ACS Sustainable Resource Management

pubs.acs.org/acssrm

Article

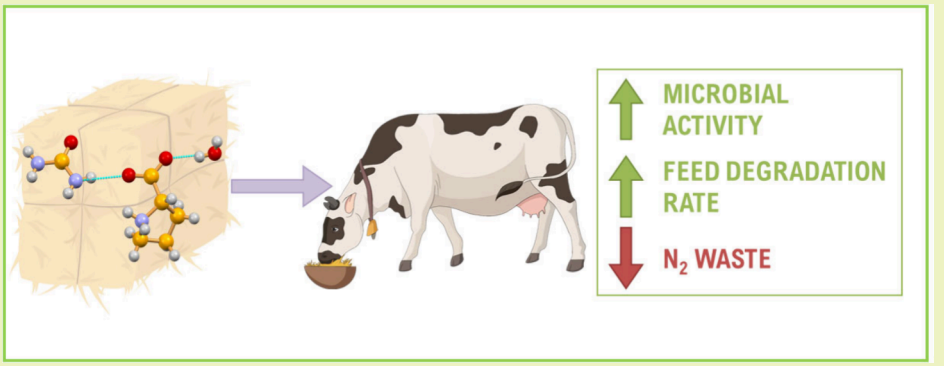
Mechanochemical Synthesis of a Urea•Proline Cocrystal as Dairy Cattle Feed and Its Effect on Ruminal Degradation

Laura Contini, Diptajyoti Gogoi, Ana Carolina Fluck, Júlia Gomes Farias, Clinton Williams, Ranijt Thakuria,* Jonas Baltrusaitis,* and Fabrizia Grepioni*

Cite This: *ACS Sustainable Resour. Manage.* 2025, 2, 1672−1680

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



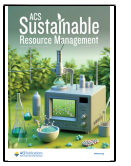
ABSTRACT: Global nitrogen cycle management is critical due to its central role in food production. Engineering new, sustainable technologies and products that facilitate all facets of nitrogen activation, uptake, and transport is thus critical in reducing the amount of accumulation of activated nitrogen species that contribute to negative environmental impacts. In particular, developing next-generation cattle feed additives or using alternative protein sources can lead to reduced greenhouse gas emissions. In this work, sustainable and scalable mechanochemical methods were utilized for the synthesis of urea cocrystals with the amino acids DL- and L-proline resulting in the formation of the new cocrystals urea•L-proline•H_2O, urea$_2$•L-proline, and urea•DL-proline•H_2O, which were fully characterized by X-ray diffraction and thermal analysis. The obtained highly soluble hydrated L-proline cocrystal was utilized as an N supplement to enhance the nutritional value of dairy cattle feed, contributing to minimizing environmental contamination by reducing microbial degradation during ruminant digestion. The results presented in this work show that crystal engineering using sustainable mechanochemical methods can be applied to develop new formulations used in cattle supplements to optimize nitrogen use efficiency.

KEYWORDS: *Urea•Proline Cocrystal, cattle feed supplement, mechanochemistry, microbial degradation*

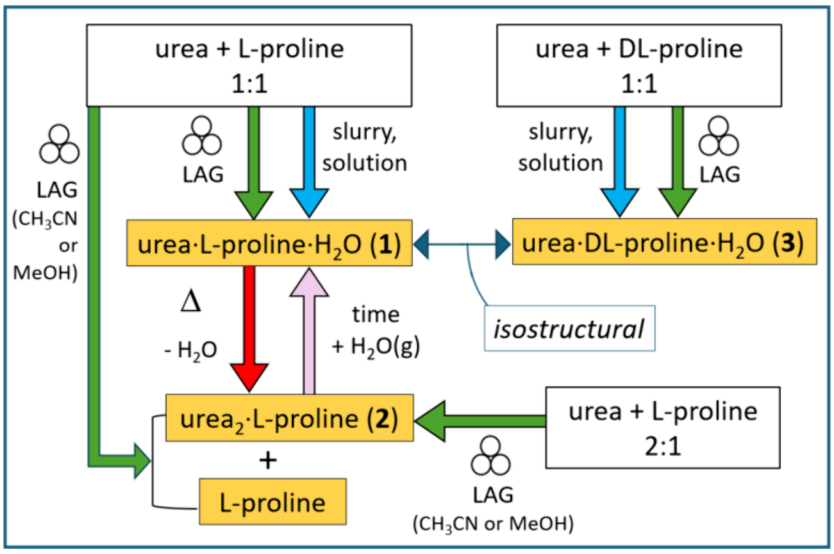
INTRODUCTION

Nitrogen (N) is a critical nutrient since it is a key component of proteins which make up animal muscles, plants, and other ultimate products, such as milk. Proteins are vital nitrogen containing nutrients that help ruminants digest fiber. The relationship between dietary protein intake and milk production in dairy cattle is well-documented, with increased protein consumption shown to enhance milk yield.[1] However, overfeeding cows with nitrogen poses significant economic and environmental challenges. Excess nitrogen from dietary protein leads to higher nitrogen losses in the form of ammonia emissions and leaching, contributing to environmental pollution.[1−6] A mathematical model by Kohn *et al.* showed a strong correlation between ruminant N utilization and N losses, highlighting the impact of optimizing cattle management practices on reducing N pollution.[7] Efforts to optimize N intake include rational formulation and precise N feeding according to animal requirements, as well as enhancing microbial protein synthesis in the rumen.[8] Microbial protein synthesis plays a pivotal role in capturing recycled nitrogen and utilizing the end products of protein breakdown in the rumen, thereby improving the efficiency of absorbed amino acid utilization.[9,10] Numerous studies have shown that a way to stimulate ruminal microbiota growth rates and microbial protein synthesis is through amino acids and peptide intake.[11−13]

Received: March 6, 2025
Revised: August 5, 2025
Accepted: August 6, 2025
Published: August 22, 2025



Scheme 1. Solution, Slurry, and LAG Syntheses of the Cocrystals Described in This Work[a]



[a]Solvent is water unless explicitly stated. Neat grinding, though explored, never resulted in quantitative reactions.

Wu defines essential amino acids, including proline, as critical components involved in key metabolic pathways responsible for synthesizing various physiological substances, such as hormones.[14] Additionally, these amino acids enhance nutrient utilization efficiency, growth rates, and overall production performance in ruminants. This is achieved by strategically manipulating dietary amino acid profiles, allowing for the reformulation of dietary requirements tailored to specific species, categories, and physiological stages of these animals. McCoard and Pacheco further emphasize that dietary supplementation with amino acids directly enhances endogenous synthesis, which, in turn, supports the survival, growth, development, reproduction, and overall health of ruminants.[15] Notably, the significance of amino acids in dietary manipulation remains pronounced, even in the absence of limiting factors such as ammonia and carbohydrates. Surprisingly, their role is crucial even when ammonia and carbohydrates are not limiting factors. Among amino acids, L-proline is particularly promising, due to its critical role in dairy milk protein synthesis and mammary gland function, with studies suggesting that its use as a supplement may reduce the need for other amino acids, such as arginine.[14,16] Research on lactating goats and Israeli-Friesian cows has demonstrated that proline supplementation can improve milk fat content and yield, highlighting its potential as a valuable feed additive.[17] Additionally, L-proline plays a role in early pregnancy by promoting myogenic differentiation, so its use as a supplement during pregnancy could benefit milk production and animal health during critical physiological stages.[18,19] The costs associated with animal feeding are substantial, particularly for ruminants, where these expenses can be significantly influenced by the efficiency of substrate utilization by the ruminal microbiota.[20] This efficiency underscores the importance of effective digestion, which relies on the availability of essential nutrients, including nitrogen, energy, and minerals. Undigested feed and microbial biomass produced in the rumen continue through the digestive tract for further digestion in the abomasum and small intestine, facilitated by enzymatic and additional microbial processes in the large intestine. The effective utilization of this biomass in ruminants is strongly contingent upon the rates of forage digestion in the rumen, as microbial activity plays a crucial role in this digestive process.[21]

