Effects of a commercial fermentation byproduct or urea on milk production, rumen metabolism, and omasal flow of nutrients in lactating dairy cattle

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Hi Sotirios,

I hope you are fine. Regarding our meeting on Tuesday I wonder if we can postpone it. As you know, it is Kathari Deutera this Monday, and I will be at my parents house in Poros. The first boat starts at 7:15 and I will not make it to be back at 9 for our meeting.

Can we make it on Wednesday at 9?

By the way, I did not receive your detailed notes for our discussion.

Moreover, I will try to have my students at the farm the following week. Probably, on Thursday or Friday. I will call them on Tuesday to see if it is OK for them. Due to some difficulties in organizing sampling and communicating with the nutritionists at the farms, we decided not to perform the detailed protocol with faeces, urine and body weight. Thus, we will collect only feeds, TMR and then evaluate the diet. Therefore, I will need the latest diet(s) fed at the farm.

Best regards,

Andreas

Interpretive Summary

Byproducts of human food production can be used to improve the environmental and economic sustainability of milk protein production in dairy cattle. Efficient milk protein production requires optimization of the balance between rumen protein degradation and microbial protein synthesis. The objective of this study was to evaluate the effect of a commercial fermentation byproduct on ruminal nitrogen metabolism and omasal flow of nutrients. Fermentation byproduct inclusion reduced overall degradation of protein in the rumen and allowed for more efficient fermentation of fiber. Results indicated that stimulation of microbial populations does not always increase microbial protein flow to the omasum.

Effects of commercial fermentation byproduct or urea on milk production, rumen metabolism, and omasal flow of nutrients in lactating dairy cattle

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Abstract

The objective of this study was to evaluate the effects of a fermentation byproduct on rumen fermentation and microbial yield in high producing lactating dairy cattle. Eight ruminally cannulated multiparous Holstein cows averaging 60 ± 10 DIM and 637 ± 38 kg of BW were

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assigned to one of two treatment sequences in a switchback design. Treatment diets contained (dry matter basis) 44% corn silage, 13% alfalfa silage, 12% ground corn, and 31% premix containing either a control mix of urea and wheat middlings (**CON**) or a commercial fermentation byproduct meal (Fermenten, Arm and Hammer Animal Nutrition, Princeton, NJ) at 3% diet inclusion rate (**EXP**). Diets were formulated to be iso-nitrogenous and iso-caloric, with similar levels of neutral detergent fiber and starch. The trial consisted of three 28 d experimental periods, where each period consisted of 21 d of diet adaptation and 7 d of data and sample collection. Omasal nutrient flows were determined using a triple-marker technique and doubly-labeled $^{15}N^{15}N$-urea. The EXP diet provided 18 g/d more non-ammonia N vs. the CON diet, representing 3.0% of total N intake. Energy corrected milk yield (41.7 and 43.1 kg/d for CON and EXP, respectively), milk fat and protein yield and content did not differ between treatments. Total dry matter intake was similar between treatments (25.5 and 26.4 kg/d for CON and EXP, respectively). Ammonia N concentration and pool size in the rumen was greater in cows fed the EXP diet. No differences were observed in rumen or total tract dry matter, organic matter, or neutral detergent fiber digestibility. Ruminal degradation of feed N was 15% lower in cows fed EXP diets, resulting in differences in omasal N flows. Results demonstrated the fermentation byproduct meal had a sparing effect on degradable feed protein, but did not increase microbial N flow from the rumen.

