Effect of combinations of feed-grade urea and slow-release urea in a finishing beef diet on fermentation in an artificial rumen system

Daryoush Alipour,† Atef Mohamed Saleem,‡ Haley Sanderson,§ Tassilo Brand,|| Laize V. Santos,¶ Mahdi Mahmoudi-Abyane,† Mohammad Reza Marami,|| and Tim Angus McAllister||,†

†Department of Animal Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran; ‡Department of Animal and Poultry Production, Faculty of Agriculture, South Valley University, Qena 83523, Egypt; ||Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, Alberta T1J 4B1, Canada; $Department of Animal Sciences, Ruminant Nutrition, University of Göttingen, Göttingen 37073, Germany; and ¶Department of Agricultural Science, State University of Southwestern of Bahia, Bahia 45700-000, Brazil

ABSTRACT: This study evaluated the effect of combinations of feed-grade urea and slow-release urea (SRU) on fermentation and microbial protein synthesis within two artificial rumens (Rusitec) fed a finishing concentrate diet. The experiment was a completely randomized, dose–response design with SRU substituted at levels of 0% (control), 0.5%, 1%, or 1.75% of dry matter (DM) in place of feed-grade urea, with four replicate fermenters per dosage. The diet consisted of 90% concentrate and 10% forage (DM basis). The experiment was conducted over 15 d, with 8 d of adaptation and 7 d of sampling. Dry matter and organic matter disappearances were determined after 48 h of incubation from day 9 to 12, and daily ammonia (NH₃) and volatile fatty acid (VFA) production were measured from day 9 to 12. Microbial protein synthesis was determined on days 13–15. Increasing the level of SRU quadratically affected total VFA (Q, P = 0.031) and ammonia (Q, P = 0.034), with a linear increment in acetate (L, P = 0.01) and isovalerate (L, P = 0.05) and reduction in butyrate (L, P = 0.05). Disappearance of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was quadratically affected by levels of SRU, plateauing at 1% SRU. Inclusion of 1% SRU resulted in the highest amount of microbial nitrogen associated with feed particles (Q, P = 0.037). Responses in the efficiency of microbial protein synthesis fluctuated (L, P = 0.002; Q, P = 0.001) and were the highest for 1% SRU. In general, the result of this study showed that 1% SRU in combination with 0.6% urea increased NDF and ADF digestibility and total volatile fatty acid (TVFA) production.

Key words: beef, finishing diet, ¹⁵N, rumen, Rusitec, slow-release urea

INTRODUCTION

One of the challenges in high-producing ruminants is to synchronize the availability of energy and nitrogen (N) in the rumen in a manner that captures the value of both dietary components.

Feed-grade urea is a nonprotein N (NPN) source that is rapidly hydrolyzed by rumen bacterial ureases to ammonia (Van Soest, 1994) and is considered to be available as a N source immediately after ingestion (Sniffen et al., 1992). However, urea is poorly utilized as a N source for microbial protein synthesis if rapidly digestible carbohydrates are not available to provide the carbon skeletons needed for the synthesis of amino acids by
rumen microorganisms. To overcome this problem, attempts have been made to modify urea so as to slow its rate of ruminal hydrolysis and increase the efficiency of N capture by rumen microorganisms (Taylor-Edwards et al., 2009). To achieve this goal, alternative N sources, such as biuret and starea, as well as oil-coated, calcium-bound, and polymer-coated sources of urea have been developed (Taylor-Edwards et al., 2009). However, even with these approaches, the rate of N release in some experiments was faster or slower than the degradation of feed sources of protein, such as grains (Holder, 2012; Sinclair et al., 2012). In theory, synchronization of the rates of urea release and carbohydrate degradation should enhance the efficiency of microbial protein synthesis. Feed carbohydrates are comprised of rapidly degradable (mostly starch) and slowly degradable (cell wall) components. Providing N that could be synchronized with both rapidly and slowly degradable carbohydrates could enhance microbial protein synthesis and possibly increase feed digestibility (López-Soto et al., 2014). A combination of readily and slowly available N sources (i.e., mixture of feed-grade urea and slow-release products) is likely optimal for microbial protein synthesis.

A slow-release urea (SRU) N source was developed, which has a rate of N release of 7%/h (Mahmoudi-Abyane et al., 2017), a rate similar to the degradation rate of corn starch (6%/h; Batajoo and Shaver, 1998). It is hypothesized that a combination of urea and SRU will enhance microbial protein synthesis. Feed carbohydrates are comprised of rapidly degradable (mostly starch) and slowly degradable (cell wall) components. Providing N that could be synchronized with both rapidly and slowly degradable carbohydrates could enhance microbial protein synthesis and possibly increase feed digestibility (López-Soto et al., 2014). A combination of readily and slowly available N sources (i.e., mixture of feed-grade urea and slow-release products) is likely optimal for microbial protein synthesis.