Cocrystals, defined as multicomponent solids containing two or more molecular or ionic compounds that are solid at ambient conditions as pure forms, and are present in stoichiometric ratio, have been the subject of intense investigation and utilization in these last decades.[22−28] Cocrystals possess different and improved physicochemical properties with respect to the separate components (color, thermal properties, solubility, morphology, and so on), and for this reason, they can have huge potential applications in the pharmaceutical, agrochemical and materials fields.[29] Cocrystals containing urea have been explored in the last few years as sustained release nitrogen fertilizers.[30−34] Urea can serve as coformer for cocrystal formation as it is also used as a non-protein source of nitrogen (NPN) in ruminant nutrition; no reports on urea•proline multi-component solids in the Cambridge Structural Database (CSD) are present up to date.[35,36] Moreover, the functional groups present in both the compounds made urea-proline multi-component system a suitable candidate for co-crystallization based on complementary hydrogen bonding interactions. The use of urea in ruminant feed is convenient as it does not result in an increased environmental nitrogen load compared to dietary proteins.[37,38] Additionally, its high solubility in water makes it a good coformer for the cocrystallization with L-proline. By mantaining the solubility of L-proline while providing a digestible nitrogen source, this novel supplement has the potential to improve microbial protein synthesis, optimize feed utilization, and support sustainable milk production practices. In order to explore the crystal structure landscape, DL-proline was also considered as coformer during liquid-assisted grinding (LAG) as well as slurry crystallization.

To the best of our knowledge, this is the first report on amino-acid cocrystals with urea with full crystal structure determination that has been investigated as an alternative dietary supplement for rumen. Here, in this study we have successfully synthesized three novel cocrystals of urea with proline viz. urea•L-proline•H_2O (**1**), $urea_2$•L-proline (**2**) and urea•DL-proline•H_2O (**3**) using mechanochemical and sol-

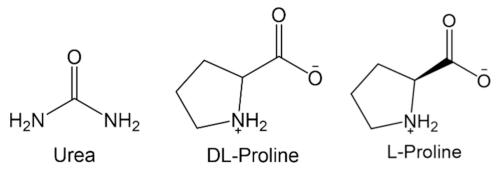
https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680

ution methods and investigated their stability as well as the effect of cocrystal formation on rumen feed and nitrogen degradation in *in vitro* ruminal digestibility technique.

MATERIALS AND METHODS

Instruments and Chemicals. The chemical route for the preparation of the target cocrystals is reported in Scheme 1. The reagents (See Chart 1) and solvents employed for the synthesis of proline cocrystals were purchased from Sigma-Aldrich and used without further purification. Corn silage for *in vitro* ruminal digestibility was provided by Silo Forte®, Venâncio Aires, Brazil.

Chart 1. Reagents Used in This Work[a]



[a]Both L- and DL-proline are in their zwitterionic form.

Synthesis via Slurry of Urea•L-Proline•H_2O (1) and Urea•DL-Proline•H_2O (3). Urea (120 mg; 2 mmol) and L-proline (230 mg; 2 mmol) were added to a vial containing 0.5 mL of solvent (either water or acetonitrile). The slurry of urea (120 mg; 2 mmol) with DL-proline (230 mg; 2 mmol) was conducted in methanol. The homogeneous suspension thus obtained was kept stirring for 1 day. The products obtained were filtered, dried at room temperature and characterized via powder X-ray diffraction.

Synthesis via Liquid Assisted Grinding (LAG) of Urea•L-Proline•H_2O (1), Uurea$_2$•L-Proline (2), and Urea•DL-Proline•H_2O (3). For the LAG synthesis of (**1**) and (**3**) urea (120 mg; 2 mmol) and L or DL-proline (230 mg; 2 mmol) were ground in a 5 mL stainless steel (SS) jar in the presence of 80 μL of water and two SS balls with a 7 mm diameter and an average mass of 1.39(2) g. To perform the synthesis a Retsch MM400 ball miller was employed, operating at the frequency of 25 Hz for 45 min. The product, a viscous liquid, was left to dry at room temperature and characterized, without further purification, via powder X-ray diffraction. The same mechanochemical conditions were employed for the preparation of (**2**); in this case a 2:1 stoichiometric ratio of urea and L-proline was used, and water was replaced by either acetonitrile or methanol. The product, a wet white powder, was left to dry at room temperature and characterized, without further purification, via powder X-ray diffraction.

Synthesis from Solution of Urea•L-Proline•H_2O (1) and Urea•DL-Proline•H_2O (3). *Urea and L-Proline.* With the aim of obtaining single crystals of the two crystal forms obtained via slurry and LAG, 50 mg of the two compounds in powder forms were placed in a vial dissolved in water and allowed to recrystallize at room temperature. All recrystallization attempts invariably yielded single crystals of the form later characterized as urea•L-proline•H_2O (**1**), irrespective of the stoichiometric ratio of the reagents.

Urea and DL-Proline. Both recrystallization of the LAG product from water and the slow drying of the viscous liquid obtained via LAG yielded single crystals of urea•DL-proline•H_2O (**3**).

Powder X-ray Diffraction (PXRD) at Room and Variable Temperature. X-ray diffraction patterns were collected in the 3°−40° 2θ range in Bragg−Brentano geometry on a PANalytical X'Pert Pro automated diffractometer equipped with an X'celerator detector, using Cu−Kα radiation (λ = 1.5418 Å) without monochromator (continuous scan mode, step size 0.033°; time/step: 20 s; Soller slit 0.04 rad, antiscatter slit: 1/2, divergence slit: 1/4; 40 mA•40 kV). For variable temperature measurements, an Anton Paar TTK 450 system was also employed.

For the structural solution of **2**, powder XRD patterns were collected, in the 5-60° 2θ range, on a Panalytical X'Pert PRO automated diffractometer, equipped with an X'celerator detector, using Cu−Kα radiation (λ = 1.5418 Å) without monochromator (continuous scan mode; step size 0.0114°; counting time 200.025 s; Soller slits 0.02; antiscatter slit 1/4; divergence slit 1/8; 40 mA × 40 kV). Four diffraction patterns were recorded and summed, to enhance the signal-to-noise ratio. For structural solution powder diffraction data was analyzed with the software EXPO2014,[39] which is designed to analyze both monochromatic and non-monochromatic data. Selected peaks were chosen in the 5°−50° 2θ range, and unit cell parameters were found using the algorithm N-TREOR09.[40] Structural solution from powder data was then carried out via simulated annealing, using molecular fragments retrieved from the structure of **1**, solved previously via single crystal X-ray diffraction (SCXRD). Twenty runs for simulated annealing trials were set, and a cooling rate (defined as the ratio T_n/T_{n-1}) of 0.95 was used. The best solution was chosen for a preliminary Rietveld refinement, which was performed using the software EXPO2014. A Chebyshev function with 20 parameters was used to fit the background. Geometry optimization of the refined structure was carried out using the Dmol3 module[41,42] in Materials Studio using the Perdew−Burke−Ernzerhof generalized gradient approximation exchange-correlation density functional and Density functional Semicore Pseudo Potentials, with atomic orbital basis set-DNP.[43] Optimizations were considered complete when energies converged to values lower than 1 × 10^{-5} Ha per atom, atomic displacements to 5 × 10^{-3} Å, and maximum forces to 2 × 10^{-2} Ha $Å^{-1}$. The lattice parameters were kept fixed during the minimizations. A final Rietveld refinement was then carried out on the optimized structure using the software EXPO2014 (see Table SI-1 and Figure SI-5). Crystalline powders resulting from the LAG experiments were used, after grinding, for all the VT-PXRD measurements.