Keywords: omasal sampling, soluble protein, CNCPS, microbial protein synthesis, Fermenten

INTRODUCTION

Protein is one of the most expensive macronutrients in dairy cattle rations, and overfeeding degradable protein results in excessive N losses to the environment (Huhtanen and Hristov, 2009). Efficient use of feed N can be achieved by first meeting the requirements of the rumen

71 microbial population, followed by balancing diets to meet the AA requirements of the cow. To
72 decrease competition for quality protein that could otherwise be fed to humans, dairy cattle are
73 fed byproducts of human food production, thereby converting waste product streams into highly
74 valuable milk protein. One such byproduct of commercial AA production is Fermenten (Arm and
75 Hammer Animal Nutrition, Princeton, NJ). Commercial AA production is performed using
76 bacterial cultures, resulting in a waste stream with high amounts of soluble nitrogenous
77 compounds (Fessenden, 2016). These compounds of bacterial origin are in the highly available
78 forms of ammonia, free AA, small peptides, and purines. Amino acids and peptides have been
79 hypothesized to increase the flow of microbial protein from the rumen through stimulation of
80 microbial protein synthesis (Cotta and Russell, 1982, Lean et al., 2005). Increased microbial N
81 flow also reduces reliance on expensive rumen undegradable dietary protein sources commonly
82 used to provide AA to high producing dairy cattle. Previous research with varying sugar levels
83 suggested that these fermentation byproducts might only affect certain microbial populations
84 (Penner et al., 2009).
85 Mathematical models such as the Cornell Net Carbohydrate and Protein System (**CNCPS**)
86 (Higgs et al., 2015; Van Amburgh et al., 2015) have been successfully used to optimize rumen
87 microbial output and meet nutrient requirements of cattle while reducing N losses to the
88 environment (Tylutki et al., 2008). Successful use of such models requires accurate
89 characterization of the metabolizable AA outflows from the rumen. The omasal sampling
90 technique developed by Huhtanen et al. (1997) and modified by Ahvenjärvi et al. (2000)
91 provides useful data to assess the accuracy of model predictions of ruminal digestion and flow of
92 AA to the small intestine (Fessenden and Van Amburgh, 2016).

The hypothesis of this study was that inclusion of a fermentation byproduct with soluble AA and peptides vs. a wheat middlings and urea control blend would increase post-ruminal non-ammonia N flow at the omasal canal. The specific objectives of this study were to 1) evaluate the effect of urea or soluble AA and peptides from a commercial fermentation byproduct on rumen digestion and omasal flows of nutrients, and 2) provide comparisons of model predicted vs. measured values for rumen N outflows.

MATERIALS AND METHODS

The experiment was conducted from April to July 2014 at the Cornell University Ruminant Center in Harford, NY. All animals involved in this experiment were cared for according to the guidelines of the Cornell University Animal Care and Use committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

Eight ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 60 ± 10 DIM and 637 ± 38 kg of BW were enrolled in a 3 wk pre-trial acclimation period where all animals were managed and housed in a tie-stall and individually fed a common diet. The pre-trial diet consisted of 42% corn silage, 11% alfalfa silage, 15% ground corn, and 32% protein premix. At the end of the 3 wk period, animals were stratified by pre-trial milk production and randomly assigned to one of two treatment sequences in a switchback design. The trial consisted of three 28 d experimental periods, where each period contained 21 d for diet adaptation and 7 d of data and sample collection. All cows were injected on d 1 with bovine somatotropin (500 mg of Posilac, Elanco Animal Health, Greenfield, IN) and at 14 d intervals thereafter for the entire trial. Cows were milked 3x daily at 06:00, 14:00, and 22:00 h through a parlor except during sampling

115 periods, when cattle were milked in the tie-stalls. Milk yield was recorded and milk samples
116 taken at each milking on d 21, 22, and 23 of each period and analyzed for fat, true protein,
117 lactose, somatic cell count, total solids, and milk urea nitrogen at a commercial laboratory
118 (DairyOne, Ithaca, NY). Body weights were measured weekly after the 14:00 h milking, and
119 body condition score was recorded weekly as the average of two trained scorers. Change in body
120 weight and BCS were calculated as the difference between measurements taken on d 28 of each
121 period.