Experimental Apparatus and Incubations

Each Rusitec apparatus was equipped with eight 920-mL fermenters. Each fermenter had an inlet for the infusion of buffer and an effluent outlet. Fermenters were immersed in a water bath at 39 °C. The four treatments were randomly assigned to duplicate fermenters within each Rusitec apparatus (four replications per treatment). The experiment was initiated by filling each fermenter with 180 mL of warmed McDougall’s buffer (McDougall, 1948); pH 6.60, containing NaH₂PO₄·H₂O (8.24 g/L) and NaHCO₃ (6.34 g/L), NaCl (0.47 g/L), KCl (0.6 g/L), CaCl₂·2H₂O (0.029 g/L), MgCl₂·6H₂O (0.06 g/L), and (NH₄)₂SO₄ (0.3 g/L). The rumen inoculum was collected from three ruminally cannulated cows 2 h after the morning feeding. Rumen contents were immediately strained through four layers of cheese cloth and transported to the laboratory where solid and liquid contents were equally pooled. Mixed contents were kept in a water bath at 39 °C prior to introduction into fermenters. Nylon bags containing 20 g of rumen solids and 10 g of diet DM were placed inside each fermenter. From day 2 onward, fermenters were opened daily to replace the bag that had been incubated for 48 h. Artificial saliva was continuously infused into the fermenters at a dilution rate of 2.9%/h. During bag exchange, each fermentation vessel was flushed with O₂-free CO₂ to promote anaerobicity. The effluent was collected in a 2.0-L Erlenmeyer flask and the volume was measured daily during feed bag exchange.

MATERIAL AND METHODS

The present experiment was conducted at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre, Lethbridge, Alberta, Canada. Donor-cannulated steers used in the experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009) and protocols were reviewed and approved by the Lethbridge Research and Development Centre Animal Care Committee.

Experimental Design and Diets

The experiment was a completely randomized design with four treatments (ruminal inoculum) carried out in 16 Rusitec fermenters (n = 4/treatment). The experiment consisted of 8 d of adaptation (days 1–8) and 7 d of sampling (days 9–15). Treatments consisted of increasing levels of SRU as a replacement for feed-grade urea at (1) 0% (control), 0.5%, 1%, and 1.75% SRU (dry matter [DM] basis of diet). The SRU or urea alone or in combination accounted for 33% of the crude protein (CP) content in the diet (13.2% of DM). The SRU was designed using a blend of vegetable oils and polymers and developed at the Department of Animal Science in Bu-Ali Sina University (Hamedan, Iran; Mahmoudi-Abyane et al., 2017). A matrix was used to trap urea and it generated a release rate of 7% N/h. The product contained 87% urea on DM basis. The diets consisted of 90% concentrate (corn grain, molasses, SRU, feed-grade urea, calcium carbonate, and a mineral–vitamin premix) and 10% barley silage (Table 1). Diets were mixed and ground through a 4-mm screen (Arthur H. Thomas Co., Philadelphia, PA) and mixed with SRU and urea particles prior to placement in polyester bags (100 × 200 mm; pore size = 50 μm; ANKOM; Ankom Technology Corp).

The experiment was a completely randomized design with four treatments (ruminal inoculum) carried out in 16 Rusitec fermenters (n = 4/treatment). The experiment consisted of 8 d of adaptation (days 1–8) and 7 d of sampling (days 9–15). Treatments consisted of increasing levels of SRU as a replacement for feed-grade urea at (1) 0% (control), 0.5%, 1%, and 1.75% SRU (dry matter [DM] basis of diet). The SRU or urea alone or in combination accounted for 33% of the crude protein (CP) content in the diet (13.2% of DM). The SRU was designed using a blend of vegetable oils and polymers and developed at the Department of Animal Science in Bu-Ali Sina University (Hamedan, Iran; Mahmoudi-Abyane et al., 2017). A matrix was used to trap urea and it generated a release rate of 7% N/h. The product contained 87% urea on DM basis. The diets consisted of 90% concentrate (corn grain, molasses, SRU, feed-grade urea, calcium carbonate, and a mineral–vitamin premix) and 10% barley silage (Table 1). Diets were mixed and ground through a 4-mm screen (Arthur H. Thomas Co., Philadelphia, PA) and mixed with SRU and urea particles prior to placement in polyester bags (100 × 200 mm; pore size = 50 μm; ANKOM; Ankom Technology Corp).
Table 1. Ingredients and chemical composition of experimental diets fed to the Rusitec‡

| Ingredients                  | Control    | 0.5% SRU*   | 1% SRU      | 1.75% SRU    |
|------------------------------|------------|-------------|-------------|--------------|
| Barley silage                | 10.25      | 10.3        | 10.3        | 10.25        |
| Corn grain (dent)            | 86.05      | 85.9        | 85.90       | 85.75        |
| Molasses                     | 0.18       | 0.18        | 0.18        | 0.18         |
| Urea                         | 1.45       | 1.00        | 0.60        | 0.00         |
| SRU                          | 0.00       | 0.05        | 1.00        | 1.75         |
| Calcium carbonate            | 2.00       | 2.00        | 2.00        | 2.00         |
| Mineral and vitamin mix†     | 0.07       | 0.07        | 0.07        | 0.07         |
| Chemical composition, % of DM|            |             |             |              |
| OM                           | 96.06      | 96.01       | 96.01       | 95.98        |
| CP                           | 13.25      | 13.21       | 13.27       | 13.30        |
| NDF                          | 11.4       | 11.8        | 11.4        | 11.1         |
| Metabolizable energy, MCal/kg DM | 2.96   | 2.95        | 2.95        | 2.95         |

*Slow-release urea as described by Mahmoudi-Abyane et al. (2017).
†Composed of zinc sulfate (43.5%), copper sulfate (15.8%), manganous sulfate (22.4%), EDDI 80 (0.22%), selenium (7.7%), cobalt carbonate (0.12%), vitamin A (2.6%), vitamin D (0.26%), and vitamin E (7.25%).
‡All the acronyms are defined in the text.