Single Crystal X-ray Diffraction (SCXRD). X-ray data for **1** and **3** (see Table SI-1) were collected at room temperature on an Oxford Diffraction X'Calibur diffractometer, equipped with a CCD detector, using MoKα radiation (λ = 0.71073 Å) and monochromator graphite. The structure was solved via Olex2[44] with the SHELXT[45] structure solution program, using Intrinsic Phasing, and refined with the SHELXL[46] refinement package via Least Squares minimization. One of the two independent L-proline molecules in both **1** and **3** is affected by ca. 50:50 positional disorder (see SI). Hydrogen atoms were added in calculated positions. Anisotropic displacement parameters were refined for all non-hydrogen atoms. Extensive use was made of the Program Mercury[47] for the analysis of the crystal packings. CCDC 2422986-2422988 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry (DSC) traces were recorded using a TA Instruments Q10 apparatus fitted with a standard DSC cell, equipped with a Discovery Refrigerated Cooling System (RCS90, TA Instruments), under a nitrogen atmosphere (purge flow: 20 mL min^{-1}). The samples (3 − 8 mg range) were placed in aluminum pans. Crystalline powders, as obtained from LAG experiments, were used as such for all DSC measurements. All samples were cooled down to 0 °C, then they were heated up to 130 °C. All DSC traces are reported in the Supporting Information.

Thermogravimetric Analysis (TGA). TGA measurements were performed with a PerkinElmer TGA8000 in the 30−500 °C temperature range, under N_2 gas flow at a heating rate of 10 °C min^{-1}. TGA traces are reported in the Supporting Information.

Hot-Stage Microscopy (HSM). HSM analyses were performed using an OLYMPUS BX41 microscope equipped with a VISICAM 5.0, a NIKON DS FI3 camera and a Linkam TMS-94 stage used for temperature control. Images were taken under polarized light to highlight modifications in the single crystals due to solid state transformations.

Chemical Characterization and *in Vitro* Digestibility Experiments. *Corn Silage for Digestibility Experiments.* Corn silage, supplied by Silo Forte®, Venâncio Aires, Brazil, was initially dried at 55 °C for 48 h, then ground. Treatments were applied at 1% of the

https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680

Table 1. Chemical Composition of Corn Silage Supplemented with Different Nitrogen Sources[a]

Treatment	N	SP	CP	SP_CP	ADF	NDF	Ash	OM
Corn Silage	1.39 ± 0.01	4.47 ± 0.12	9.03 ± 0.2	50.77 ± 0.6	25.8 ± 0.2	44.3 ± 0.4	5.5 ± 0.2	94.5 ± 0.2
Corn Silage + Urea	1.91 ±0.05	8.27 ± 0.31	12.40 ± 0.3	66.80 ± 1.0	25.5 ± 0.3	44.3 ± 0.2	5.0 ± 0.4	95.0 ± 0.4
Corn Silage + L-Proline	1.51 ±0.02	5.70 ± 0.00	9.80 ± 0.1	58.03 ±0.1	25.6 ± 0.1	44.5 ± 0.1	4.3 ± 0.3	95.7 ± 0.3
Corn Silage + Urea-L-	1.54 ±0.01	5.57 ± 0.12	10.01 ± 0.1	57.57 ±0.7	25.9 ± 0.4	44.2 ± 0.2	4.2 ± 1.0	95.8 ± 1.0

[a]Values are reported as percentage of dry matter, means ± St. Dev. calculated for three replicates. N: nitrogen; SP: Soluble Protein; CP: Crude Protein; SP_CP: Soluble Crude Protein; ADF: Acid Detergent Fiber, NDF: Neutral Detergent Fiber; OM: Organic Matter Nitrogen values (%) and crude protein of the chemicals used were respectively: urea (46.67; 303.4), L-proline (12.16; 79), and Urea•L-Proline•H_2O (21.75; 141.4).

dry weight. Four treatments were tested, each in triplicate: (a) untreated corn silage, (b) corn silage + urea, (c) corn silage + L-proline, and (d) corn silage + **1** (urea•L-proline•H_2O cocrystal). The results of the measured chemical composition of corn silage and *in vitro* treatments are reported in Table 1.

Chemical Characterization of Treated Corn Silage. Chemical characterization of corn silage, pure and supplemented with various sources of NPN, was performed as follows: dry matter (DM) was measured with a two-step process. First, a partial dry matter content was determined using an adapted method from Goering *et al.*[48] Next, samples were dried at 105 °C for 3 h, following the guidelines provided by the National Forage Testing Association.[49] Ash content (**Ash**) was measured according to AOAC method 942.05 (1998),[50] with modifications: 1.5 g of sample, a 4-hour ash period, and hot weighing of residues. Organic matter (**OM**) was estimated using the equation *100 - Ash*. Crude protein (**CP**) was quantified using the AOC method 978.10 (2000). Soluble protein (**SP**) was measured using the borate-phosphate procedure as described by Krishnamoorthy et al. (1982).[51] Neutral Detergent Fiber (**NDF**) and Acid Detergent Fiber (**ADF**) contents were determined following Van Soest et al. (1991),[52] with adjustments by Senger et al. (2008).[53] The protocol used 16 μm polyester bags and an autoclave at 110 °C for 40 min. Acid Detergent Lignin (**ADL**) was determined by treating ADF residue with 12 M sulfuric acid, following AOAC method 973.18 (1998).[54]

***In Vitro* Digestibility.** For the *in vitro* digestibility, 0.5 g samples from each treatment were prepared and incubated over three nonconsecutive days. Samples were incubated in rumen fluid collected from lactating dairy cows, combined with Van Soest buffer under continuous CO_2 flow, being the ratio ruminal fluid:buffer solution 1:4. Each sample was placed in a 250 ml Erlenmeyer flask in a stationary water bath. Incubation times were set at 6, 12, 24, and 48 h. At each time point, samples were removed from the water bath and chilled to stop fermentation. The contents were then filtered through 9 cm diameter, 2.4-micron glass fiber filters (Millipore) to separate residual material. Filters were subsequently dried at 55 °C for 18 h and weighed to determine dry matter loss.

Digestibility Result Processing and Statistical Analysis. The extent of rumen degradation for each sample was calculated at each incubation time to estimate the degradation rate. A nonlinear Gompertz model with discrete latency was applied to the data using the Nlin procedure.[55] The Gompertz model,[56] represented by the following eq 1, was used to model the degradation profile

$$V_t = V_f \exp\{-\exp[1 - 1 + k_e(\lambda - t)]\} + \epsilon \quad (1)$$

where: V_t represents the response variable (gas volume) as a function of time (t); V_f denotes the asymptotic gas volume reached for a single pool substrate; λ is the discrete lag time of Gompertz's growth model; and k_e is the fractional rate constant representing the cumulative degradation rate of dry matter. Each degradation measurement was treated as an independent experimental unit due to distinct and isolated incubation environments.

A completely randomized design was adopted, using the mixed model methodology. To identify the best-fit distribution and adjustment of the model, the Glimmix procedure[55] was used, selecting based on Akaike's Information Criterion (AICc).