122 ***Treatment Administration and Sample Collection***

123 Treatment diets contained (DM basis) 44% corn silage, 13% alfalfa silage, 12% ground corn,
124 and 31% premix containing either a control mix of urea and wheat middlings (**CON**) or
125 Fermenten (**EXP**) at 3% inclusion rate in the final diet (Table 1). The premixes also differed
126 slightly in mineral sources to account for a higher level of sulfates in Fermenten. EXP contained
127 calcium carbonate, while CON contained calcium sulfate (Table 1). Forages and other
128 ingredients were analyzed for chemical composition for use in the CNCPS v. 6.5 using wet
129 chemical methods by Cumberland Valley Analytical Services (Hagerstown, MD). Rumen
130 degradable protein and NH_3-N balance for CON and EXP diets as predicted by the CNCPS v. 6.5
131 were 8.2 and 7.8% of DM and 120 and 115% of NH_3-N requirement, respectively. The forage
132 and corn grain portion of the diets were mixed daily as a single batch and delivered to the cattle
133 housing facility, where the batch was split in half and either the CON or EXP protein premix was
134 added to complete the treatment diets. Final mixing was done in a Super Data Ranger (American
135 Calan Inc., Northwood, NH) and the resulting TMR was offered once daily at 07:00 h. Orts were
136 collected and weights recorded at 06:00 h and feeding rate was adjusted daily to yield orts of 5 to
137 10% of daily intake. Weekly samples of corn silage, alfalfa silage, corn grain, protein premixes,

and TMR were analyzed for DM by drying at 60 °C for 48 h and diets were adjusted to maintain intended formulation. Dried samples were ground through a 1-mm screen (Wiley no. 4 Mill, Arthur H. Thomas, Philadelphia, PA), composited by period, and analyzed for nutrient composition (Tables 1 and 2). Intake of DM was calculated from DM determinations on TMR and orts. During sampling days, daily samples of TMR and orts were processed in the same manner as above, and equal parts DM from each sampling day were combined to create a sampling period composite for each cow within period.

Marker Infusion and Omasal Sampling

During the last week of each period, cows entered the infusion and omasal sampling phase. A triple marker system using CoEDTA (Udén et al., 1980), $YbCl_3$ (modified from Siddons et al., 1985), and undegraded aNDFom (**uNDFom**) (Raffrenato et al., 2018) were used to quantify liquid, small particle, and large particle flow at the omasal canal, respectively. Cobalt-EDTA and $YbCl_3$ were dissolved in distilled water and continuously infused into the rumen at rates of 2.8 g/d Co and 3.4 g/d Yb in 2.75 L of solution/d. All animals received a 3 L priming dose of the Co and Yb solution immediately prior to infusion start, providing 3.05 g of Co and 3.71 g of Yb per cow. On d 21 of each period, cattle were fitted with an indwelling catheter (Micro-renathane tubing, Braintree Scientific Inc., Braintree, MA) in the jugular vein for infusion of the microbial marker. Double-labeled urea ($^{15}N^{15}N$-urea, 98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) in sterile saline (9 g NaCl/L) was continuously infused a rate of 150 mL/d, providing 0.675 g/d of $^{15}N^{15}N$-urea. Before starting the infusion, samples of whole ruminal contents, feces, urine, plasma, and rumen microbes were taken for determination of ^{15}N background. All markers were infused continuously from 14:00 h on d 21 until 10:00 h on d 28 of each period via peristaltic pump (Masterflex, Cole-Parmer Instrument Company, LLC,

161 Vernon Hills, IL). All cows had at least 72 h of continuous infusion to reach uniform marker
162 distribution before any sampling occurred, as suggested by Broderick and Merchen (1992) and
163 conducted previously in our laboratory (Marini and Van Amburgh, 2003; Recktenwald, et al.,
164 2014).

