), it forms the metabolizable protein (MP) that is digested and absorbed in the small intestine to satisfy the AAs requirements of ruminants (Owens et al., 2014). However, protein-rich feedstuffs like soybean meal (SBM) are primarily associated with high costs and environmental footprints prompting research to seek alternative protein sources. Consequently, incorporating non-protein nitrogen (NPN) into ruminant diets has emerged as a viable strategy that not only enhances MCP synthesis and lowers feeding costs but also mitigates food-feed-fuel competition and reduce both water and carbon footprints (Reid, 1953; Taylor-Edwards et al., 2009; Spanghero et al., 2018; Salami et al., 2021; Simoni et al., 2023).

Despite decades of research, NPN continues to be a topic of great interest and debate in ruminant nutrition (Pacheco et al., 2021; Grossi et al., 2021; Saro et al., 2023). Feed grade urea (FGU) is the most widely used NPN source among various NPN compounds. However, its application in ruminant diet is limited due to its rapid hydrolysis to ammonia-N (NH_3-N) in the rumen which results in an asynchrony between NH_3-N and fermentable energy potentially adversely affecting MCP synthesis and microbial protein outflow (Cherdthong and Wanapat, 2010; Simoni et al., 2023). Additionally, rapid hydrolysis of FGU in the rumen can decrease nitrogen (N) utilization, increase N excretion, elevate blood NH_3 levels, and increase the risk of NH_3 toxicity (Mahmoudi-Abyane et al., 2020; Heidari et al., 2022). To overcome this, several coating technologies (i.e. fat-coated, calcium-bound, and polymer-coated) have been employed over the past 40 years to develop slow-release urea (SRU) products to control the rate of urea degradation NH_3 release in the rumen (Salami et al., 2021; Zhu et al., 2022; Saro et al., 2023). An extensive review of literature (Cherdthong and Wanapat, 2010) highlighted that dietary addition of SRU for ruminant have positive effects on N retention, MCP production, and fiber digestion. Moreover, in both beef (Salami et al., 2020) and dairy cattle (Simoni et al., 2023), replacing conventional protein feed ingredients with urea, especially SRU, led to enhanced production performances and improved feed efficiency which mainly attributed to healthier, more stable, and efficient rumen function. However, the outcome of using different urea sources in ruminants remains inconclusive. For instance, a study (Mahmoudi-Abyane et al., 2020) with fattening lambs showed the superiority of SRU over FGU regarding increase in population of fibrolytic bacteria and the ruminal ammonia concentration. On the contrary, replacing FGU with SRU in beef steers diets reduced both rumen ammonia and blood urea concentrations (Taylor-Edwards et al., 2009). Also, dietary substitution of FGU with SRU in finishing steers negatively affected on body weight gain and feed conversion ratio (Tedeschi et al., 2002). On the other hand, feeding either FGU or SRU as an alternative led to similar performance and feed efficiency in growing beef (Pacheco et al., 2021), fattening lambs (Saro et al., 2023), and dairy cows (Heidari et al., 2022).

All above, feeding strategies aimed at utilizing either FGU or SRU to replace conventional protein sources must be achieved, first, without impairing rumen function and future studies are warranted to explore more suitable nitrogen sources as substitutes for protein feed. Therefore, in this paper, we evaluate the effects of nitrogen source, inclusion level, and fiber-to-starch ratio on *in vitro* rumen fermentation parameters and gas kinetics, aiming to provide a more comprehensive assessment for selecting optimal nitrogen sources in beef diets. Our findings offer valuable insights for elucidating the impacts of NPN sources on rumen fermentation profile and NH_3-N production, providing benchmark for the practical formulation of ruminant diets.

2. Material and methods

2.1. Experimental treatment

The study was carried out at the Faculty of Veterinary Medicine and Animal Husbandry of the Autonomous University of the State of Mexico, located in "El Cerrillo Piedras Blancas", Toluca, State of Mexico. Its coordinates are: 19.4074° N, 99.6899° W. The location is at an altitude of 2,632 meters above sea level. Its climate is temperate subhumid with an average annual temperature of 13.7 °C.

For review only

Average annual precipitation varies from 1,000 to 1,200 mm, and there are 80 to 140 days of frost during the cold season.

The Institutional Review Board Statement was approved by the Professional Committee on Standardization of Experimental Animals of the Universidad Autonoma del Estado de México (project UAEMex code 6663/2022 SF) and all animal cares and experiments were conducted in accordance with its relevant institutional, national, and international guidelines and regulations.

Corn stover (CS) and rolled corn (RC) were used as substrates. They were previously ground in a Willey mill to pass through a 2-mm screen and combined at a ratio of CS/RC (50/50) to make a third substrate. Subsequently, five sources of nitrogen were included, which were: 1 - soybean meal (SBM, as a reference protein source), 2 - urea 46% N (FGU, Agrocolmex, Mexico), 3 - Optigen (SRU, Optigen®, Alltech Inc., Kentucky, USA), 4 - biuret (SRU, Biuret®, VIMIFOS, Mexico), 5 - Nitrosure (SRU, NitroShure™, Balchem Encapsulates, New Hampton, NY, USA), which were added at 2 and 4% of the dry matter of the substrate. To do this, 100 g of the substrate on a DM basis were weighed, subsequently and 0, 2 and 4 g as DM of the nitrogen source were added and the samples were mixed homogeneously.

The experiment followed a 5 × 3 × 3 factorial arrangement in a completely randomized design. The treatments included five kinds of N sources, three different fiber substrates: starch sources, and three inclusion levels of the N sources (SBM, FGU, Optigen, Biuret and Nitrosure). The three energy sources were: a cellulose-rich source (CS), a starch-rich source (RC) and their 50:50 combination (CS/RC). Each N source was evaluated at three inclusion levels (0, 2, and 4% DM).

2.2. Animals and rumen fluid

Three healthy Swiss brown beef cattle [577 ± 48.75 kg body weight (BW); mean ± standard deviation (SD)] fitted with a flexible rumen fistula were used as inoculums donors of rumen fluid for the experiment. From 14 d prior to the trial, animals were fed a total mixed ration (TMR) consisting of mixture concentrate (70% corn grain and 28% soybean meal) supplemented with 2% vitamins and minerals (MultiTech (Malta) Ltd; Celaya; Mexico), and (barley hay and corn maize stover; 50:50) twice daily (08:00 and 17:00 h) with *ad libitum* access to fresh water. The animals were housed in a tall barn measuring in a 15 × 10 m barn divided into two sections: one half with a roof and a sand floor, and the other half for sun exposure, with feed and water troughs located in the outdoor area. Ruminal inoculum was collected before morning feeding (06:00 h), pooled, strained through four layers of cheesecloth and brought to the laboratory in a thermo-insulated container prefilled with CO_2.