RESULTS AND DISCUSSION

Synthesis and Characterization of Urea•L-Proline•H_2O (1) and Urea$_2$•L-Proline (2). The urea•L-proline•H_2O (**1**) cocrystal was prepared from liquid-assisted grinding or slurry with water, starting from a 1:1 stoichiometric ratio of the reactants. Recrystallization from either methanol or water solution afforded single crystals suitable for X-ray diffraction. The structural identity between the product of the solid-state synthesis and the product of the recrystallization was verified by comparing the powder X-ray diffraction (PXRD) pattern, calculated on the basis of the single-crystal structure, and the experimental pattern measured for the crystalline powder (see Figure SI-1). The crystal can be described as a layered structure (Figure 1a) formed by infinite hydrogen-bonded chains of proline molecules connected via hydrogen bonds to a

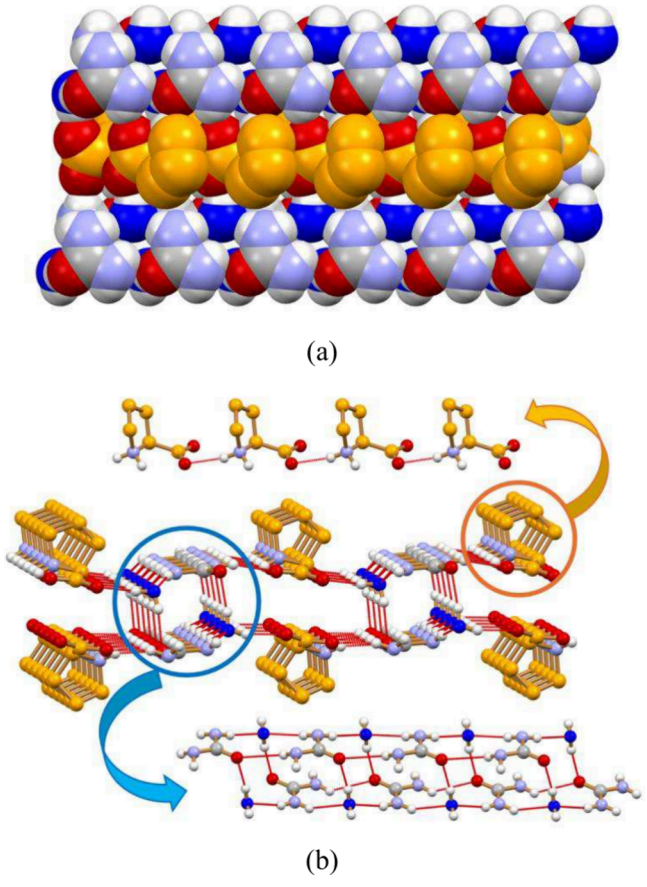


Figure 1. (a) Layered structure of crystalline Urea•L-proline•H_2O (3). (b) Hydrogen bonding network between layers of urea and L-proline. Urea C atoms in grey, L-proline C atoms in orange, O_{water} in blue; H_{CH} atoms are omitted for clarity.

https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680

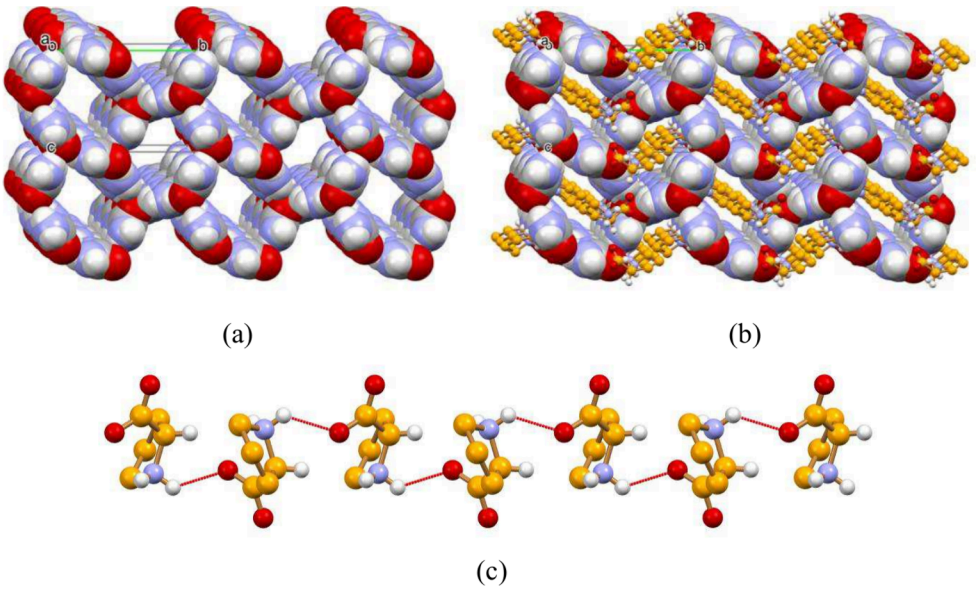


Figure 2. (a-b) Hydrogen bonding framework of urea molecules in crystalline urea$_2$•L-proline (**2**). (c) infinite threads of L-proline, interspersed in the urea framework. Urea C atoms in grey, L-proline C atoms in orange; H_{CH} atoms omitted for clarity.

further hydrogen-bonding pipe-like arrangement of urea and water molecules (Figure 1b).

Thermal stability of **1** was assessed via TGA and DSC measurements in the 30-500 °C and in the 20-130 °C ranges, respectively. DSC measurements on **1** show the presence of an endothermic event at ca. 50 °C (peak T), compatible with a congruent melting accompanied by water evaporation, as observed via TGA (Figure SI-7). Variable temperature powder XRD measurements (VT-PXRD), however, show a different thermal behavior from the one observed in DSC and TGA: a second phase is detected starting from 45 °C, with complete conversion at 50 °C. The new form is stable up to 105 °C. This new pattern corresponds to the one obtained for urea$_2$•L-proline (**2**) (Figure SI-10). This is not the first time that the thermal behavior of a crystalline solid is found to be dependent on the methodology employed for its investigation.[57] The phenomenon was further investigated via Hot-Stage Microscopy (HSM) on single crystals of **1** (Figure SI-13). Loss of water, evidenced by the formation of bubbles beneath the thin oil layer covering the crystal, is observed around 40 °C, accompanied by initial melting along the edge of the crystal. Melting is complete at ca. 50 °C and is then followed by recrystallization to urea$_2$•L-proline (**2**).

The cocrystal urea$_2$•L-proline (**2**), was prepared from liquid assisted grinding of urea and L-proline with methanol or acetonitrile in a 2:1 stoichiometric ratio of the reactants (Figure SI-2). Despite numerous attempts and techniques tested, single crystals of **2** could not be obtained. The structure was thus solved from powder data, collected on the product of the mechanochemical reaction, with the help of information derived from thermogravimetric analysis (TGA), which allowed to confirm the anhydrous nature of the cocrystal (Figure SI-8). The structure of crystalline urea$_2$•L-proline (**2**) can be described as the result of an unusual arrangement of urea molecules in a 3D hydrogen bonding framework (Figure 2a). Infinite threads (Figure 2c) of L-proline molecules are interspersed within the framework, filling its cavities (Figure 2b) and interacting with the urea framework via additional hydrogen bonds. While it has been observed that hygroscopicity of urea is lowered by cocrystallization with organic coformers[31,33] this appears not to be the case with crystalline **2**, which upon time, if exposed to air, captures water from the atmosphere, releases one urea molecule per formula unit, and transforms into the hydrated form urea•L-proline•H_2O (**1**) (Figure SI-3).

Thermal stability of **2** was assessed via TGA and DSC measurements in the 30-500 °C and in the 20-130 °C ranges, respectively. The thermal behavior of the anhydrous phase is markedly different from that of the hydrate form: DSC measurements show the presence of an endothermic event at ca. 113 °C (peak T), which, as shown by the TGA curve, is immediately followed by degradation (Figures SI-6,8). For this compound, DSC and TGA measurements agree with the results obtained during VT-PXRD measurements (Figure SI-11): no phase conversion occurs up to 110 °C, when melting is observed.

Synthesis and Characterization of Urea•DL-Proline•H_2O (3). The urea•DL-proline•H_2O (**3**) cocrystal was prepared via liquid assisted grinding or slurry with water, methanol and acetonitrile starting from a 1:1 stoichiometric ratio of the reactants. Recrystallization from either methanol or water solution afforded single crystals suitable for X-ray diffraction. The structural identity between the product of the solid-state synthesis and the product of the recrystallization was verified by comparing the powder X-ray diffraction (PXRD) pattern, calculated on the basis of the single-crystal structure, and the experimental pattern measured for the crystalline powder (Figure SI-4). The cocrystal is isostructural with **1** (Figure 3) but its thermal behavior is markedly different. The TGA curve shows an initial weight loss, starting at 39 °C, of ca. 8 wt %, compatible with the loss of one water molecule per formula unit (theoretical value = 9 wt %).