165 Omasal samples were obtained using the omasal sampling technique developed by Huhtanen
166 et al. (1997) and adapted by Reynal and Broderick (2005). Samples of whole omasal contents
167 were collected from the omasal canal at 2 h intervals during three 8 h sessions: at 16:00, 18:00,
168 20:00, and 22:00 h on d 24; at 00:00, 02:00, 04:00, and 06:00 h on d 26; and at 08:00, 10:00,
169 12:00, and 14:00 on d 27. The sampling device was removed at the end of each 8 h sampling
170 session, and re-inserted at the start of the next session. Sampling times were chosen to
171 encompass every 2 h of the average 24 h feeding cycle. During each 8 h session, a 425 mL spot
172 sample was obtained at the first 3 time points, while 675 mL were taken at the last time point.
173 Spot omasal content samples were split into subsamples of 50 mL (x2), 125 mL, and 200 mL;
174 with an additional 250 mL subsample at the last time point. One of the 50 mL samples (**OF**) was
175 filtered through cheesecloth, acidified with 1 mL of 50% H_2SO_4, combined within period, and
176 stored at −20°C for subsequent NH_3-N and VFA analysis, while the other was processed and
177 stored for a separate investigation of soluble non-ammonia N flows. The 125 mL subsamples
178 were held on ice and combined within session, yielding a 500 mL sample for bacterial isolation.
179 The 200 mL samples were combined within period and stored at −20 °C, yielding a 2.4 L
180 composite for digestion phase separation. The additional 250 mL sample obtained at the end of
181 each session was immediately processed to isolate protozoa (**OP**) using flocculation and
182 filtration techniques, as described in the companion paper (Fessenden et al., 20XXb) for
183 investigation of microbial nitrogen and AA flows.

The bacterial isolations from each 8 h session were combined within sampling period to yield an omasal bacteria (**OB**) sample for each cow within period. Isolation was performed according to Whitehouse et al. (1994) with modifications. Briefly; whole omasal contents were filtered through 4 layers of cheesecloth and solids were rinsed once with saline, and the filtrate (I) was treated with formalin (0.1% v/v in final solution) and stored at 4 °C. The solids retained on the cheesecloth were incubated for 1 h at 39 °C in a 0.1% methylcellulose solution, mixed for 1 min at low speed (Omni Mixer, Omni International, Kennesaw, GA) to detach solids associated bacteria, and held at 4°C for 24 h. The contents were then squeezed through 4 layers of cheesecloth and the filtrate (II) was treated with formalin (0.1% v/v in final solution). Filtrates I and II were then combined and centrifuged at 1,000 x *g* for 5 min at 4 °C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x *g* for 20 min at 4 °C and the bacterial pellet, representing both solid and liquid associated bacteria, was collected and stored at −20 °C until lyophilization and later analysis.

Spot fecal and rumen fluid samples were taken at the same time points as omasal spot samples. Fecal samples were composited by period and stored at −20 °C, while rumen fluid (**RF**) was filtered through cheesecloth, acidified with 50% H_2SO_4 and composited by period before storage at -20 °C. On d 24 of each period, a sample of whole rumen contents was taken 4 hours after feeding for isolation of rumen microbes. Spot urine and blood samples were taken at the second time point of each session. Blood samples were collected into tubes containing sodium heparin, centrifuged (3,000 × *g* for 20 min at 4 °C), and plasma was harvested and stored at −20 °C. Urine samples were immediately acidified to pH < 2 with 50% H_2SO_4 and stored at −20 °C. On the last day of each period, rumen contents were evacuated, weighed, mixed, and a

representative sample was obtained and stored at −20 °C. Rumen contents were returned to the cow via the rumen cannula within 30 min of evacuation.

Sample Processing and Chemical Analysis

Sampling period TMR and orts composites were analyzed for DM at 105 °C for 6 h and ash according to AOAC (2005), and for total N using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Composited TMR and orts samples were analyzed for aNDFom (Mertens, 2002), and uNDFom after 240 h of in vitro incubation with rumen fluid, according to Raffrenato et al. (2018). The 2.4 L pooled omasal composites were thawed and separated into omasal large particle (**LP**), small particle (**SP**) and liquid phases (**LQ**) as described by Reynal and Broderick (2005). All phase samples were freeze dried and either ground through a 1 mm screen on a Wiley mill (LP) or homogenized with a mortar and pestle (SP and LQ) before analysis. Concentration of Co and Yb was determined by ICM-MS in all phase samples (Cornell University Nutrient Analysis Laboratory, Ithaca, NY) and the LP and SP phases were analyzed for uNDFom as described above. All omasal phases were analyzed for DM, OM, aNDFom and total N as described previously for feed samples to determine ruminal digestion and flow parameters. Concentrations of Yb, Co, and uNDFom in each phase were used to calculate the concentration of each nutrient in a theoretical entity representing omasal true digesta (**OTD**) (France and Siddons, 1986). As such, the reported flows and concentrations of any given nutrient in OTD is a mathematical calculation based on re-constitution factors determined using the triple marker technique and measured values of the nutrient in LQ, SP, and LP. This mathematical construct is referred to in this paper as OTD. Composite fecal samples were thawed, thoroughly mixed, and a subsample was dried for 72 h at 60 °C in a forced air oven. Subsamples from rumen evacuations were freeze-dried and the dried feces and rumen contents were ground to pass a 1