Nutrients Disappearance and Fermentation

Dry matter, organic matter (OM), CP, neutral detergent fiber (NDF), and acid detergent fiber (ADF) disappearance from 48-h incubated substrate bags was determined from day 9 to 12 of the sampling period. Feed bags were withdrawn from each fermenter and washed under cold tap water until the water was clear. Bags were oven dried at 55 °C for 48 h and hot weighed to determine DM disappearance. Residues were pooled over 4 d to ensure sufficient sample for chemical analysis, ground through a 1-mm screen using a Wiley mill (Standard Model 4, Arthur Thomas Co., Philadelphia, PA), and analyzed for OM, total N, NDF, and ADF. Ash content was determined by combustion at 550 °C for 5 h, and OM was calculated as 100 minus the proportion of ash. Total N was determined using a combustion analyzer (NA 2100, Carlo Erba Instruments, Milan, Italy), with CP calculated as N × 6.25. The NDF content was determined using the sequential method with ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY) using reagents as described by Van Soest et al. (1991) with heat-stable α-amylase (Termamyl 120 L, Novo Nordisk Biochem, Franklinton, NC) and sodium sulfate included in the NDF analysis. The ADF content was determined based on Association of Official Analytical Chemists (2000) official method 973.18. Neutral detergent fiber and ADF were expressed exclusive of residual ash. Disappearance of OM, CP, NDF, ADF, and starch was determined as the difference between the amount of these components in the substrate before incubation and the amount remaining in the residue after incubation.

Total gas production was recorded daily throughout the experiment using a drum-type gas meter (Model DM3A, Alexander-Wright, London, UK). A volume of 20 mL gas was sampled from the septum of each collection bag daily using a 20-mL syringe and injected and compressed into evacuated 6.8-mL exetainers (Labco Ltd., Wycombe, UK). Methane concentration was determined using a Varian 4900 gas chromatograph equipped with GS-Carbon PLOT 30 m × 0.32 mm × 3 μm column and thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada) at an isothermal oven temperature of 35 °C with helium as the carrier gas (27 cm/s).

The pH of the fluid from each fermenter was recorded (Orion model 260A, Fisher Scientific, Ottawa, Ontario, Canada) daily (days 1–15) at the time of feed bag exchange. To determine VFA concentration in fermenter effluent, subsamples (5 mL) were collected directly from the effluent flasks at the time of feed bag exchange. To determine VFA concentration in fermenter effluent, subsamples (5 mL) were collected directly from the effluent flasks at the time of feed bag exchange. Samples were placed in screw-cap vials, preserved with 1 mL of 25% (w/w) metaphosphoric acid, and immediately frozen at −20 °C until analyzed. At the same time, subsamples (5 mL) of effluent were also collected, placed in screw-cap vials, and preserved with 1 mL of H₂SO₄ (1%, vol/vol) for the determination of NH₃-N. The concentrations of VFA and NH₃-N (mmol/L) were multiplied by daily effluent production (L/d) to estimate VFA (mmol/day) and NH₃ (mg/day) production. The tungstic acid and
trichloroacetic acid soluble N were determined as described by Winter et al. (1964). Results were used to calculate (mg/100 mL) large peptide N (= trichloroacetic acid N – tungstic acid N) and small peptide plus amino acids N (= tungstic acid N – NH3-N) as described by Licitra et al. (1996).

**Microbial Protein Synthesis**

Microbial protein synthesis in the fermenters was estimated using 15N. On day 8, 0.3 g/L (NH4)2SO4 in McDougall’s buffer was replaced with 0.3 g/L 15N-enriched (NH4)2SO4 (Sigma Chemical Co., St. Louis, MO; minimum 15N enrichment 1 g/L) until the end of the experiment. On days 13–15, daily effluent samples were preserved with 3 mL of a sodium azide solution (20%; wt/vol) and 40 mL were subsampled for the isolation of liquid-associated bacteria.

To determine 15N concentration, effluent liquid samples were centrifuged (20,000 × g, 30 min, 4 °C) and the resulting pellets were washed using phosphate buffer and centrifuged three times (20,000 × g, 30 min, 4 °C) to analyze liquid-associated bacteria (LAB). The pellets were then resuspended in distilled water and frozen at −20 °C until lyophilized. The feed-particle associated (FPA) microbes were suspended in 1.4 mL of a sodium azide solution (20%; wt/vol) and 40 mL were subsampled for the isolation of liquid-associated bacteria.

The feed-particle associated (FPA) microbes were pelleted for the isolation of liquid-associated bacteria. The pellets were then resuspended in distilled water and frozen at −20 °C until lyophilized. The supernatant was decanted and centrifuged (20,000 × g, 30 min, 4 °C) to isoalte liquid-associated bacteria (LAB). The pellets were then resuspended in distilled water and stored at −20 °C. To obtain feed particle-bound (FPB) bacteria, washed feed residues were dried at 55 °C for 48 h, weighed for DM determination, and, after ball grinding (MM 400; Retsch Inc., Newtown, PA), analyzed for total N and 15N by combustion analysis using a mass spectrometer (NA 1500, Carlo Erba Instruments).