Thermal degradation starts at 125 °C and ends at ca. 350 °C (Figure SI-9). The DSC curve in the 20-120 °C range features a single endothermic event at 43 °C (T_{onset}), with peak maximum at 47 °C (Figure SI-6). The shape of the peak is that of a melting endotherm, and the thermal range is the same as the one observed in TGA and attributed to water loss; this

https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680

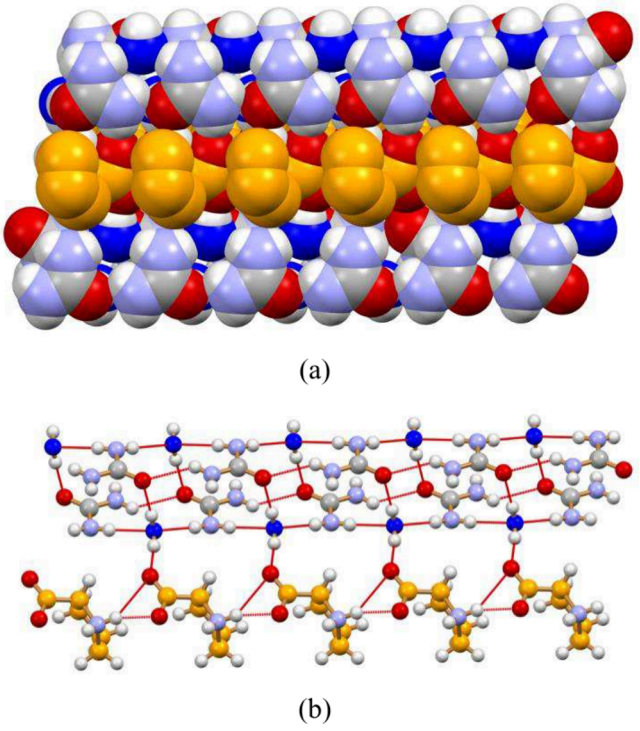


Figure 3. (a) Layered structure of crystalline Urea•DL-proline•H_2O (**3**) (H_{CH} atoms omitted for clarity.). (b) Hydrogen bonding network between layers of urea and DL-proline. Urea C atoms in grey, L-proline C atoms in orange, O_{water} in blue.

could be explained with congruent melting at low temperature immediately followed by water evaporation.

VT-PXRD confirms this hypothesis (Figure SI-12): heating the sample up to 40 °C (green pattern) causes the melting of the cocrystal. Melting can be also confirmed visually by observing the sample inside the sample holder. No recrystallization event was detected on increasing the temperature or after cooling the sample back to room temperature. Due to the low thermal stability of **3**, we decided to focus the in vitro experiments only on crystalline **1**.

Urea•L-Proline•H_2O Efficacy in *in Vitro* Rumen Degradability and Degradation Rates. The efficiency of nitrogen utilization is critical in ruminant nutrition. While increasing crude protein (CP) content in dairy cow diets can enhance milk production, it often results in elevated ruminal ammonia and blood urea nitrogen levels, contributing to greater nitrogen losses through urine[58,59] highlight the critical importance of formulating an efficient diet, which emphasizes the necessity of balancing degradable nitrogen sources. This balance is essential for optimizing microbial protein production while simultaneously minimizing nitrogen waste. Urea•L-proline•H_2O was tested as a nitrogen source for rumen *in vitro* to ascertain its efficiency and compared to other nitrogen sources, such as urea or L-proline. The untreated corn silage served as a baseline for comparison with samples supplemented with nitrogen sources (urea, L-proline, and **1**). Treatments showed minimal variations in dry matter (DM) and organic matter (OM) content. However, the addition of nitrogen sources increased crude protein (CP) and soluble protein (SP) fractions. The NDF and ADF contents remained relatively consistent across treatments, indicating that the structural carbohydrate fractions were unaffected by supplementation.

The extent of rumen degradation for each sample was calculated at each incubation time to estimate the degradation rate and fitted using a nonlinear Gompertz model.[56] *In vitro* degradability curve (6 to 48 h) of dry matter (DM) is shown in Figure SI-14. The resulting modeling outcome of nitrogen supplementation on *in vitro* rumen degradability and degradation rates are presented in Table 2. Supplementing

Table 2. Estimation of Ruminal Parameters *in Vitro* for 48 h Using the Gompertz Model[a]

Ruminal Parameters	Corn Silage	Corn Silage + Urea	Corn Silage + L-Proline	Corn Silage + Urea•L-Proline•H_2O	p-value
Rumen degradability (% 48 h)	68.43	68.26	68.26	67.23	0.8123
Degradation rate (per hour)	0.070	0.074	0.076	0.080	0.0236
Lag time (per hour)	0.36	0.43	0.57	0.69	0.0261

[a]Values are mean ±STDEV of samples (n=3).

corn silage with nitrogen sources at 1% of dry matter did not significantly impact rumen degradability over 48 h. However, nitrogen supplementation significantly increased the hourly degradation rate (Pr>F 0.0236), with the highest rate observed for the treatment with **1**, which improved the rate by 14% compared to untreated corn silage.

Interestingly, both L-proline and **1** had a similar impact as conventional urea, regardless of the considerable difference in crude protein (CP) content among them. Notably, conventional urea contains approximately twice as much CP as **1** and nearly four times as much as L-proline (Table 1). The requirement for peptides is linked to the efficiency of microbial growth, which, in turn, depends on energy sources and amino acids to ensure ruminal growth. This suggests that amino acids and peptides act as catalysts for microbial growth, potentially explaining the higher degradation rates observed. The observed increase in degradation rates detected when silage is treated with nitrogen supplements aligns with the results obtained by Argyle and Baldwin in their *in vitro* experiments.[60] This study demonstrated the stimulating effect of AA (amino acid) and peptides on bacterial growth, doubling bacterial proliferation under optimal conditions. Amino acids, like proline, act as catalysts for microbial activity, enhancing ruminal degradation rates when paired with an energy source and this potentially explains the higher degradation rates observed in Table 2. Reducing nitrogen intake through low-crude protein (CP) diets enhances the transfer of endogenous urea from the bloodstream to the portal-drained viscera.[61,62] An *in situ* study with rumen-fistulated Holstein cows evaluated soybean meal, canola meal, high-protein corn, and wheat dried distillers grains, revealing that the ruminal disappearance of essential and total amino acids mirrored the overall CP degradation pattern. Furthermore, the extent of amino acid disappearance varied among individual amino acids and protein supplements,[63] indicating that supplements rich in essential amino acids can effectively modulate ruminal digestibility.

Nitrogen Degradability Assessment. Nitrogen degradability based on the results presented in Table 2 is reported in Figure 4. Silage supplemented with L-proline or **1** tended to reduce nitrogen degradability compared to the urea-treated

https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680

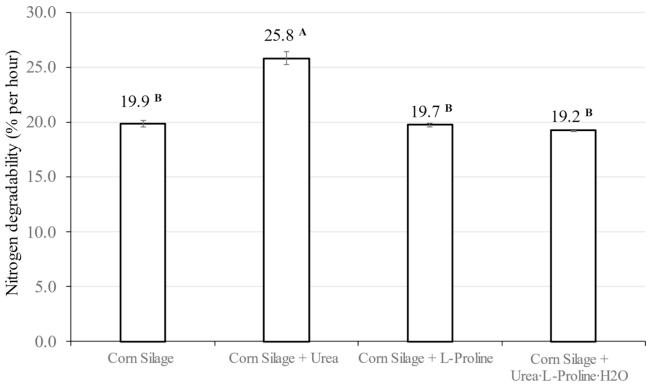


Figure 4. Nitrogen degradability per hour, *in vitro* incubation (48 h). Different capital letters indicate significant differences among different treatments ($p < 0.05$).

one. Reducing nitrogen degradability while maintaining high ruminal digestion is advantageous, as it allows for more efficient utilization of dietary nitrogen. This minimizes nitrogen excretion as waste while ensuring adequate nitrogen availability for microbial protein synthesis, ultimately improving animal performance and reducing the environmental impact of excess nitrogen emissions.[8]

The findings of this study highlight the potential of urea•L-proline•H_2O (**1**) as a rumen supplement. We attribute this increased utilization of the dietary nitrogen from urea•L-proline•H_2O (**1**) to the high solubility of the cocrystal (see the SI). By balancing nitrogen degradability and microbial activity, this additive may reduce nitrogen waste while maintaining or enhancing feed efficiency. Lowering nitrogen degradability through AA-containing supplements can support microbial protein production and reduce the environmental burden associated with excessive nitrogen emissions.