2.3. *In vitro* incubation procedure

2.3.1. *In vitro* gas production

In vitro gas production (IVGP) and fermentation profile were carried out as described by Theodorou et al. (1994). Briefly, 0.800 g DM from each substrate was incubated in 125 ml calibrated glass bottles in triplicate for four incubation runs with 100 mL of incubation medium that contained 10 mL of filtered rumen fluid and 90 mL of buffer solution. Bottles (12 bottles of each substrate) were placed vertically in a water bath at 39°C with gentle manual mixing at each sampling time. Three bottles without substrate used as blanks. The volume of gas produced (mL of gas/g DM) was manually recorded after 0, 2, 4, 6, 8, 12, 16 and 20 h of incubation using a pressure transducer [Delta Model HD 8804, Caselle di Salvezzano (PD), Italy]. After incubations, the dry matter disappearance (DMD) was determined.

Moreover, the kinetic parameters of IVGP were estimated through an iterative procedure of non-linear regression analysis (PROC NLIN, SAS Institute, version 9.0; SAS Institute Inc., Cary, NC, USA) according to the model proposed by France et al. (1993):

$$Y = A\{1 - \exp[-B(t - T) - C(\sqrt{t} - A/T)]\} \quad (1)$$

For review only

in which Y is the cumulative gas production (mL), t is the incubation time (h^{-1}), A is the asymptote curve (mL), B (h^{-1}) and C ($h^{-1/2}$) are the gas production constants, T is the time of delay (hours) for microbial colonization to begin fermentation.

Metabolizable energy (MJ/kg DM) was estimated according to Menke et al. (1979) as:

$$ME = 2.20 + 0.136\ GP\ (mL/0.5\ g\ DM) + 0.057\ crude\ protein\ (CP)\ (g/kg\ DM) \quad (2)$$

Microbial biomass production (MCP) was calculated according to Blümmel et al. (1997) as:

$$MCP\ (mg/g\ DM) = mg\ DMD - (mL\ gas \times 2.2\ mg/mL) \quad (3)$$

in which 2.2 mg/mL is a stoichiometric factor which expresses mg of C, H and O required for the short chain fatty acids gas associated with production of one mL of gas (Blümmel et al., 1997).

2.3.2. *In vitro* NH_3-N and pH determination

Ruminal inoculums was collected as previously described above. Ruminal ammonia (NH_3-N) concentration and pH were determined in four incubation runs in triplicate according to Theodorou et al. (1994). From each flask, 1 mL of incubation fluid was collected at 0, 0.5, 1, 2, 4, 8 and 16 h of incubation and acidified with 0.2 mL of 0.2 N HCl, and stored at −20 °C. Immediately before analysis, samples were thawed and centrifuged (15,300 × *g* for 20 min at 4 °C) and supernatants were used to determine ammonia (NH_3-N, mg/dL), using a phenol-hypochlorite method (Chaney and Marbach, 1962). The pH was determined in duplicate at the same NH_3-N sampling time (Conductronic model pH130, Puebla, Mexico).

2.4. Chemical composition analysis

Individual ingredients (SBM, FGU, Biuret, Optigen, Nitrosure, CS, RC) were analyzed (in triplicates) according to AOAC methods (AOAC, 2015) for dry matter (DM, method number 945.15), ash (method number 954.02), with crude protein calculated as N × 6.25 (CP, method number 954.01). All experimental diets, were then pooled, ground in a hammer mill with a 1-mm screen (Arthur Hill Thomas Corporation, Philadelphia, Pennsylvania, USA), and were analyzed (in triplicates) according to AOAC methods (AOAC, 2015) for dry matter (DM, method number 945.15), ash (method number 954.02), crude protein (CP, method number 954.01), and ether extract (EE, method number 973.18). Ash-free neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed following Van Soest et al. (1991).

After the incubation period, residues were analyzed for DM disappeared (DMD), and N multiplied by 6.25 and determined Rumen degradable protein (RDP), and undegradable intake protein (UIP) by difference.

2.5. Statistical analysis

Data from the three runs conducted within each individual samples were averaged before statistical analysis. The mean values of each individual sample were used as the experimental unit. Results of chemical composition, *in vitro* GP and rumen fermentation parameters were analyzed as a factorial experiment using the PROC GLM procedure of SAS (version 9.0; SAS Institute Inc., Cary, NC, USA) (SAS, 2004) as follows:

$$Y_{ijkl} = \mu + S_i + A_j + L_k + SA_{ij} + SL_{ik} + AL_{jk} + SAL_{ijk} + \varepsilon_{ijkl}$$

in which Y_{ijkl} is every observation of the i-th substrate (S_i) when incubated with the j-th additive types (A: type of Additive; SBM, Urea, Optigen, Biuret, Nitrosure), and dose level (L: 0, 2, 4% inclusion level of the additive), μ is the general mean, S_i (i = 1-3) is the substrate (CS, CS/RC 50/50, RC), A_j is the additive type effect (j = 1-5), L_k is the inclusion level of the additive (n = 1-3), $(S\times A)_{ij}$, $(S\times L)_{ik}$, $(A\times L)_{jk}$, $(S\times A\times L)_{ijk}$ is the interaction between substrate, the additive and inclusion dose level and ε_{ijkl} is residual error. Normality and homogeneity of variances were evaluated by the Shapiro–Wilk and Levene tests,

For review only

respectively. Tukey-Kramer test was used to compare least square means (LSM). Significance was declared at $P<0.05$, and $0.05 \leq P \leq 0.10$ indicated a significant trend. Orthogonal polynomial contrasts were also used to test statistical significance of the linear and quadratic components of treatments.

3. Results

3.1. Chemical composition

Among the substrate feed sources, RC exhibited the highest N content at 14.4 g/kg DM, while urea had the highest N content among the N additives at 448 g/kg DM (Table 1). Conversely, CS contained the lowest N content among the substrate feed sources at 8 g/kg DM, with SBM showing the lowest N content among the N additives at 70.4 g/kg DM (Table 1).

Corn stover, as a fibrous source, had the highest NDF content among the substrates, at 701 g/kg DM, while SBM exhibited the highest NDF content among the N additives at 123 g/kg DM (Table 1). Regarding starch content, RC had the highest level among the substrates, with 680 g/kg DM, and SBM contained approximately 45 g/kg DM of starch among the N additives (Table 1).