Total effluent microbial N (MN) production (mg/day) was calculated using the N concentration (%) of the microbial pellet multiplied by the microbial weight in the total effluent (mg/day). Microbial weight in the total effluent was calculated by multiplying daily effluent production (milliliters) by the microbial density (milligrams per milliliter) in a 40-mL subsample. Microbial N production from FPA fraction was calculated by multiplying daily effluent production (milligrams per day) was calculated as: 

\[ \text{MN} = \left( \frac{\text{APE in RN}}{\text{APE in MN}} \right) \times \text{RN} \]

where APE in RN = the percentage excess of 15N in the residue N, and APE in FPA microbial pellet was used as the source of APE in MN. Total MN production (milligrams per day) was calculated as the sum of microbial production in the effluent, FPA, FPB of straw residues, and FPB of concentrate residues. The ratio of MN (milligrams) to fermented OM (grams) was considered a measure of the efficiency of microbial protein synthesis (EMPS).

**Real-time PCR** Total DNA was extracted from FPA pellets using a QiaGen QIAamp DNA Stool mini kit (Toronto, ON) according to manufacturer instruction. Approximately, 30 mg of each sample was suspended in 1.4 mL of ASL buffer (Stool lysis buffer; QiaGen Inc.) with 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) and homogenized for 3 min at maximum speed (30/s) using a QiaGen Tissue Lyser II (Qiagen Inc.). The suspension was heated at 95 °C and gently mixed in a thermomixer (Eppendorf-Thermomixer comfort, Eppendorf Ltd, Mississauga, Ontario, Canada) for 5 min prior to processing according to the manufacturer’s protocol. Total DNA was eluted in 200 μL of Buffer AE (Elution buffer; QiaGen Inc.) and quantified using a Quant-iTTM PicoGreen dsDNA Assay Kit (Invitrogen Canada Inc., Burlington, Ontario, Canada) with a NanoDrop 3300 fluorometer (Thermo Scientific, Wilmington, DE) and a Nanodrop One C (Thermo Scientific). Real-time PCR was used to quantify total bacteria (16S rRNA) and total methanogens (mcrA), as well as 16S rRNA sequences specific to Fibrobacter succinogenes and Selenomonas ruminantium. Primers and PCR conditions are the same as those reported by Saleem et al., (2019).

**Statistical Analysis**

Data were analyzed using the MIXED procedure of SAS (SAS Inc., Cary, NC). The model included the fixed effects of treatments, day and treatment × day interaction with the day of sampling from each fermenter treated as a repeated measure and individual fermenter as the experimental unit. The minimum values of Akaike’s information criterion were used to select the covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured, and banded for each parameter. The effect of day and its interactions were removed from the model when day was not significant and samples were combined for analysis. Orthogonal polynomial contrasts were performed to determine if replacing feed-grade urea
with increasing levels (0%, 0.5%, 1%, and 1.75%) of SRU resulted in a linear or quadratic effect on measured parameters. Real-time PCR of gene copy number was log-transformed to obtain normality and analyzed as described above. Significance was declared at \( P \leq 0.05 \), and a trend was discussed when \( 0.05 \leq P \leq 0.10 \).

RESULTS

The inclusion of SRU did not affect (\( P > 0.10 \)) pH, total gas, or methane production (Table 2). A quadratic effect was observed on total VFA (Q, \( P = 0.03 \)) with a linear increase in the daily production of acetate (L, \( P = 0.01 \)). Inclusion of SRU led to linear reduction in butyrate (L, \( P = 0.05 \)) and linear increase in isovalerate (L, \( P = 0.05 \)). The ratio of acetate to propionate was linearly increased (L, \( P = 0.049 \)) with increasing SRU. The daily production of propionate, isobutyrate, valerate, and caproate was not affected (\( P > 0.10 \)). The daily production of ammonia responded quadratically (Q, \( P = 0.034 \)), whereas large peptide and small peptide plus amino acids were not affected by the level of SRU.

SRU had no effect (\( P > 0.10 \)) on DM digestibility (DMD), OM digestibility (OMD), or CP digestibility (CPD; Table 3). A quadratic effect was observed for NDF digestibility (NDFD; Q, \( P = 0.017 \)) and ADF digestibility (ADFD; Q, \( P = 0.015 \)) to SRU, with 1% SRU exhibiting higher values as compared to the control (NDFD: 401.86 vs. 333.43 g/kg DM; ADFD: 337.06 vs. 272.73 g/kg DM). Total MN, FPB, and LAB were not affected by SRU (\( P > 0.10 \)), while 1% SRU resulted in the highest FPA as compared to other treatments (Q, \( P = 0.037 \)). The EMPS fluctuated in response to increasing levels of SRU (L, \( P = 0.0027 \); Q, \( P = 0.001 \)) and 1% SRU. SRU had no effect on absolute quantities or relative quantities of copy numbers associated with bacteria, methanogens, \textit{F. succinogenes}, or \textit{S. ruminantium} (Table 4).

DISCUSSION

Gas production is a reflection of OM degradation in in vitro systems (Cone and van Gelder, 1999) a parameter that was not affected by the inclusion of SRU in the current experiment. A previous study that used polyurethane-coated urea as a source of N in a dual-flow continuous culture system also found no effect on pH in comparison with feed-grade urea (Xin et al., 2010). Higher production of TVFA in SRU receiving treatment suggest an increase in substrate fermentation, even though this was not supported by more gas production. Also, increased TVFA was not supported by higher DMD.