While the *in vitro* assays reported in this study provide valuable insights into feed degradability, they do not fully replicate the complex digestive processes of ruminants. Future *in vivo* studies are required to provide a clearer understanding of the impact of these supplements in altering microbial activity. Indeed, in vivo studies have shown that additives can significantly influence nitrogen recycling and microbial protein synthesis reinforcing the need for further research.[64]

CONCLUSIONS

The utilization of feed by the ruminal microbiota is closely linked to the nitrogen supply available to these microorganisms. Research has demonstrated that the inclusion of L-proline alongside silage enhances microbial activity, thereby improving the in vitro degradation efficiency of silage.

Novel cocrystals of urea with proline were synthesized both mechanochemically as well as by slurry in water. All cocrystals were fully characterized either by single crystal or powder X-ray diffraction. The highly soluble cocrystal Urea•L-proline•H_2O (**1**) was further investigated as a supplement to improve efficiency and sustainability in dairy cattle feeding practices. Efficient digestion and microbial activity in the rumen depend on the availability of key nutrients, particularly nitrogen, energy, and minerals. Undigested feed and microbial biomass synthesized in the rumen pass through the digestive tract for further breakdown. In the abomasum and small intestine, enzymatic digestion occurs, followed by additional microbial fermentation in the large intestine. The efficiency of feed utilization in ruminants depends heavily on the rate of forage digestion in the rumen. Microbial activity plays a pivotal role in this process.[21] The enhanced degradation rates observed with silage treated with nitrogen supplements, particularly **1**, can thus be explained as the outcome of increased microbial activity. This finding aligns with the understanding that amino acids and peptides, such as those derived from L-proline, can enhance microbial growth and fiber digestion.[11] This is important as, forage degradability is also influenced by fiber and lignin content, as they can act as barriers to microbial fermentation.[65] While the fiber content of corn silage remained consistent across treatments, the improved degradation rates observed in treated silage suggest that the addition of amino acids like L-proline may facilitate fiber digestion. These results emphasize the potential of supplements to enhance rumen function by balancing nitrogen availability while supporting microbial protein synthesis. This experiment demonstrated that L-proline and urea•L-proline•H_2O can be utilized as an N supplement and can provide equal performance as conventional urea supplements avoiding the potential hazards associated with feed-grade urea and maintaining favorable fermentation parameters.

These findings suggest that supplementing amino acids can not only enhance the nutritional value for ruminants but also play a key role in reducing environmental contamination by reducing microbial degradation during ruminant digestion. Even though *in vitro* analysis is a great tool to analyze and predict feed degradability, it does not consider the digestive process as a whole. This study is a starting point for greater and more in-depth research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssusresmgt.5c00125.

Additional experimental data that supports the findings discussed in the main text; Powder X-ray diffraction patterns, Crystallographic tables and Rietveld refinement results for structural analysis, DSC and TGA, Variable Temperature X-ray diffraction, Hot Stage Microscopy images for detailing the thermal behavior, Raw digestibility data of urea•L-proline•H_2O (**1**), Solubility measurements to support the physicochemical property study, and Bibliography (PDF)

AUTHOR INFORMATION

Corresponding Authors

Ranjit Thakuria − *Department of Chemistry, Gauhati University, Guwahati, Assam 781014, India;* orcid.org/0000-0002-0325-3316; Email: ranjit.thakuria@gmail.com

Jonas Baltrusaitis − *Department of Chemical and Biomolecular Engineering, Lehigh University, Bethlehem, Pennsylvania 18015, United States;* orcid.org/0000-0001-5634-955X; Email: job314@lehigh.edu

Fabrizia Grepioni − *Department of Chemistry "Giacomo Ciamician", University of Bologna, 40129 Bologna, Italy;* orcid.org/0000-0003-3895-0979; Email: fabrizia.grepioni@unibo.it

Authors

Laura Contini − *Department of Chemistry "Giacomo Ciamician", University of Bologna, 40129 Bologna, Italy*

Diptajyoti Gogoi − *Department of Chemistry, Gauhati University, Guwahati, Assam 781014, India*
Ana Carolina Fluck − *Universidade Tecnológica Federal do Paraná, Dois Vizinhos, PR 85660-000, Brasil*
Júlia Gomes Farias − *USDA-ARS, US Arid Land Agricultural Research Center, Maricopa, Arizona 85138, United States;* orcid.org/0000-0002-2268-5501
Clinton Williams − *USDA-ARS, US Arid Land Agricultural Research Center, Maricopa, Arizona 85138, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssusresmgt.5c00125

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the USDA-ARS through NACA # 58-2020-2-013 funding. R.T. acknowledges the project funded by the Science and Engineering Research Board under the Teachers Associateship for Research Excellence (TARE) grant (Project No. TAR/2021/000251). F.G. acknowledges the project "NICE − Nature Inspired Crystal Engineering" (PRIN2020) and financial support (RFO) from the University of Bologna. A PNRR DM 351/2022 PhD project (L.C.) is also acknowledged.