Table 1 - Chemical composition of the ingredients (g/kg DM)

Item	CS	CS:RC (50/50)	RC	SBM	Urea	Optigen	Biuret	Nitrosure
Dry matter	937	917	897	915	999	962	970	998
Organic matter	930	958	985	934	1000	1000	1000	1000
Nitrogen	8	11	14.4	70.4	448	410	448	409
Crude protein	50	70	90	440	2800	2562	2800	2556
RDP	35	56	76.5	92.5	2300	2300	2300	2300
UIP	15	14	13.5	15	0	0	0	0
NDF	701	396	90	123	0	0	0	0
ADF	441	236	30	82	0	0	0	0
Ether extract	13	28	43	20	0	0	0	0
Starch	220	450	680	45	0	0	0	0
ME (MJ/kg DM)	9	12	15	16	0	0	0	0

CS - corn stover; RC - rolled corn; SBM - soybean meal; RDP - rumen degradable protein; UIP - undegradable intake protein; NDF - neutral detergent fiber; ADF - acid detergent fiber; ME - metabolizable energy; DM - dry matter.

For review only

Substrate sources were differing in OM, CP, RDP, UIP, NDF, ADF, and EE ($P<0.001$; Table 2). Different N additives where not affected the chemical composition ($P>0.05$), however, the additive dose (i.e. 0, 2, and 4) linearly increased ($P<0.001$) the concentration of CP and RDP. Additionally, the interaction effects of substrate × additive ($P<0.001$), substrate × dose ($P<0.001$), substrate × level, additive × dose ($P<0.001$), and substrate × additive × dose were significant for NDF, ADF, and EE (Table 2).

3.2. *In vitro* gas production and fermentation kinetics

The highest ($P<0.001$) *in vitro* gas production (IVGP) was observed in RC in all incubation periods, followed by CS/RC, and CS (Table 3). While different N additives where not affected IVGP ($P>0.05$), increasing the additive dose linearly decreased IVGP after 2 h ($P<0.001$), 4 h ($P = 0.003$), 6 ($P = 0.015$), and 8 h ($P = 0.019$) of incubation. Overall, there were interaction effects of substrate × additive ($P<0.001$), substrate × dose level ($P<0.001$), and additive × dose level ($P<0.001$) for IVGP at 2, 4, 6, 8, 12, 16 and 20 h of incubation (Table 3).

Table 2 - Proximate analysis (g/kg DM) of experimental treatments with different non-protein nitrogen (NPN) sources by substrate, additive, and inclusion level

Item	Chemical composition (g/kg DM)						
	OM	CP	RDP	UIP	NDF	ADF	EE
Substrate							
Corn stover (CS)	930.49c	107.24b	92.88b	15.07b	694.19a	436.75a	13.07c
CS/RC 50/50	957.50b	127.28ab	112.59ab	16.15a	407.14b	243.41b	26.50b
Rolled corn (RC)	985.07a	147.29a	134.28a	13.55c	90.92c	30.99c	42.81a
SEM	1.76	3.91	3.96	0.09	19.60	13.19	1.00
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Additive							
SBM	957.60	127.39	112.67	14.95	406.78	245.27	24.41
Urea	957.73	127.19	114.39	14.84	395.79	235.68	28.08
Optigen	957.61	127.39	113.64	14.87	395.50	235.29	28.06
Biuret	957.68	127.30	112.34	15.07	395.33	235.37	28.09
Nitrosure	957.73	127.19	112.96	14.86	395.30	235.24	28.05
SEM	1.76	3.91	3.96	0.09	19.60	13.19	1.00
P-value	1.00	1.00	1.00	0.98	1.00	1.00	0.87
Dose level							
0	957.60	70.05c	55.39c	14.93	395.51	235.46	28.09
2	957.72	127.20b	113.17b	14.94	390.91	232.83	27.83
4	957.74	184.56a	171.18a	14.90	405.83	242.85	26.46
SEM	1.76	3.91	3.96	0.09	19.60	13.19	1.00
P-value	0.999	<0.001	<0.001	0.980	0.950	0.950	0.780
Linear	0.970	<0.001	<0.001	0.900	0.830	0.820	0.510
Quadratic	0.99	0.97	0.970	0.890	0.820	0.820	0.790
Overall							
SEM	0.10	0.31	0.28	0.04	0.15	0.10	0.01
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Substrate × Additive	0.995	0.999	0.997	0.286	<0.001	<0.001	<0.001
Substrate × Level	0.127	0.933	0.113	0.997	<0.001	<0.001	<0.001
Additive × Level	0.937	0.999	0.322	0.521	<0.001	<0.001	<0.001
Substrate × Additive × Level	0.999	0.999	0.999	0.857	<0.001	<0.001	<0.001

OM - organic matter; CP - crude protein; RDP - rumen degradable protein; UIP - undegradable intake protein; NDF - neutral detergent fiber; ADF - acid detergent fiber; EE - ether extract; SBM - soybean meal; SEM - standard error of the mean.
Values followed by different letters are indicating significant difference (P≤0.05).

For review only

Corn stover and CS/RC mixture were superior to RC in term of total gas produced (A) (P<0.001, Table 4). The highest (P<0.001) DMD was for RC. Different N additives where not affected (P>0.05) for gas production parameters (A, B, and C), DMD, microbial crude protein (MCP), and metabolizable energy (ME), however, the lowest (P = 0.03) lag time was for SBM (Table 4). With increasing the additive dose level, total gas production (A, P<0.001), lag time (P<0.001), and MCP (P<0.001) were linearly increased. In addition, there were interaction effects (P<0.001) of substrate × additive, substrate × dose level, additive × dose level, substrate × additive × dose level and for DMD, PF16, and MCP (Table 4).

3.3. Rumen NH_3-N concentration

The CS/RC showed the highest (P<0.001) NH_3-N concentration after 0, 0.5, 1, and 16 h of incubation, followed by RC (Table 5). Different N additives where not affected NH_3-N concentration at 0, 1, 2, and 16 h of incubation (P>0.05). However, the addition of SRU (Biuret) led to lower *in vitro* NH_3-N at 4 h (P = 0.026), 6 h (P = 0.004), and 8 h (P = 0.009) with a tendency (P = 0.099) of decrease at 0.5 h of incubation. In addition, increasing the additive dose (i.e. 0, 2, and 4) linearly increased (P<0.001)

Table 3 - *In vitro* gas production (mL gas/g DM) values of diets with different non-protein nitrogen (NPN) sources)