Table 2. Effect of different inclusion levels of SRU on ruminal fermentation, methane production, and nitrogen fractions in a Rusitec fed a finishing beef diet

| Item                      | Inclusion levels of SRU, % | \( P \) value |
|---------------------------|---------------------------|---------------|
| Item                      | 0                      | 0.5          | 1          | 1.75         | SEM          | Linear | Quadratic |
| pH                        | 6.25                    | 6.21         | 6.24       | 6.26         | 0.007        | 0.64   | 0.66       | 0.46       |
| Gas, L/d                  | 1.59                    | 1.54         | 1.68       | 1.63         | 0.032        | 0.92   | 0.7        | 0.89       |
| Gas, mL/g DM fermented    | 227.56                  | 226.59       | 245.02     | 229.54       | 5.214        | 0.181  | 0.14       | 0.24       |
| \( \text{CH}_4 \), mL/d   | 32.1                    | 25.4         | 32.2       | 30.9         | 6.043        | 0.24   | 0.68       | 0.48       |
| \( \text{H}_2 \), mL/d    | 7.5                     | 7.3          | 8.6        | 8.2          | 1.191        | 0.72   | 0.61       | 0.34       |
| \( \text{CO}_2 \), mL/d   | 549.1                   | 622.1        | 515.8      | 517.1        | 82.21        | 0.58   | 0.62       | 0.80       |
| Total VFA, mmol/d         | 41.62                   | 45.49        | 46.85      | 44.33        | 0.614        | 0.07   | 0.22       | 0.03       |
| Acetate, mmol/d           | 17.75\textsuperscript{a} | 19.49\textsuperscript{b} | 21.01\textsuperscript{b} | 20.88\textsuperscript{b} | 0.675        | 0.02   | 0.01       | 0.11       |
| Propionate, mmol/d        | 12.04                   | 11.99        | 11.26      | 10.61        | 0.721        | 0.47   | 0.11       | 0.82       |
| Butyrate, mmol/d          | 12.01                   | 11.72        | 11.01      | 10.44        | 0.594        | 0.12   | 0.05       | 0.92       |
| Isobutyrate, mmol/d       | 0.21                    | 0.22         | 0.24       | 0.22         | 0.009        | 0.24   | 0.52       | 0.15       |
| Valerate, mmol/d          | 3.32                    | 3.47         | 3.83       | 3.54         | 0.185        | 0.15   | 0.31       | 0.19       |
| Isovalerate, mmol/d       | 0.87                    | 1.06         | 1.23       | 1.22         | 0.121        | 0.17   | 0.05       | 0.31       |
| Caproate, mmol/d          | 0.48                    | 0.36         | 0.39       | 0.26         | 0.092        | 0.44   | 0.15       | 0.94       |
| A:P\textsuperscript{a}    | 1.51                    | 1.65         | 1.83       | 1.91         | 0.129        | 0.13   | 0.049      | 0.63       |
| Large peptides, mg N/100 mL | 8.15            | 9.77         | 8.98       | 9.52         | 0.783        | 0.41   | 0.39       | 0.57       |
| Small peptides, mg N/100 mL | 9.47             | 8.28         | 8.43       | 11.40        | 1.967        | 0.54   | 0.44       | 0.33       |
| Ammonia, mg/d             | 84.52                   | 106.48       | 102.97     | 100.40       | 4.423        | 0.06   | 0.119      | 0.034      |

\*Acetate to propionate ratio.
\textsuperscript{a}All the acronyms are defined in the text.
\textsuperscript{b}Means with different superscript differ (\( P < 0.05 \)).
or OMD. This discrepancy might be partly due to the higher production of NH$_3$ with SRU. Ammonia neutralizes the fatty acids, which can increase the pH and lower the liberation of CO$_2$ from buffered rumen fluid and, as a result, decrease gas production (Spanghero et al., 2018). Production of VFA in the rumen mainly depends on the type of substrate fermented, the microbial species involved, and the rumen environment (i.e., pH and redox potential) during fermentation (Griswold et al., 1996). In contrast to our results, some researchers found a lack of effect of SRU on VFA production. In a Rustitec fed a diet composed of 40% steam-flaked corn meal and 58.5% forage and 1.5% of N consisting of either polyurethane-coated urea or feed-grade urea, no difference was observed in total or individual VFAs (Xin et al., 2010). Using SRU (Agri-Nutrients Technology Group, Petersburg, VA) in the diet of beef steers fed 90% corn silage showed no effects on total or individual concentrations of VFAs in the rumen (Taylor-Edwards et al., 2009). However, the SRU structure and rate of release were not clear in this report. Also, Ceconi et al. (2015) reported that the inclusion of two sources of SRU (Optigen II or NitroShure) in the diet of feedlot cattle did not change VFA concentrations. The inclusion of urea and SRU (i.e., Optigen II) every 2 h (using automated feeder) or once daily in the diet of beef steers

### Table 3. Effect of different inclusion levels of SRU on true DM, NDF, ADF, and N disappearance and microbial N production in a Rusitec fed a finishing beef diet†

| Item                     | Inclusion levels of SRU, % | Treatment | Contrasts |
|--------------------------|-----------------------------|-----------|-----------|
| DMD                      | 0, 0.5, 1, 1.75, SEM        | P value   | Linear Quadratic |
| OMD                      | 78.85, 78.01, 75.69, 78.95, 1.391 | 0.35      | 0.89 0.13 |
| NDFD                     | 33.34, 37.69, 40.18, 36.18, 2.624  | 0.06      | 0.21 0.017 |
| ADFD                     | 27.27, 31.71, 33.70, 28.22, 3.004  | 0.09      | 0.76 0.015 |
| CPD                      | 88.91, 88.06, 88.32, 88.57, 1.419  | 0.36      | 0.23 0.62 |