■ REFERENCES

(1) Emery, R. S. Feeding for Increased Milk Protein1. *Journal of Dairy Science* **1978**, *61* (6), 825−828.
(2) Jonker, J. S.; Kohn, R. A.; High, J. Use of Milk Urea Nitrogen to Improve Dairy Cow Diets1. *Journal of Dairy Science* **2002**, *85* (4), 939−946.
(3) Castillo, A. R.; Kebreab, E.; Beever, D. E.; Barbi, J. H.; Sutton, J. D.; Kirby, H. C.; France, J. The Effect of Protein Supplementation on Nitrogen Utilization in Lactating Dairy Cows Fed Grass Silage Diets. *J. Anim. Sci.* **2001**, *79* (1), 247−253.
(4) Lassaletta, L.; Billen, G.; Garnier, J.; Bouwman, L.; Velazquez, E.; Mueller, N. D.; Gerber, J. S. Nitrogen Use in the Global Food System: Past Trends and Future Trajectories of Agronomic Performance, Pollution, Trade, and Dietary Demand. *Environ. Res. Lett.* **2016**, *11* (9), No. 095007.
(5) Blair, R.; Jacob, J. P.; Ibrahim, S.; Wang, P. A Quantitative Assessment of Reduced Protein Diets and Supplements to Improve Nitrogen Utilization. *J. Appl. Poult. Res.* **1999**, *8* (1), 25−47.
(6) Shurson, G. C. We Can Optimize Protein Nutrition and Reduce Nitrogen Waste in Global Pig and Food Production Systems by Adopting Circular, Sustainable, and One Health Practices. *Journal of Nutrition* **2025**, *155*, 367.
(7) Kohn, R. A.; Dou, Z.; Ferguson, J. D.; Boston, R. C. A Sensitivity Analysis of Nitrogen Losses from Dairy Farms. *Journal of Environmental Management* **1997**, *50* (4), 417−428.
(8) Hristov, A. N.; Bannink, A.; Crompton, L. A.; Huhtanen, P.; Kreuzer, M.; McGee, M.; Nozière, P.; Reynolds, C. K.; Bayat, A. R.; Yáñez-Ruiz, D. R.; Dijkstra, J.; Kebreab, E.; Schwarm, A.; Shingfield, K. J.; Yu, Z. Invited Review: Nitrogen in Ruminant Nutrition: A Review of Measurement Techniques. *J. Dairy Sci.* **2019**, *102* (7), 5811−5852.
(9) Committee on Nutrient Requirements of Dairy Cattle; Board on Agriculture and Natural Resources; Division on Earth and Life Studies; National Academies of Sciences, Engineering, and Medicine. *Nutrient Requirements of Dairy Cattle*; Eighth Revised ed.; National Academies Press: Washington, D.C., 2021; DOI: 10.17226/25806.
(10) Hackmann, T. J.; Firkins, J. L. Maximizing Efficiency of Rumen Microbial Protein Production. *Front. Microbiol.* **2015**, *6*, 465.
(11) Soto, R. C.; Muhammed, S. A.; Newbold, C. J.; Stewart, C. S.; Wallace, R. J. Influence of Peptides, Amino Acids and Urea on Microbial Activity in the Rumen of Sheep Receiving Grass Hay and on the Growth of Rumen Bacteria in Vitro. *Animal Feed Science and Technology* **1994**, *49* (1), 151−161.
(12) Carro, M. D.; Miller, E. L. Effect of Supplementing a Fibre Basal Diet with Different Nitrogen Forms on Ruminal Fermentation and Microbial Growth in an in Vitro Semi-Continuous Culture System (RUSITEC). *Br J. Nutr* **1999**, *82* (2), 149−157.
(13) Kajikawa, H.; Mitsumori, M.; Ohmomo, S. Stimulatory and Inhibitory Effects of Protein Amino Acids on Growth Rate and Efficiency of Mixed Ruminal Bacteria. *Journal of Dairy Science* **2002**, *85* (8), 2015−2022.
(14) Wu, G. Functional Amino Acids in Nutrition and Health. *Amino Acids* **2013**, *45* (3), 407−411.
(15) McCoard, S. A.; Pacheco, D. The Significance of N-Carbamoylglutamate in Ruminant Production. *Journal of Animal Science and Biotechnology* **2023**, *14* (1), 48.
(16) Auestad, N.; Layman, D. K. Dairy Bioactive Proteins and Peptides: A Narrative Review. *Nutr Rev.* **2021**, *79* (Supplement_2), 36−47.
(17) Bruckental, I.; Ascarelli, I.; Yosif, B.; Alumot, E. Effect of Duodenal Proline Infusion on Milk Production and Composition in Dairy Cows. *Animal Science* **1991**, *53* (3), 299−303.
(18) Wu, G. D.; Chen, J.; Hoffmann, C.; Bittinger, K.; Chen, Y.-Y.; Keilbaugh, S. A.; Bewtra, M.; Knights, D.; Walters, W. A.; Knight, R.; Sinha, R.; Gilroy, E.; Gupta, K.; Baldassano, R.; Nessel, L.; Li, H.; Bushman, F. D.; Lewis, J. D. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science* **2011**, *334* (6052), 105−108.
(19) Chowdhury, M. R.; Wilkinson, R. G.; Sinclair, L. A. Reducing Dietary Protein and Supplementation with Starch or Rumen-Protected Methionine and Its Effect on Performance and Nitrogen Efficiency in Dairy Cows Fed a Red Clover and Grass Silage−Based Diet. *Journal of Dairy Science* **2024**, *107* (6), 3543−3557.
(20) Matthews, C.; Crispie, F.; Lewis, E.; Reid, M.; O'Toole, P. W.; Cotter, P. D. The Rumen Microbiome: A Crucial Consideration When Optimising Milk and Meat Production and Nitrogen Utilisation Efficiency. *Gut Microbes* **2019**, *10* (2), 115−132.
(21) Jouany, J. P. Methods of manipulating the microbial metabolism in the rumen. *Ann. Zootech.* **1994**, *43* (1), 49−62.
(22) Kavanagh, O. N.; Croker, D. M.; Walker, G. M.; Zaworotko, M. J. Pharmaceutical Cocrystals: From Serendipity to Design to Application. *Drug Discov Today* **2019**, *24* (3), 796−804.
(23) Jones, W.; Motherwell, W. D. S.; Trask, A. V. Pharmaceutical Cocrystals: An Emerging Approach to Physical Property Enhancement. *MRS Bull.* **2006**, *31* (11), 875−879.
(24) Thakuria, R.; Delori, A.; Jones, W.; Lipert, M. P.; Roy, L.; Rodríguez-Hornedo, N. Pharmaceutical Cocrystals and Poorly Soluble Drugs. *Int. J. Pharm.* **2013**, *453* (1), 101−125.
(25) Duggirala, N. K.; Perry, M. L.; Almarsson, Ö.; Zaworotko, M. J. Pharmaceutical Cocrystals: Along the Path to Improved Medicines. *Chem. Commun.* **2016**, *52* (4), 640−655.
(26) Bolla, G.; Nangia, A. Pharmaceutical Cocrystals: Walking the Talk. *Chem. Commun.* **2016**, *52* (54), 8342−8360.
(27) Bolla, G.; Sarma, B.; Nangia, A. K. Crystal Engineering of Pharmaceutical Cocrystals in the Discovery and Development of Improved Drugs. *Chem. Rev.* **2022**, *122* (13), 11514−11603.
(28) Schultheiss, N.; Newman, A. Pharmaceutical Cocrystals and Their Physicochemical Properties. *Cryst. Growth Des.* **2009**, *9* (6), 2950−2967.
(29) Ammar, M.; Ashraf, S.; Gonzalez-Casamachin, D. A.; Baltrusaitis, J. Sustainable by (Crystal) Design: Novel Materials for Agriculture via Active Ingredient Cocrystallization. *RSC Sustain.* **2025**, *3*, 781.
(30) Parakatawella, S.; Gogoi, D.; Deka, P.; Xu, Y.; Sandaruwan, C.; Jayasundera, A. C. A.; Arhangelskis, M.; Thakuria, R.; Adassooriya, N. M. Mechanochemical Synthesis of Polymorphic Urea · Adipic Acid Cocrystal as a Sustained-Release Nitrogen Source. *ChemSusChem* **2022**, *15* (3), No. e202102445.
(31) Rajbongshi, T.; Parakatawella, S.; Gogoi, D.; Deka, P.; Adassooriya, N. M.; Thakuria, R. Mechanosynthesis of Urea−