Item	Time of incubation (hour, h)						
	2 h	4 h	6 h	8 h	12 h	16 h	20 h
Substrate							
Corn stover (CS)	−0.79c	1.04c	4.74c	8.45c	18.57c	28.70c	37.24c
CS/RC 50/50	1.76b	10.08b	22.24b	35.65b	60.42b	85.39b	110.84b
Rolled corn (RC)	7.26a	20.87a	40.79a	67.17a	107.45a	147.85a	191.36a
SEM	0.36	0.83	1.41	2.24	3.34	4.44	5.74
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Additive							
SBM	3.98	14.09	27.59	43.42	70.42	97.20	125.88
Urea	2.22	9.59	21.14	35.45	60.60	85.58	110.70
Optigen	2.38	9.94	21.69	36.29	61.46	86.37	111.99
Biuret	3.15	10.77	22.41	36.58	61.46	86.54	112.42
Nitrosure	1.98	8.94	20.15	33.75	56.81	80.91	104.76
SEM	0.36	0.83	1.41	2.24	3.34	4.44	5.74
P-value	0.41	0.32	0.50	0.71	0.78	0.84	0.84
Dose level							
0	4.97a	14.83a	28.09a	45.13a	72.82	100.36	130.12
2	1.33b	8.26b	19.97b	33.86ab	58.37	82.88	107.56
4	1.91b	8.90b	19.71b	32.28b	55.27	78.70	101.75
SEM	0.36	0.83	1.41	2.24	3.34	4.44	5.74
P-value	<0.001	0.001	0.021	0.037	0.072	0.107	0.103
Linear	<0.001	0.003	0.015	0.019	0.032	0.047	0.044
Quadratic	0.004	0.034	0.182	0.301	0.420	0.478	0.488
Overall							
SEM	0.07	0.13	0.191	0.246	0.375	0.462	0.568
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive	<0.001	<0.001	0.011	0.062	<0.001	0.005	0.002
Subtrate × Level	<0.001	<0.001	<0.001	<0.001	0.011	<0.001	<0.001
Additive × Level	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive × Level	0.005	0.045	0.059	0.018	0.001	<0.001	<0.001

SBM - soybean meal; SEM - standard error of the mean.
Values followed by different letters are indicating significant difference (P≤0.05).

For review only

NH_3-N concentration. There was an interaction effects of substrate × additive (P<0.001), substrate × dose level (P<0.001), and additive × dose level (P<0.001) for NH_3-N at incubation periods of 0, 0.5, 1, 2, 4, 6, 8, and 16 h (Table 5).

3.4. Rumen pH values

The pH values of CS were significantly higher (P<0.001) than that for the other two substrates at 2, 4, 6, and 8 h of incubation (Table 6). While the highest (P<0.001) pH at 0.5 h and 1 h was for RC. Neither FGU nor SRU altered *in vitro* pH values at 0.5, 1, 6, 8, and 16 h (P>0.05). However, the highest pH at 2 h (P = 0.006) after incubation was for FGU and Biuret, respectively. Additionally, the interaction of substrate × additive (P<0.001), additive × dose level (P<0.001), and substrate × additive × dose level (P<0.001) showed a significant difference at all incubation periods (Table 6).

Table 4 - *In vitro* gas production parameters and rumen fermentation profile values of diets with different non-protein nitrogen (NPN) sources

Item	A	B	C	Lag time	DMD	PF_{16}	MCP	ME
Substrate								
Corn stover (CS)	1559.84a	0.003c	−0.006a	3.52a	175.36c	171.19b	94.86	9.85c
CS/RC 50/50	1419.30a	0.014b	−0.017b	2.08b	407.70b	211.46ab	89.92	12.02b
Rolled corn (RC)	699.06b	0.025a	0.031c	1.54c	711.42a	249.03a	82.61	14.66c
SEM	77.22	0.001	0.001	0.11	21.02	10.87	4.43	0.23
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.01	0.52	<0.001
Additive								
SBM	1022.41	0.017	−0.021	1.80b	382.43	298.80a	84.73	12.23
Urea	1227.00	0.013	−0.016	2.78a	449.96	183.16b	89.90	12.59
Optigen	1201.61	0.012	−0.017	2.50a	453.48	186.49b	89.50	12.17
Biuret	1398.60	0.010	−0.015	2.18ab	447.71	195.39b	89.58	12.04
Nitrosure	1280.75	0.015	−0.020	2.67a	423.89	188.99b	91.96	11.89
SEM	77.22	0.001	0.001	0.11	21.02	10.87	4.43	0.23
P-value	0.65	0.36	0.64	0.03	0.82	0.002	0.99	0.91
Dose level								
0	636.84b	0.015	−0.019	1.66b	427.78	237.03a	26.00c	11.64
2	1469.25a	0.014	−0.020	2.61a	373.82	246.66a	91.03b	11.93
4	1572.13a	0.012	−0.016	2.89a	492.88	147.98b	150.36a	12.96
SEM	77.22	0.001	0.001	0.11	21.02	10.87	4.43	0.23
P-value	<0.001	0.34	0.37	<0.001	0.069	<0.001	<0.001	0.061
Linear	<0.001	0.155	0.368	<0.001	0.203	<0.001	<0.001	0.025
Quadratic	0.014	0.734	0.284	0.129	0.052	0.014	0.052	0.459
Overall								
SEM	49.46	0.005	0.005	0.301	3.949	3.475	0.204	0.157
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.311
Subtrate × Level	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	<0.001
Additive × Level	0.394	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.616
Subtrate × Additive × Level	0.075	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.940

DMD - dry matter disappearance; PF_{16} - partitioning factor 16 h (mL gas 16 h/g DMD); MCP - microbial biomass production; ME - metabolizable energy (Mj/kg DM); SBM - soybean meal; SEM - standard error of the mean.
Values followed by different letters are indicating significant difference (P≤0.05).

For review only

4. Discussion

Urea has been explored as a cost-effective alternative to plant proteins such as SBM (Pacheco et al., 2021; Simoni et al., 2023). Given the risks associated with rapid NH_3-N release from FGU, the SRU products have been developed to improve N synchrony and reduce environmental N losses (Grossi et al., 2021; Simoni et al., 2023). Nevertheless, responses to FGU and SRU remain inconsistent across both *in vitro* and *in vivo* studies (Mahmoudi-Abyane et al., 2020; Pacheco et al., 2021; Saro et al., 2023). In this work, we evaluated how increasing levels of FGU or SRU affect *in vitro* rumen fermentation and gas kinetics when supplied with fermentable corn-based substrates.

Our data indicate that the different N additives did not affect chemical composition, DMD, microbial crude protein (MCP) and metabolizable energy (ME), although they affected lag time which was lower for SBM than for urea, Optigen, and Nitrosure. In addition, rumen NH_3-N concentration *in vitro* was reduced at 4 and 6 h with biuret compared with Nitrosure, whereas SBM resulted in lower NH_3-N concentrations than NitroShure at 6 and 8 h.