mm screen on a Wiley mill. DM, OM, total N, aNDFom and uNDFom was determined on the dried ground feces for total tract digestibility, while rumen contents were analyzed for DM and OM. Ammonia N concentration was determined in RF and OF using the colorimetric method of Chaney and Marbach (1962). Urea N concentration was determined in plasma and urine using an enzymatic colorimetric assay based on a commercial kit (No. 640, Sigma-Aldrich, St. Louis, MO). Volatile fatty acid concentration in RF and OF was determined by HPLC (Agilent 1100 series HPLC, Agilent Technologies, Santa Clara, CA) using crotonic acid as an internal standard (Siegfried et al., 1984).

Samples of OB, OP, rumen contents and omasal digesta phases were analyzed for non-ammonia N (**NAN**) concentration and ^{15}N enrichment as follows: 20 μg of N from each sample was weighted into tin capsules and 10 μL of 72 m*M* K_2CO_3 were added and incubated at 60°C overnight to volatilize ammonia. Samples were then analyzed for NAN and ^{15}N using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY). Samples of rumen bacteria, protozoa, and contents for natural abundance of ^{15}N were prepared and analyzed separately in the same manner as the enriched samples.

Calculations

Total digesta N entering the omasal canal was calculated using the triple marker technique and partitioned into three fractions: Ammonia N, microbial N, and non-ammonia non-microbial N (**NANMN**). Ammonia N flow was determined using the concentration of ammonia N in the OF sample and the flow of liquid determined using the triple marker system. Total NAN flow was calculated as the difference between total N and ammonia N. Microbial N flow was determined using ^{15}N atom percent excess (**APE**) in OTD and ^{15}N APE of the OB and OP

252 samples. The APE was calculated for digesta and microbial samples for each cow within period
253 as follows:

254 ^{15}N APE = (enriched ^{15}N-atom% - mean natural ^{15}N-atom%) / mean natural ^{15}N-atom%

255 Mean natural abundance of ^{15}N in rumen bacteria, protozoa, and contents was 0.3684 ± 0.0002
256 (mean ± SD). The natural abundance of ^{15}N in rumen bacteria, protozoa, and contents was
257 assumed to be representative of OB, OP, and OTD, respectively. Protozoa biomass flow was
258 calculated using gravimetric determinations of protozoa DM in omasal liquid multiplied by
259 protozoa NAN content and daily omasal liquid flow. The ^{15}N APE in protozoa and bacteria was
260 then used to calculate total microbial N flow:

261 Omasal protozoa NAN flow (g/d) = OP DM flow (g/d) x OP NAN (g/g DM)

262 Omasal bacteria NAN flow (g/d) = ([OTD NAN flow (g/d) x OTD ^{15}N APE (g/g N)] – [OP
263 NAN flow (g/d) x OP ^{15}N APE (g/g NAN)]) / OB ^{15}N APE (g/g NAN)

264 Microbial NAN flow (g/d) = OP NAN flow (g/d) + OB NAN flow (g/d)

265 The NAN content (g/g DM) of the OB and OP samples was used to calculate the flow of total
266 microbial biomass. Flow of NANMN was calculated as the difference between total NAN flow
267 and microbial NAN flow. Endogenous N flows were not determined in this study, as such all
268 NANMN was assumed to be dietary in origin. Therefore, RUP flow was estimated by
269 multiplying NANMN by 6.25, neglecting any contribution of non-^{15}N endogenous N
270 contributions (Lapierre et al., 2008). Rumen degradable protein supply was calculated as total N
271 intake minus RUP flow. Apparent and true ruminal digestibility of DM, OM, aNDFom and N
272 were calculated as follows:

Apparent digestibility = nutrient intake – omasal nutrient flow

True digestibility = nutrient intake – (omasal nutrient flow – microbial nutrient flow)

where all intakes and flows are g/d. Apparent total tract digestibility of DM and OM was determined using the fecal composite with uNDFom as an internal marker. Rumen and total tract digestibility of aNDFom can be considered true digestibility, as the use of sodium sulfite in the aNDFom procedure reduces microbial contamination (Van Soest, 2015).

A comparison of observed values and CNCPS predictions was performed. Cattle characteristics, diet composition and actual DMI were entered into the CNCPS v. 6.5, and the model was used to predict total omasal N flow, microbial N flow, and rumen undegraded protein flow. These values were then compared to the observed values to evaluate the model's ability to predict post ruminal nutrient flow. Due to individual animal variation and the limited number of independent observations, observed vs. predicted flow comparisons are on a numerical basis only.

Statistical Analyses

All data were analyzed using SAS version 9.3 (SAS Institute Inc. Cary, NC). Diet chemical composition was analyzed using PROC GLM and means were compared using the LSMEAN statement. All other data were analyzed using the MIXED procedure of SAS version 9.3. Due to slight negative effect of omasal sampling procedure on intake, milk production and associated intake were determined as the mean of 3 d before the infusion period began, while omasal parameters and associated nutrient intake were determined from data collected during the omasal sampling period. All variables were analyzed according to the following model:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + ST_{il} + \varepsilon_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, P_k = fixed effect of period k, T_l = fixed effect of treatment l, ST_{il} = fixed interaction effect of sequence i and treatment l, and ε_{ijkl} = residual error. Degrees of freedom were calculated using the Kenward-Roger option. Means were determined using the least squares means statement, and treatment means were compared using the PDIFF option. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Diets, animal performance, and rumen concentration of metabolites

Corn silage, alfalfa silage, and Fermenten chemical composition is reported in Table 1. Experimental diets were formulated to be iso-nitrogenous and iso-energetic. Model predicted RDP was decreased in EXP diets compared with CON (8.4 vs. 8.0% of DM; $P < 0.01$) as calculated by the CNCPS v. 6.5 and this was intended in diet formulation. Concentration of aNDFom tended to be greater in EXP diets (30.9 vs. 31.2% of DM; $P = 0.08$), although this is likely of limited biologic significance given typical variation in feeding management. All other analyzed nutrients were not different between diets ($P > 0.05$; Table 2).

Body weight change over the trial followed typical patterns for peak lactation dairy cattle, and was not affected by treatment. Body condition score similarly was not affected (data not shown); all cows averaged 2.25 ± 0.14 (mean ± SD) for the duration of the trial. Rumen degradable N source had no effect on intake or daily milk, protein or fat production (Table 3); however this trial was not specifically designed to assess effects on milk production. Milk urea N and plasma urea N concentration increased ($P = 0.01$) in cows fed the EXP diet and the relationship between rumen NH_3-N and plasma urea N is described in Figure 1. The slopes of the

lines were not different between treatments (0.63 for both CON and EXP; $P = 0.67$) and were similar to the results observed by Recktenwald et al. (2014). This suggests that Fermenten does not alter the rate of uptake of rumen NH_3-N. Rumen NH_3-N pool size and concentration was also increased ($P < 0.01$) in EXP cows (Table 4). This, combined with the increase in PUN and MUN in the cattle fed the EXP diet, indicated a possible reduction in utilization of rumen ammonia by microbes and subsequently greater ureagenesis and excretion of N in milk.