Hydroxybenzoic Acid Cocrystals as Sustained-Release Nitrogen Fertilizer. *RSC Sustain.* **2023**, *1* (6), 1416−1422.
(32) Mazzei, L.; Broll, V.; Casali, L.; Silva, M.; Braga, D.; Grepioni, F.; Baltrusaitis, J.; Ciurli, S. Multifunctional Urea Cocrystal with Combined Ureolysis and Nitrification Inhibiting Capabilities for Enhanced Nitrogen Management. *ACS Sustainable Chem. Eng.* **2019**, *7* (15), 13369−13378.
(33) Casali, L.; Mazzei, L.; Shemchuk, O.; Sharma, L.; Honer, K.; Grepioni, F.; Ciurli, S.; Braga, D.; Baltrusaitis, J. Novel Dual-Action Plant Fertilizer and Urease Inhibitor: Urea·Catechol Cocrystal. Characterization and Environmental Reactivity. *ACS Sustainable Chem. Eng.* **2019**, *7* (2), 2852−2859.
(34) Casali, L.; Mazzei, L.; Shemchuk, O.; Honer, K.; Grepioni, F.; Ciurli, S.; Braga, D.; Baltrusaitis, J. Smart Urea Ionic Co-Crystals with Enhanced Urease Inhibition Activity for Improved Nitrogen Cycle Management. *Chem. Commun.* **2018**, *54* (55), 7637−7640.
(35) Kertz, A. F. Review: Urea Feeding to Dairy Cattle: A Historical Perspective and Review. *Professional Animal Scientist* **2010**, *26* (3), 257−272.
(36) Oltjen, R. R.; Slyter, L. L.; Kozak, A. S.; Williams, E. E. Evaluation of Urea, Biuret, Urea Phosphate and Uric Acid as NPN Sources for Cattle. *Journal of Nutrition* **1968**, *94* (2), 193−202.
(37) *NONPROTEIN NITROGEN IN THE NUTRITION OF RUMINANTS*. https://www.fao.org/4/AC149E/AC149E05.htm (accessed 2024-09-28).
(38) Mutsvangwa, T.; Davies, K. L.; McKinnon, J. J.; Christensen, D. A. Effects of Dietary Crude Protein and Rumen-Degradable Protein Concentrations on Urea Recycling, Nitrogen Balance, Omasal Nutrient Flow, and Milk Production in Dairy Cows. *Journal of Dairy Science* **2016**, *99* (8), 6298−6310.
(39) Altomare, A.; Cuocci, C.; Giacovazzo, C.; Moliterni, A.; Rizzi, R.; Corriero, N.; Falcicchio, A. EXPO2013: A Kit of Tools for Phasing Crystal Structures from Powder Data. *J. Appl. Cryst.* **2013**, *46* (4), 1231−1235.
(40) Altomare, A.; Campi, G.; Cuocci, C.; Eriksson, L.; Giacovazzo, C.; Moliterni, A.; Rizzi, R.; Werner, P.-E. Advances in Powder Diffraction Pattern Indexing: N-TREOR09. *J. Appl. Cryst.* **2009**, *42* (5), 768−775.
(41) Delley, B. From Molecules to Solids with the DMol3 Approach. *J. Chem. Phys.* **2000**, *113* (18), 7756−7764.
(42) Delley, B. An All-electron Numerical Method for Solving the Local Density Functional for Polyatomic Molecules. *J. Chem. Phys.* **1990**, *92* (1), 508−517.
(43) Perdew, J. P.; Burke, K.; Ernzerhof, M. Generalized Gradient Approximation Made Simple. *Phys. Rev. Lett.* **1996**, *77* (18), 3865−3868.
(44) Dolomanov, O. V.; Bourhis, L. J.; Gildea, R. J.; Howard, J. a. K.; Puschmann, H. OLEX2: A Complete Structure Solution, Refinement and Analysis Program. *J. Appl. Cryst.* **2009**, *42* (2), 339−341.
(45) Sheldrick, G. M. SHELXT - Integrated Space-Group and Crystal-Structure Determination. *Acta Crystallogr. A Found Adv.* **2015**, *71* (1), 3−8.
(46) Sheldrick, G. M. Crystal Structure Refinement with SHELXL. *Acta Cryst. C* **2015**, *71* (1), 3−8.
(47) Macrae, C. F.; Sovago, I.; Cottrell, S. J.; Galek, P. T. A.; McCabe, P.; Pidcock, E.; Platings, M.; Shields, G. P.; Stevens, J. S.; Towler, M.; Wood, P. A. Mercury 4.0: From Visualization to Analysis, Design and Prediction. *J. Appl. Cryst.* **2020**, *53* (1), 226−235.
(48) Goering, H. K.; Van Soest, P. J. *Forage Fiber Analyses: Apparatus, Reagents, Procedures, and Some Applications*; USDA-ARS Agricultural Handbook 379, 1970; Vol. Washington DC.
(49) Shreve, B.; Thiex, N.; Wolf, M. National Forage Testing Association Reference Method: Dry Matter by Oven Drying for 3 Hours at 105°C. *NFTA Reference Methods*.
(50) Thiex, N.; Novotny, L.; Crawford, A. Determination of Ash in Animal Feed: AOAC Official Method 942.05 Revisited. *Journal of AOAC INTERNATIONAL* **2012**, *95* (5), 1392−1397.
(51) Krishnamoorthy, U.; Muscato, T. V.; Sniffen, C. J.; Van Soest, P. J. Nitrogen Fractions in Selected Feedstuffs. *Journal of Dairy Science* **1982**, *65* (2), 217−225.
(52) Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74* (10), 3583−3597.
(53) Singer, M. D.; Robinson, P. H.; Salem, A. Z. M.; DePeters, E. J. Impacts of Rumen Fluid Modified by Feeding Yucca Schidigera to Lactating Dairy Cows on in Vitro Gas Production of 11 Common Dairy Feedstuffs, as Well as Animal Performance. *Animal Feed Science and Technology* **2008**, *146* (3−4), 242−258.
(54) Soest, P. J. V. Use of Detergents in the Analysis of Fibrous Feeds. II. A Rapid Method for the Determination of Fiber and Lignin. *Journal of Association of Official Agricultural Chemists* **1963**, *46* (5), 829−835.
(55) *Statistical Analysis System (SAS/STAT)*, 2018.
(56) Gompertz, B. On the Nature of the Function Expressive of the Law of Human Mortality, and on a New Mode of Determining the Value of Life Contingencies on JSTOR. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **1825**, *182*, 513−585.
(57) Braga, D.; Grepioni, F.; Maini, L.; Capucci, D.; Nanna, S.; Wouters, J.; Aerts, L.; Quéré, L. Combining Piracetam and Lithium Salts: Ionic Co-Crystals and Co-Drugs? *Chem. Commun.* **2012**, *48* (66), 8219−8221.
(58) Zhao, X.; Zheng, N.; Zhang, Y.; Wang, J. The Role of Milk Urea Nitrogen in Nutritional Assessment and Its Relationship with Phenotype of Dairy Cows: A Review. *Animal Nutrition* **2025**, *20*, 33.
(59) Colmenero, J. J. O.; Broderick, G. A. Effect of Dietary Crude Protein Concentration on Milk Production and Nitrogen Utilization in Lactating Dairy Cows. *J. Dairy Sci.* **2006**, *89* (5), 1704−1712.
(60) Argyle, J. L.; Baldwin, R. L. Effects of Amino Acids and Peptides on Rumen Microbial Growth Yields. *Journal of Dairy Science* **1989**, *72* (8), 2017−2027.
(61) Marini, J. C.; Van Amburgh, M. E. Nitrogen Metabolism and Recycling in Holstein Heifers. *J. Anim Sci.* **2003**, *81* (2), 545−552.
(62) Chibisa, G. E.; Mutsvangwa, T. Effects of Feeding Wheat or Corn-Wheat Dried Distillers Grains with Solubles in Low- or High-Crude Protein Diets on Ruminal Function, Omasal Nutrient Flows, Urea-N Recycling, and Performance in Cows. *J. Dairy Sci.* **2013**, *96* (10), 6550−6563.
(63) Maxin, G.; Ouellet, D. R.; Lapierre, H. Effect of Substitution of Soybean Meal by Canola Meal or Distillers Grains in Dairy Rations on Amino Acid and Glucose Availability. *J. Dairy Sci.* **2013**, *96* (12), 7806−7817.
(64) Lima, J.; Ingabire, W.; Roehe, R.; Dewhurst, R. J. Estimating Microbial Protein Synthesis in the Rumen—Can. 'Omics' Methods Provide New Insights into a Long-Standing Question? *Veterinary Sciences* **2023**, *10* (12), 679.
(65) Fluck, A. C.; Kozloski, G. V.; Martins, A. A.; Mezzomo, M. P.; Zanferari, F.; Stefanello, S. Relationship between Chemical Components, Bacterial Adherence and in Vitro Fermentation of Tropical Forage Legumes. *Ciênc. agrotec.* **2013**, *37* (5), 457−463.

https://doi.org/10.1021/acssusresmgt.5c00125
ACS Sustainable Resour. Manage. 2025, 2, 1672−1680