Table 5 - *In vitro* ammonia-N ($N\text{-}NH_3$) concentration of diets with different non-protein nitrogen (NPN) sources

Item	Time of incubation (hour, h)							
	0 h	0.5 h	1 h	2 h	4 h	6 h	8 h	16 h
Substrate								
Corn stover (CS)	17.03b	18.37b	11.76b	22.03	24.90a	23.79	20.87	10.37b
CS/RC 50/50	21.35a	22.78a	17.29a	23.39	19.77b	20.65	21.94	17.43a
Rolled corn (RC)	17.18b	18.47b	12.20b	21.73	24.39a	22.82	21.01	17.40a
SEM	0.40	0.44	0.51	0.77	0.69	0.81	0.78	0.73
P-value	<0.001	<0.001	<0.001	0.645	0.003	0.258	0.832	<0.001
Additive								
SBM	16.35	17.45	13.04	19.97	20.48ab	18.08b	17.02b	12.02
Urea	18.29	19.51	13.87	22.35	25.62ab	24.55ab	22.56ab	16.20
Optigen	18.96	20.44	14.30	21.73	21.68ab	20.90ab	19.79ab	13.91
Biuret	18.53	19.66	12.07	20.75	19.71b	18.72b	18.61ab	12.86
Nitrosure	19.30	20.66	15.29	24.30	25.69a	26.45a	25.39a	18.00
SEM	0.04	0.44	0.51	0.77	0.69	0.81	0.78	0.73
P-value	0.155	0.099	0.558	0.307	0.026	0.004	0.009	0.107
Dose level								
0	15.17c	16.03c	8.59c	15.76c	17.00c	15.25c	13.98c	11.62b
2	19.37b	20.75b	15.02b	23.40b	23.21b	21.41b	20.32b	15.71a
4	21.51a	23.38a	18.33a	28.78a	29.41a	31.56a	30.58a	17.92a
SEM	0.40	0.44	0.51	0.77	0.69	0.81	0.78	0.73
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Linear	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Quadratic	0.163	0.183	0.066	0.406	0.997	0.126	0.104	0.525
Overall								
SEM	0.24	0.26	0.18	0.28	0.24	0.24	0.27	0.24
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Level	0.004	0.012	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Additive × Level	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive × Level	0.028	0.042	0.016	0.007	<0.001	<0.001	<0.001	<0.001

SBM - soybean meal; SEM - standard error of the mean.
Values followed by different letters are indicating significant difference (P≤0.05).

For review only

Additionally, ruminal pH value and NH_3-N release in the rumen is affected by the dose and type of N source as well as by the interaction between N source inclusion level and substrate The primary factor impact driving these effects is the differential rate of nitrogen hydrolysis among the N sources (FGU, SRU, and SBM) and their synchronization with the substrate fermentation dynamics. SRU exhibits slower N release, leading to lower NH_3-N accumulation and more stable pH compared with FGU, which rapidly increases NH_3-N, particularly at early fermentation stages (Garcia-Gonzalez et al., 2007; Salami et al., 2021). The substrate carbohydrate profile (e.g., steam-flaked corn) further modulates these effects by providing fermentable energy that interacts with N availability (Getachew et al., 2004). It has been well established that the relationship between ruminal pH and NH_3-N influences both N metabolism and microbial health. Ammonia exists in equilibrium between the ionized (NH_4^+) and unionized (NH_3) forms, with the proportion of toxic NH_3 increasing as pH rises above 6.0 (Abdoun et al., 2006; Jin et al., 2018). Because NH_3 is highly lipophilic, it readily diffuses across the rumen epithelium, potentially leading to systemic toxicity when its concentrations is high and pH is elevated. Conversely, rapid NH_3-N release from FGU can transiently raise pH by consuming protons during urea hydrolysis, creating a feedback loop that further favors NH_3 formation (Zurak et al., 2023). In our study, the early pH increase with FGU is consistent with this mechanism, whereas the slower NH_3-N release from

Table 6 - *In vitro* pH values of diets with different non-protein nitrogen (NPN) sources

Item	Time of incubation (hour, h)							
	0 h	0.5 h	1 h	2 h	4 h	6 h	8 h	16 h
Substrate								
Corn stover (CS)	6.34c	6.04b	5.93b	6.98a	7.02a	6.99a	6.96a	6.85a
CS/RC 50/50	6.88a	6.80a	6.89a	6.88b	6.96a	6.82b	6.67b	6.53b
Rolled corn (RC)	6.54b	6.90a	6.90a	6.89b	6.84b	6.63c	6.53c	6.43c
SEM	0.02	0.04	0.04	0.01	0.01	0.01	0.02	0.02
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Additive								
SBM	6.54	6.57	6.52	6.85b	6.88	6.80	6.73	6.59
Urea	6.61	6.64	6.59	6.96a	6.90	6.84	6.78	6.66
Optigen	6.58	6.58	6.58	6.94a	6.93	6.82	6.73	6.61
Biuret	6.58	6.55	6.53	6.89ab	6.95	6.81	6.69	6.52
Nitrosure	6.55	6.63	6.61	6.93a	6.87	6.82	6.78	6.66
SEM	0.02	0.04	0.04	0.01	0.02	0.01	0.02	0.02
P-value	0.738	0.910	0.978	0.006	0.236	0.971	0.119	0.271
Dose level								
0	6.57ab	6.52	6.60	6.85c	6.89	6.80	6.69	6.57
2	6.66a	6.65	6.54	6.99a	6.97	6.83	6.74	6.60
4	6.52b	6.58	6.59	6.92b	6.97	6.82	6.74	6.64
SEM	0.02	0.04	0.04	0.01	0.02	0.01	0.02	0.02
P-value	0.038	0.313	0.806	<0.001	0.069	0.732	0.457	0.336
Linear	0.369	0.529	0.903	<0.001	0.044	0.538	0.305	0.141
Quadratic	0.016	0.165	0.519	<0.001	0.258	0.621	0.475	0.967
Overall								
SEM	0.01	0.01	0.01	0.02	0.01	0.02	0.03	0.01
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Level	<0.001	<0.001	<0.001	0.215	0.658	<0.001	<0.001	<0.001
Additive × Level	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Subtrate × Additive × Level	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

SBM - soybean meal; SEM - standard error of the mean.
Values followed by different letters are indicating significant difference (P≤0.05).

For review only

SRU avoided these fluctuations, supporting safer N utilization. Ruminal pH also affects fiber digestion, as cellulolytic bacteria function best near at pH 6.0–6.8 and are inhibited by more alkaline conditions (Abdoun et al., 2006; Zurak et al., 2023). Although pH in our study remained within a generally functional range, the transient rise with FGU may have briefly impaired microbial efficiency, limiting DMD despite high NH_3-N. In contrast, SRU promoted more stable pH and NH_3-N concentrations release, supporting consistent fibrolytic activity (Cherdthong and Wanapat, 2010).