Ammonia-N concentrations of CON diets were very close to the minimal optimal concentration of 5 mg NH_3-N/dL to support efficient microbial growth as recommended by Satter and Slyter (1974). This is consistent with the desired formulation of rumen available N in order to determine the effect of the fermentation byproduct on microbial N use. The compositing of samples done in the current experiment limit the ability to investigate temporal fluctuations in rumen NH_3-N concentrations; it is possible that both diets experienced some time below 5 mg/dL. However, it is unlikely that rumen ammonia concentration significantly reduced microbial activity in this study, as no differences were observed in VFA concentration, pool size, or ruminal digestibility of DM, OM or NDF between treatments (Tables 4 and 5).

Rumen and total tract digestion of DM, OM, and NDF

Intake during the omasal sampling period was not different between diets ($P > 0.05$; Table 5). A slight disturbance of the cattle during sampling procedures might have reduced DMI during the omasal sampling period, therefore separate intakes are reported for the milk production data vs. the omasal sampling data (Tables 3 and 5, respectively). The average ruminal DM and OM digestibility in the experiment was 59.9 and 67.7%, respectively, and were not different between treatments. Rumen aNDFom digestibility averaged 31.2 and 33.4% for diets CON and EXP, respectively ($P = 0.24$) when expressed as a percent of total aNDFom. Digestion of the

potentially digestible pool was not different among treatments, and averaged 44.9 and 47.4% for diets CON and EXP, respectively ($P = 0.36$). True OM and DM digestion in the rumen was within the range reported by Huhtanen et al. (2010). Rumen aNDFom digestion was slightly lower than the mean determined by Huhtanen et al. (2010), however values observed in this study were similar those reported in studies performed with typical North American diets (Brito et al., 2006; 2007). The difference between total tract and rumen aNDFom digestibility in this experiment indicates that approximately 20% of the total-tract NDF digestion occurred post-ruminally. This is at the lower end of the range reported by Huhtanen et al. (2010), yet similar to the summary by Firkins (1997). The discrepancy could be related to the differences in datasets and methodologies represented in each summary. This study utilized a relatively high level of corn silage as the forage source and would be more similar to the results of Firkins (1997) and the North American diets represented in the Hutanen et al. (2010) review. North American diets represented the lower end of ruminal NDF digestion presented in the review, likely due to intake and forage type considerations.

The lack of response in aNDFom digestion to ruminal protein source has been observed previously when degradable protein sources were compared (Robinson, 1997; Brito et al., 2007). Brito et al., 2007 reported no difference in apparent ruminal NDF digestion when urea, soybean meal, cottonseed meal, and canola meal were fed; averaging 31% across diets. It is likely that diets in this study and Brito et al. (2007) provided enough RDP that NDF digestion was not negatively affected. Arroquy et al. (2004) also reported no effect of RDP source on NDF or OM digestion in steers fed low quality forage. Apparent total tract OM digestibility tended to be lower in cows fed EXP diets (70.9 vs. 69.2 % for CON and EXP respectively; $P = 0.07$). This could be related to the difference Ca sources used in the study, as $CaSO_4$ has a slightly lower

363 absorption coefficient than $CaC0_3$ (NRC, 2001) which might have affected the ash digestibility.
364 Diet Ca content averaged 0.87 % and 0.88 % of DM for CON and EXP, respectively, and were
365 formulated to meet or slightly exceed the requirements of a mid-lactation cow according to the
366 NRC (2001). It is unlikely that Ca sources had meaningful influences on the results of this study.