Microbial nitrogen, mg/day

| Item          | Inclusion levels of SRU, % | Treatment | Contrasts |
|---------------|-----------------------------|-----------|-----------|
| FPB           | 9.11, 7.52, 10.48, 7.81, 1.044  | 0.56      | 0.74 0.48 |
| FPA           | 11.58a, 10.91b, 14.18a, 10.52a, 0.751  | 0.02      | 0.76 0.037 |
| LAB           | 55.42, 52.09, 51.27, 50.90, 4.578  | 0.41      | 0.51 0.71 |
| Total         | 76.11, 70.53, 78.85, 69.23, 4.316  | 0.38      | 0.47 0.57 |

### Table 4. Effect of different inclusion levels of SRU on absolute and relative quantities of rumen microbes in a Rusitec fed a finishing beef diet‡

| Item                     | Inclusion levels of SRU, % | Treatment | Contrasts |
|--------------------------|-----------------------------|-----------|-----------|
| Absolute quantities*     |                             |           | Linear Quadratic |
| Total Bacteria 16S rRNA copies, $\times 10^7$ | 7.84, 5.74, 8.76, 4.88, 1.430  | 0.36      | 0.506 0.674 |
| Methanogens, mcrA copies, $\times 10^4$    | 1.58, 1.05, 3.47, 2.24, 0.921  | 0.41      | 0.410 0.792 |
| Fibrobacter succinogenes, 16S rRNA copies, $\times 10^5$ | 1.49, 0.30, 1.20, 0.91, 0.210  | 0.69      | 0.988 0.512 |
| Selenomonas ruminantium, 16S rRNA copies, $\times 10^5$ | 5.12, 2.95, 7.26, 3.39, 1.140  | 0.45      | 0.778 0.669 |
| Relative quantities†     |                             | 0.25      | 0.519 0.941 |
| Methanogens, $\times 10^{-4}$% | 20.1, 19.0, 32.5, 46.7, 9.21  | 0.52      | 0.213 0.833 |
| Fibrobacter succinogenes, % | 0.19, 0.05, 0.14, 0.19, 0.034  | 0.75      | 0.691 0.325 |
| Selenomonas ruminantium, % | 0.25, 0.11, 0.55, 0.31, 0.095  | 0.53      | 0.519 0.941 |

*Absolute quantities are the average quantities of the target before normalization based on 16S rRNA total bacteria target.
†Relative quantities of the target are the average populations calculated as a percentage of the total bacterial 16S rRNA.
‡All the acronyms are defined in the text.
receiving 60% forage diet led to a decrease and an increase in the molar proportion of acetate and propionate, respectively (Alvarez Almora et al., 2012). Increased production of acetate in the present study is likely due to higher activity of fibrolytic bacteria, a possibility given the increase in NDFD and ADFD with 1% SRU. However, we did not see an increase in the copy numbers associated with F. succinogenes. The theory behind using SRU is to provide a steady and concurrent supply of N with the liberation of carbon skeletons within the rumen that can support the synthesis of amino acids by rumen microorganisms. In an in vitro trial which studied hydrolysis rate of urea on ruminal bacterial diversity using acethydroxamic acid as an agent to slow the release rate of urea promoted the growth of total bacteria and F. succinogenes during the early stages of fermentation (Wang et al., 2018). It has been proposed that a slower and steadier supply of N may support slow-growing fibrolytic bacteria (Ceconi et al., 2015), even though no differences were observed in the copy number of F. succinogenes in the current experiment.

Ammonia concentration can influence fiber degradation, but there are disagreements among some authors on what levels are optimal for fiber degradation. Belasco (1954) reported that the maximum digestion of cellulose occurs when ammonia concentration in the rumen is approximately 43 mg/dL. Conversely, some authors suggest that this concentration should be between 19 and 23 mg/dL (Mehrez et al., 1977). As the capacity of microbial protein synthesis and ammonia use relates to the rate and extent of carbohydrate fermentation (Bach et al., 2005), it can be speculated that, for each diet, there is an optimal ammonia concentration. In the current experiment, the maximum concentration of ammonia reached was around 13 mg NH₃-N/dL, which was below the recommended levels. Approximately, 30% of total CP in the current experiment was supplied by urea and SRU. It was expected that the concentration of NH₃-N would be higher in 0% SRU in comparison with other treatments. This discrepancy could be due to the mechanism of urea metabolism in the Rusitec. Most of the urease activity in the rumen is associated with bacteria that adhere to the rumen epithelial (Dehority, 2003). Most of the ureolytic activity is present in microorganisms that are loosely adhered to solid feed particles (Cheng and McAllister, 1997). The presence of urea in this compartment results in higher ureolytic activity and, therefore, higher ammonia concentration, which can flow to microorganisms tightly bound to solid substrates where the ammonia concentration is low as it is under high demand for microbial protein synthesis. (Cheng and McAllister, 1997). With SRU, sustained release of urea into this microenvironment (i.e., incubation bags) during incubation probably led to a more consistent supply of ammonia for microbial activity that increased NDFD and ADFD at optimal concentrations. It was expected that this would be reflected in an increase in the quantity of total bacteria and fibrolytic bacteria in parallel with increasing the amount of FPA, NDFD, and ADFD. In the current study, no statistical differences were observed in microbial growth in response to the increasing level of SRU in the diet. However, the population of total bacteria, methanogens, and S. ruminantium were numerically higher with 1% SRU. In contrast to our results, Cherdthong et al. (2011) found that incubation of SRU as a mixture of urea–calcium with cassava chips or corn meal increased NDFD and also the population of total bacteria and F. succinogenes as compared to feed-grade urea. Even though F. succinogenes plays an important role in ruminal fiber degradation, other members of the fibrolytic bacterial population may have been responsible for the improvement in fiber digestion as fiber digestion requires multispecies bacterial consortia (McAllister et al., 1994).