In this study, different N additives did not alter the chemical composition. However, increasing the additive dose linearly elevated the concentration of CP and RDP concentrations. This trend is consistent with previous reports in which increasing urea levels (0, 0.5, and 1%) in *Leucaena* silageenhanced CP content (Phesatcha and Wanapat, 2016). Regarding fermentation, *in vitro* gas production (IVGP) was higher for CS and the CS/RC mixture than for RC. IVGP is known to originate from the microbial digestion of feed ingredients as well as the buffering of acids generated during fermentation (Getachew et al., 2004; Menke and Steingass, 1988), reflecting greater digestion of soluble and insoluble carbohydrates in CS and the CS/RC in the current study. In this experiment, steam-flaked corn served as the substrate. The steam flaking process disrupts the protein matrix that encases the starch granules, thereby increasing the surface area and facilitating starch gelatinization. This modification enhances

the accessibility of amylose and amylopectin to rumen microorganisms, thereby increasing the starch susceptibility to enzymatic hydrolysis (DePeters et al., 2003; Rafiee and Darabighane, 2021).

It has been reported that the strategies aimed at reformulating ruminant diets by incorporating NPN sources must not impair ruminal fermentation to avoid deleterious impacts on animal productivity (Spanghero et al., 2018; Salami et al., 2021). The present *in vitro* results revealed that the different N additives where did not affect IVGP, fermentation parameters (A, B, and C), DMD, microbial crude protein (MCP), or metabolizable energy (ME). Similarly, Salami et al. (2021) showed that the *in vitro* total gas production remained unaffected by different nitrogen sources (SBM, SRU and FGU) in high-forage diets. In contrast, Muthia et al. (2021) showed that the biuret- and urea- treated treated straw enhanced DMD, because nitrogen and energy were available simultaneously and in the proportions required by rumen microbes, thus promoting diet digestion. Likewise, Cherdthong et al. (2011) showed that SRU addition to cassava chip (as an energy source) led to enhanced IVGP. Supplementation with a urea-calcium mixture in a high-quality feed block also increased IVGP in buffalo rumen fluid (Cherdthong and Wanapat, 2014). Cone and van Gelder (1999) reported that the gas production data from samples differing in CP content can present conflicting results. Typically, IVGP implies inferior DMD; nevertheless, feedstuffs rich in CP usually produce lesser gas during fermentation, regardless of whether their degree of degradation is high. However, in line with our results, Zhu et al. (2022) reported that the different N supplementation levels did not affect *in vitro* degradability of DM, CP, ADF, and NDF. In the present study, the SRU (Biuret) numerically reduced the B fraction compared with FGU, which agrees with previous reports (Sinclair et al., 2012). It has been well established that the SRU moderates ruminal nitrogen release rates while ensuring its availability within the rumen (Pacheco et al., 2021). Our data indicate a linear increase in MCP with increasing supplementation level, which agrees with the findings of Zhang et al. (2016) who reported an increased MCP production when dietary urea increased from 0% to 2% (DM basis).

The present findings revealed that SRU (especially Biuret) resulted in NH_3-N concentrations similar to those of SBM, but significantly lower than those of FGU, confirming that SRU reduced diminished urea hydrolysis under *in vitro* conditions. This may be attributed to the fact that both SRU and SBM have similar N disappearance rates in the rumen, in contrast to the rapid rate exhibited by FGU (Cherdthong and Wanapat, 2010). Similarly, Garcia-Gonzalez et al. (2007) reported that the dissolution rate of SRU in the rumen is lower than that of inferior to FGU, leading to lower ruminal NH_3-N levels in steers. Consistent with the present results, previous studies have shown that the addition of FGU increased NH_3-N concentration in high-forage diets, whereas no differences were found between SRU and SBM (Salami et al., 2021). It has also reported that the lower NH_3-N accumulation with SRU could mitigate the risk of ammonia toxicity and enhance N utilization efficiency by ensuring better synchronization between ammonia release and available fermentable energy in the rumen (Cherdthong and Wanapat, 2010). Our data also indicate a linear relationship between NH_3-N concentration and NPN level, meaning that as NPN levels increase, NH_3-N concentrations also increase. This is in line with the results reported by Spanghero et al. (2018), who observed a linear trend of *in vitro* NH_3-N accumulation with increasing doses of urea. Similarly, increasing FGU doses (0, 0.3, 0.6, and 0.9% of diet DM) have been reported to increase ruminal NH_3-N concentrations in vivo (Boucher et al., 2007). A recent meta-analysis (Simoni et al., 2023) of 44 published studies on the effects of dietary supplementation with FGU and SRU in high-producing dairy cattle showed that both FGU and SRU increased ruminal NH_3-N concentrations, with greater increases observed for FGU.

It is well known (Abdoun et al., 2006; Zhu et al., 2022) that elevated ruminal pH accelerates the toxicity of urea, as an increase in ruminal pH enhances thepermeability of the rumen epithelium to NH_3-N. Ruminal pH provides an overall indication of the fermentation process. However, it can be influenced by various factors, and maintaining a relatively stable pH value is important for optimal animal health and feed utilization (Bao et al., 2011). In the current study, the pH range supported normal ruminal fermentation. Moreover, pH values remained unchanged with the addition of FGU and/or SRU during the 16-h fermentation period, except an increase at 2 h post-incubation with FGU. These findings align with previous studies showing that FGU and/or SRU did not alter ruminal pH (Garcia-Gonzalez, et al.,

For review only

2007; Taylor-Edwards et al., 2009; Mahmoudi-Abyane et al., 2020; Heidari et al., 2022). In line with our results, Salami et al. (2021) showed that the different N supplements (including SBM, FGU, and SRU) in high-forage diets did not affect *in vitro* pH values during 24 h of fermentation. Overall, the decrease in NH_3-N levels and the transient increase in pH with FGU addition suggests that this NPN source was unable to sustain NH_3-N levels required for optimal cellulolytic bacterial activity.

5. Conclusions

The current *in vitro* study investigated the effects of different N sources (including SBM, FGU and SRU) on ruminal NH_3-N concentration and pH values in beef cattle. Our results demonstrated that pH values increased at 2 h and 4 h post-incubation with FGU, likely due to rapid ammonia release, which may reduce the efficiency of cellulolytic bacterial activity, although it did not impair overall rumen fermentation. In contrast, SRU maintained stable pH throughout 16 hours of fermentation, supporting better synchronization of nitrogen and energy for microbial activity. Notably, SRU (especially Biuret) resulted in NH_3-N concentrations similar to those SBM, but significantly lower than those of FGU implying its viability as an NPN source for partially replacing vegetable protein sources, without negatively affecting *in vitro* rumen fermentation under the current experimental conditions. However, future studies, both *in vitro* and/or *in vivo*, are warranted to better explore the suitability of alternative NPN sources for replacing conventional proteins in ruminant diets.

Data availability

All data generated or analyzed during this study are included in this published article.

Author contributions

Conceptualization: Gonzalez-Ronquillo, M. and Robles-Jiménez, L. E. **Data curation:** Gonzalez-Ronquillo, M.; Darabighane, B. and Robles-Jiménez, L. E. **Formal analysis:** Gonzalez-Ronquillo, M.; Darabighane, B.; Narvaez-Lopez, A. C.; Zaragoza, R.; Osorio-Avalos, J. and Robles-Jiménez, L. E. **Funding acquisition:** Gonzalez-Ronquillo, M. **Investigation:** Gonzalez-Ronquillo, M. **Methodology:** Gonzalez-Ronquillo, M. and Robles-Jiménez, L. E. **Project administration:** Gonzalez-Ronquillo, M. **Resources:** Gonzalez-Ronquillo, M. **Supervision:** Gonzalez-Ronquillo, M. and Robles-Jiménez, L. E. **Validation:** Gonzalez-Ronquillo, M.; Narvaez-Lopez, A. C.; Zaragoza, R.; Osorio-Avalos, J. and Robles-Jiménez, L. E. **Visualization:** Gonzalez-Ronquillo, M. and Robles-Jiménez, L. E. **Writing – original draft:** Gonzalez-Ronquillo, M.; Medina, E.; Ghavipanje, N.; Narvaez-Lopez, A. C.; Zaragoza, R.; Osorio-Avalos, J. and Robles-Jiménez, L. E. **Writing – review & editing:** Gonzalez-Ronquillo, M.; Medina, E.; Ghavipanje, N.; Darabighane, B. and Robles-Jiménez, L. E.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Universidad Autónoma del Estado de México with a grant number 6663/2022 SF.

References

Abdoun, K.; Stumpff, F. and Martens, H. 2006. Ammonia and urea transport across the rumen epithelium: a review. Animal Health Research Reviews 7:43-59. https://doi.org/10.1017/S1466252307001156

AOAC. 2015. Official methods of analysis. Association of Official Analytical Chemists. 18th ed. AOAC, Arlington, VA, p.806-814.

For review only

Bao, L.; Huang, Q.; Chang, L.; Zhou, J. and Lu, H. 2011. Screening and characterization of a cellulase with endocellulase and exocellulase activity from yak rumen metagenome. Journal of Molecular Catalysis B: Enzymatic 73:104-110. https://doi.org/10.1016/j.molcatb.2011.08.006

Blümmel, M.; Makkar, H. P. S. and Becker, K. 1997. *In vitro* gas production: a technique revisited. Journal of Animal Physiology and Animal Nutrition 77:24-34. https://doi.org/10.1111/j.1439-0396.1997.tb00734.x

Boucher, S. E.; Ordway, R. S.; Whitehouse, N. L.; Lundy, F. P.; Kononoff, P. J. and Schwab, C. G. 2007. Effect of incremental urea supplementation of a conventional corn silage-based diet on ruminal ammonia concentration and synthesis of microbial protein. Journal of Dairy Science 90:5619-5633. https://doi.org/10.3168/jds.2007-0012

Calsamiglia, S.; Ferret, A.; Reynolds, C. K.; Kristensen, N. B. and van Vuuren, A. M. 2010. Strategies for optimizing nitrogen use by ruminants. Animal 4:1184-1196. https://doi.org/10.1017/S1751731110000911

Chaney, A. L. and Marbach, E. P. 1962. Modified reagents for determination of urea and ammonia. Clinical Chemistry 8:130-132. https://doi.org/10.1093/clinchem/8.2.130

Cherdthong, A. and Wanapat, M. 2010. Development of urea products as rumen slow-release feed for ruminant production: a review. Australian Journal of Basic and Applied Sciences 4:2232-2241.

Cherdthong, A.; Wanapat, M. and Wachirapakorn, C. 2011. Influence of urea–calcium mixtures as rumen slow-release feed on *in vitro* fermentation using a gas production technique. Archives of Animal Nutrition 65:242-254. https://doi.org/10.1080/1745039X.2011.568277

Cherdthong, A. and Wanapat, M. 2014. *In vitro* gas production in rumen fluid of buffalo as affected by urea-calcium mixture in high-quality feed block. Animal Science Journal 85:420-426. https://doi.org/10.1111/asj.12168

Cone, J. W. and van Gelder, A. H. 1999. Influence of protein fermentation on gas production profiles. Animal Feed Science and Technology 76:251-264. https://doi.org/10.1016/S0377-8401(98)00222-3

DePeters, E. J.; Getachew, G.; Fadel, J. G.; Zinn, R. A.; Taylor, S. J.; Pareas, J. W.; Hinders, R. G. and Aseltine, M. S. 2003. In vitro gas production as a method to compare fermentation characteristics of steam-flaked corn. Animal Feed Science and Technology 105:109-122. https://doi.org/10.1016/S0377-8401(03)00042-7

France, J.; Dhanoa, M. S.; Theodorou, M. K.; Lister, S. J.; Davies, D. R. and Isac, D. 1993. A model to interpret gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. Journal of Theoretical Biology 163:99-111. https://doi.org/10.1006/jtbi.1993.1109

Garcia-Gonzalez, R.; Tricarico, J. M.; Harrison, G. A.; Meyer, M. D.; McLeod, K. R.; Harmon, D. L. and Dawson, K. A. 2007. Optigen® is a sustained release source of non-protein nitrogen in the rumen. Journal of Dairy Science 90:98-98.