The diet with 1% SRU exhibited the highest NDFD and ADFD among treatments. The 1% SRU treatment included 0.6% urea as well. It is hypothesized that urea, which is immediately soluble, supported the growth of fiber-degrading bacteria during the early stages of incubation and provided more favorable conditions for microbes to colonize feed particles and carry out fiber digestion. A higher value of FPA in 1% SRU shows the enhancement of microbial colonization, which is an important step in ruminal digestion of plant cell walls (Miron et al., 2001). The quadratic response of total VFAs, NDFD, and ADFD (i.e., the highest value for 1% SRU) suggested that levels of SRU higher than 1% did not further promote plant cell wall digestibility.

The inclusion of 1% SRU also resulted in the highest level of FPA. It has been reported that solid-associated fractions account for 70–80% of the microbial biomass in the rumen (Craig et al., 1987). The microbes associated with rumen fluid are directly involved in the digestion of insoluble feed particles (Latham et al., 1979). However, total microbial protein synthesis did not differ among treatments. Similarly, Galo et al. (2003) reported that feeding dairy cows Optigen 1200 (Controlled Release N; CPG Nutrients, Inc., Syracuse, NY) did not alter microbial protein production. The
EMPS in the current study was slightly lower than the range of 12–24 mg N/g OMD as proposed by NRC (2001). In contrast to our results, Xin et al. (2010) found that the supplementation of diet with polymer-coated urea increased EMPS as compared to feed-grade urea (13.0 vs. 11.3 g N/kg OMD). However, their experimental diet consisted of 58.5% forage as compared to the 10% forage used in our study. Low-forage diets result in a higher population of nonfibrolytic bacteria, which require more peptides and amino acids for optimal growth (Bach et al., 2005). The low EMPS value in the current study might be due to a limited supply of N or lack of available N sources (peptide or amino acids) in the fermenters during incubation (Cherdthong and Wanapat, 2010). This possibility is supported by the low concentration of ammonia in the effluent.

In most in vivo experiments that have used various forms of SRU, no effects on DMD and OMD have been reported (Bourg et al., 2012; Ceconi et al., 2015). The behavior of SRU likely depends on the rate of urea release, amount of inclusion, type of diets, productivity and the nature of the rumen microbiota, and host traits, such as absorption of ruminal NH3 and liquid and solid passage rates.

In conclusion, the results of this study showed that, in spite of lack of effects on DMD and OMD using 1% SRU NDFD and ADFD, TVFA and acetate were increased compared to other treatments. The experimental diet, composed of 90% concentrate ingredients, contained a high amount of readily available carbohydrate and, therefore, necessitate providing N sources (i.e., NPN and true protein, such as peptides and amino acids) for rumen microorganisms. With 1% SRU, the presence of urea as an immediately soluble nitrogen source along with SRU as a steady provider of N could have improved the efficiency of microbial protein synthesis and enhanced populations of FPA bacteria.

Conflict of interest statement. The authors declare that there is no conflict of interest as this study was pursued strictly for academic reasons with no commercial interest.

LITERATURE CITED

Alvarez Almora, E. G., G. B. Huntington, and J. C. Burns. 2012. Effects of supplemental urea sources and feeding frequency on ruminal fermentation, fiber digestion, and nitrogen balance in beef steers. Anim. Feed Sci. Technol. 171:136–145. doi:10.1016/j.anifeedsct.2011.10.012.

Association of Official Analytical Chemists. 2000. Official methods of analysis. 17th ed. Gaithersburg (MD): The Association of Official Analytical Chemists.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9–E21. doi:10.3168/jds.S0022-0302(05)73133–7.

Batajoo, K. K., and R. D. Shaver. 1998. In situ dry matter, crude protein, and starch degradabilities of selected grains and by-product feeds. Anim. Feed Sci. Technol. 71:165–176. doi:10.1016/S0377-8401(97)00132-6.

Belasco, J. I. 1954. New nitrogen feed compounds for ruminants—a laboratory evaluation. J. Anim. Sci. 13:601–610. doi:10.2527/jas1954.133601x.

Bourg, B. M., L. O. Tedeschi, T. A. Wickersham, and J. M. Tricarico. 2012. Effects of a slow-release urea product on performance, carcass characteristics, and nitrogen balance of steers fed steam-flaked corn. J. Anim. Sci. 90:3914–3923. doi:10.2527/jas.2011-4832.

Canadian Council on Animal Care. 2009. Guide to the care and use of farm animals in research, teaching and testing. Ottawa (Canada): CCAC.

Ceconi, I., M. J. Ruiz-Moreno, N. Dilorenzo, A. Dickostanzo, and G. I. Crawford. 2015. Effect of urea inclusion in diets containing corn dried distillers grils on feedlot cattle performance, carcass characteristics, ruminal fermentation, total tract digestibility, and purine derivatives-to-creatinine index. J. Anim. Sci. 93:357–369. doi:10.2527/jas.2014-8214.