Getachew, G.; Robinson, P. H.; DePeters, E. J. and Taylor, S. J. 2004. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. Animal Feed Science and Technology 111:57-71. https://doi.org/10.1016/S0377-8401(03)00217-7

Grossi, S.; Compiani, R.; Rossi, L.; Dell'Anno, M.; Castillo, I. and Sgoifo Rossi, C. A. 2021. Effect of slow-urea release administration on production performance, health status, diet digestibility, and environmental sustainability in lactating dairy cows. Animals 11:2405. https://doi.org/10.3390/ani11082405

Heidari, M.; Ghorbani, G. R.; Hashemzadeh, F.; Ghasemi, E.; Panahi, A. and Rafiee, H. 2022. Feed intake, rumen fermentation and performance of dairy cows fed diets formulated at two starch concentrations with either conventional urea or slow-release urea. Animal Feed Science and Technology 290:115366. https://doi.org/10.1016/j.anifeedsci.2022.115366

Jin, D.; Zhao, S.; Zheng, N.; Beckers, Y. and Wang, J. 2018. Urea metabolism and regulation by rumen bacterial urease in ruminants – A review. Annals of Animal Science 18:303-318. https://doi.org/10.1515/aoas-2017-0028

Mahmoudi-Abyane, M.; Alipour, D. and Moghimi, H. R. 2020. Effects of different sources of nitrogen on performance, relative population of rumen microorganisms, ruminal fermentation and blood parameters in male feedlotting lambs. Animal 14:1438-1446. https://doi.org/10.1017/S175173111900291X

Menke, K. H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D. and Schneider, W. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor *in vitro*. The Journal of Agricultural Science 93:217-222. https://doi.org/10.1017/S0021859600086305

Menke, K. H. and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development 28:7-55.

Muthia, D.; Ridla, M.; Laconi, E. B.; Ridwan, R.; Fidriyanto, R.; Abdelbagi, M.; Harahap, R. P. and Jayanegara, A. 2021. Effects of ensiling, urea treatment and autoclaving on nutritive value and *in-vitro* rumen fermentation of rice straw. Advances in Animal and Veterinary Sciences 9:655-661. https://doi.org/10.17582/journal.aavs/2021/9.5.655.661

Owens, F. N.; Qi, S. and Sapienza, D. A. 2014. Invited Review: Applied protein nutrition of ruminants—Current status and future directions. The Professional Animal Scientist 30:150-179. https://doi.org/10.15232/S1080-7446(15)30102-9

Pacheco, R. F.; Machado, D. S.; Viana, A. F. P.; Teixeira, J. S. and Milani, L. 2021. Comparison of the effects of slow-release urea vs conventional urea supplementation on some finishing cattle parameters: A meta-analysis. Livestock Science 250:104549. https://doi.org/10.1016/j.livsci.2021.104549

For review only

Phesatcha, K. and Wanapat, M. 2016. Improvement of nutritive value and *in vitro* ruminal fermentation of *Leucaena* silage by molasses and urea supplementation. Asian-Australasian Journal of Animal Sciences 29:1136-1144. https://doi.org/10.5713/ajas.15.0591

Rafiee, H. and Darabighane, B. 2021. A meta-analysis and meta-regression of the effect of substitution of steam flaked corn instead of finely ground corn on dry matter intake, nutrients digestibility, lactation performance, and rumen fermentation in dairy cows. Animal Feed Science and Technology 280:115043. https://doi.org/10.1016/j.anifeedsci.2021.115043

Reid, J. T. 1953. Urea as a protein replacement for ruminants: a review. Journal of Dairy Science 36:955-996. https://doi.org/10.3168/jds.S0022-0302(53)91586-0

Salami, S. A.; Devant, M.; Apajalahti, J.; Holder, V.; Salomaa, S.; Keegan, J. D. and Moran, C. A. 2021. Slow-release urea as a sustainable alternative to soybean meal in ruminant nutrition. Sustainability 13:2464. https://doi.org/10.3390/su13052464

Salami, S. A.; Moran, C. A.; Warren, H. E. and Taylor-Pickard, J. 2020. A meta-analysis of the effects of slow-release urea supplementation on the performance of beef cattle. Animals 10:657. https://doi.org/10.3390/ani10040657

Saro, C.; Degeneffe, M. A.; Andrés, S.; Mateo, J.; Caro, I.; López-Ferreras, L.; Horst, E. H.; López, S. and Giráldez, F. J. 2023. Conventional feed-grade or slow-release coated urea as sources of dietary nitrogen for fattening lambs. Animals 13:3465. https://doi.org/10.3390/ani13223465

Simoni, M.; Fernandez-Turren, G.; Righi, F.; Rodríguez-Prado, M. and Calsamiglia, S. 2023. A network meta-analysis of the impact of feed-grade and slow-release ureas on lactating dairy cattle. Journal of Dairy Science 106:3233-3245. https://doi.org/10.3168/jds.2022-22786

Sinclair, L. A.; Blake, C. W.; Griffin, P. and Jones, G. H. 2012. The partial replacement of soyabean meal and rapeseed meal with feed grade urea or a slow-release urea and its effect on the performance, metabolism and digestibility in dairy cows. Animal 6:920-927. https://doi.org/10.1017/S1751731111002485

Spanghero, M.; Nikulina, A. and Mason, F. 2018. Use of an in vitro gas production procedure to evaluate rumen slow-release urea products. Animal Feed Science and Technology 237:19-26. https://doi.org/10.1016/j.anifeedsci.2017.12.017

Taylor-Edwards, C. C.; Hibbard, G.; Kitts, S. E.; McLeod, K. R.; Axe, D. E.; Vanzant, E. S.; Kristensen, N. B. and Harmon, D. L. 2009. Effects of slow-release urea on ruminal digesta characteristics and growth performance in beef steers. Journal of Animal Science 87:200-208. https://doi.org/10.2527/jas.2008-0912

Tedeschi, L. O.; Baker, M. J.; Ketchen, D. J. and Fox, D. G. 2002. Performance of growing and finishing cattle supplemented with a slow-release urea product and urea. Canadian Journal of Animal Science 82:567-573. https://doi.org/10.4141/A02-018

Theodorou, M. K.; Williams, B. A.; Dhanoa, M. S.; McAllan, A. B. and France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48:185-197. https://doi.org/10.1016/0377-8401(94)90171-6

Van Soest, P. V.; Robertson, J. B. and Lewis, B. A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74:3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Zhang, S.; Cheng, L.; Guo, X.; Ma, C.; Guo, A. and Moonsan, Y. 2016. Effects of urea supplementation on rumen fermentation characteristics and protozoa population *in vitro*. Journal of Applied Animal Research 44:1-4. https://doi.org/10.1080/09712119.2014.978779

Zhu, J.; Ren, A.; Jiao, J.; Shen, W.; Yang, L.; Zhou, C. and Tan, Z. 2022. Effects of non-protein nitrogen sources on *in vitro* rumen fermentation characteristics and microbial diversity. Frontiers in Animal Science 3:891898. https://doi.org/10.3389/fanim.2022.891898

Zurak, D.; Kljak, K. and Aladrović, J. 2023. Metabolism and utilisation of non-protein nitrogen compounds in ruminants: a review. Journal of Central European Agriculture 24:1-14. https://doi.org/10.5513/JCEA01/24.1.3645

For review only