Cheng, K.-J., and T. A. McAllister. 1997. Compartmentation in the rumen. In: Hobson, P. N. and C. S. Stewart, editors. The rumen microbial ecosystem. Dordrecht (The Netherlands): Springer; p. 492–522 – [accessed March 28, 2019]. Available from: https://doi.org/10.1007/978-94-009-1453-7_12.

Cherdthong, A., and M. Wanapat. 2010. Development of urea products as rumen slow-release feed for ruminant production: a review. Aust. J. Basic Appl. Sci. 4:2232–2241.

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

Cone, J. W, and A. H. van Gelder. 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76:251–264. doi:10.1016/S0377-8401(98)00222-3.

Craig, W. M., G. A. Broderick, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J. Nutr. 117:56–62. doi:10.1093/jn/117.1.56.

Dehority, B. A. 2003. Rumen microbiology. Nottingham (UK): Nottingham University Press.

Galo, E., S. M. Emanuele, C. J. Sniffen, J. H. White, and J. R. Knapp. 2003. Effects of a polymer-coated urea product on nitrogen metabolism in lactating Holstein dairy cattle. J. Dairy Sci. 86:2154–2162. doi:10.3168/jds.S0022-0302(03)73805-3.

Griswold, K. E., W. H. Hoover, T. K. Miller, and W. V. Thayne. 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. J. Anim. Sci. 74:483–491. doi:10.2527/1996.742483x.

Holder, V. B. 2012. The effects of slow release urea on nitrogen metabolism in cattle – [accessed June 19, 2019]. Available from: https://uknowledge.uky.edu/animalsci_etds/6/.

Latham, M. J., D. G. Hobbs, and P. J. Harris. 1979. Adsorption of rumen bacteria to alkali-treated plant stems. Ann. Rech. Vet. 10:244–245.
Slow-release urea in the Rusitec

Licitra, G., T. M. Hernandez, and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57:347–358. doi:10.1016/0377-8401(95)00837-3.

López-Soto, M. A., C. R. Rivera-Méndez, J. A. Aguilar-Hernández, A. Barreras, J. F. Calderón-Cortés, A. Plascencia, H. Dávila-Ramos, A. Estrada-Angulo, and Y. S. Valdes-Garcia. 2014. Effects of combining feed grade urea and a slow-release urea product on characteristics of digestion, microbial protein synthesis and digestible energy in steers fed diets with different starch:ADF ratios. Asian-Australas. J. Anim. Sci. 27:187–193. doi:10.5713/ajas.2013.13395.

Mahmoudi-Abyane, M., D. Alipour, and H. R. Moghimi. 2017. Effect of using different sources of nitrogen on digestibility and nitrogen balance in Mehraban male lambs. Anim. Prod. Res. 6:27–38. doi:10.22124/AR.2018.7628.1219.

McAllister, T. A., H. D. Bae, G. A. Jones, and K. J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72:3004–3018. doi:10.2527/1994.72113004x.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep’s saliva. Biochem. J. 43:99–109. [accessed June 19, 2019]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16748377.

McAllister, T. A., H. D. Bae, and G. A. Jones. 1994. Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72:3004–3018. doi:10.2527/1994.72113004x.

NRC. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. Washington (DC): The National Academies Press – [accessed June 19, 2018]. Available from: https://www.nap.edu/catalog/9825/nutrient-requirements-of-dairy-cattle-seventh-revised-edition-2001.

Saleem, A. M., G. O. Ribeiro, H. Sanderson, D. Alipour, T. Brand, M. Hünerberg, W. Z. Yang, L. V. Santos, and T. A. McAllister. 2019. Effect of exogenous fibrolytic enzymes and ammonia fiber expansion on the fermentation of wheat straw in an artificial rumen system (RUSITEC). J. Anim. Sci. 97:3535–3549. doi:10.1093/jas/skz224.

Sinclair, L. A., C. W. Blake, P. Griffin, and G. H. Jones. 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. doi:10.1017/S1751731111002485.

Sniffen, C. J., J. D. O’Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70:3562–3577. doi:10.2527/1992.70113562x.

Spanghero, M., A. Nikulina, and F. Mason. 2018. Use of an in vitro gas production procedure to evaluate rumen slow-release urea products. Anim. Feed Sci. Technol. 237:19–26. doi:10.1016/j.anifeedsci.2017.12.017.

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

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd ed. New York (NY): Cornell University Press.

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

Wang, P., S. Zhao, X. Nan, D. Jin, and J. Wang. 2018. Influence of hydrolysis rate of urea on ruminal bacterial diversity level and cellulolytic bacteria abundance in vitro. PeerJ. 6:e5475. doi:10.7717/peerj.5475.

Winter, K. A., R. R. Johnson, and B. A. Dehority. 1964. Metabolism of urea nitrogen by mixed cultures of rumen bacteria grown on cellulose. J. Dairy Sci. 47:793–797. doi:10.3168/jds.S0022-0302(64)88766-X.

Xin, H. S., D. M. Schaefer, Q. P. Liu, D. E. Axe, and X. Q. Meng. 2010. Effects of polyurethane coated urea supplement on in vitro ruminal fermentation, ammonia release dynamics and lactating performance of holstein dairy cows fed a steam-flaked corn-based diet. Asian-Australas. J. Anim. Sci. 23:491–500. doi:10.5713/ajas.2010.90153.

Translate basic science to industry innovation