367 ***Omasal Nitrogen Flows and Ruminal N Digestibility***

368 Nitrogen intake was similar between the 2 diets (Table 6). Compared to CON diets, the
369 inclusion of the fermentation byproduct in EXP diets shifted 18 g/d of N from the ammonia N
370 pool (PA1) to the soluble AA and peptide pool (PA2) and true protein pool (PB1), according to
371 the CNCPS v. 6.5 protein fractionation scheme (Higgs et al. 2015; Van Amburgh et al., 2015).
372 This shift represents approximately 3% of total nitrogen intake. The flow of NAN was not
373 different between diets. Non-ammonia non-microbial N flow was tended to increase in cows fed
374 the EXP diet, while Microbial NAN flow as a percent of total flow was similar between diets.
375 Brito et al. (2007) reported an opposite effect when soluble true protein replaced urea in diets,
376 and the differences between diets are not immediately clear. A key difference between studies is
377 the diet composition, especially regarding fermentable starch sources and diet NDF content.
378 Diets in the Brito et al., (2007) study averaged 23.9 % NDF content, and the control diet
379 contained considerably more high-moisture ear corn than experimental diets. This might have led
380 to depression of microbial CP synthesis due to possible reduction in rumen pH and soluble AA N
381 availability. The lack of increased microbial N flow in the current study also contrasts with the
382 pre-trial expectation of increased microbial flow due to stimulation of microbes with soluble AA
383 and peptides from the byproduct. A meta-analysis of continuous culture studies with
384 fermentation byproducts has previously shown positive effects on microbial N flow from diets
385 containing the commercial fermentation byproducts Fermenten and BioChlor (Lean et al., 2005).

386 The meta-analysis reported a 0.271 g/d (15.7%) increase in microbial N flow with fermentation
387 byproduct inclusion using purines as a microbial marker. Assuming a mean purine concentration
388 of 952 mg/g of microbial N, as reported by Illg and Stern (1994), the increased microbial N flow
389 would have been calculated from approximately 258 mg/d of additional purines flowing from the
390 fermenters fed fermentation byproduct.

391 Commercial fermentations byproducts are derived from microbial fermentations, and as such
392 could contain relatively high levels of microbial purines. The Fermenten in this study had a N
393 content of 8.17 % of DM (51.1 % CP / 6.25). Assuming 75% of the N in the fermentation
394 byproduct was of microbial origin (Broderick et al., 2000), this would correspond to 0.0613 g of
395 microbially derived N / g of product. Commercial fermentations are typical performed using *E.*
396 *Coli*, or *C. glutamicum* and purine content can be estimated to be 10% of cell DM under
397 commercial growth conditions (Neidhart, 1996). Assuming a cellular N content of 8.5% of DM,
398 this would correspond to 1,176 mg purines / g of microbial N (10 / 8.5). Therefore, it is possible
399 that fermentation byproducts could contain approximately 72.1 mg of purines / g of product
400 (0.0613 g microbial N / g of product * 1,176 mg / g of microbial N). Lean et al., (2005) used an
401 average inclusion rate of 3.6 g/d of fermentation byproduct, which might corresponds to an input
402 of 260 mg/d more dietary purines relative to control diets; which is very similar to the estimated
403 increase in purine outflow of 258 mg/d associated with the 0.271 g/d increase in apparent
404 microbial nitrogen flow reported by Lean et al., (2005). This clearly presents the opportunity for
405 bias toward over-estimation of microbial protein flow when using purines as a microbial marker.
406 It is uncertain if this possible bias would be present using purines or purine derivatives during in
407 vivo experiments with fermentation byproducts. Broderick et al., (2000) tested several
408 fermentation byproducts in vivo vs urea control and reported no significant change in microbial

409 CP flows as estimated using purine derivatives. Use of purine derivatives may have limited the
410 ability to pick up significant difference in that study relative to the current study, as these
411 methods have lower precision and accuracy compared to techniques using ^{15}N, and tend to
412 underestimate microbial N flow in high producing cows (Reynal et al., 2005).

413 In the present study, the 65 gram difference in NANMN outflow was more than 3 times the
414 18 g difference in true soluble protein inflow associated with diet composition and intake. This
415 indicates that the inclusion of the fermentation byproduct in EXP diets had an associative effect
416 on protein degradation of other feedstuffs in the rumen, in effect, sparing rumen degradable
417 protein allowed more feed true protein to escape the rumen. Thus, when using NANMN to
418 calculate diet rumen undegraded protein concentration, diets contained 5.0 and 6.7% of diet DM
419 as RUP in CON and EXP diets, respectively ($P = 0.04$). True ruminal N digestibility was 15%
420 lower in EXP diets (68.7 vs. 58.3% for CON and EXP, respectively; $P = 0.05$). No differences
421 were observed in efficiency of microbial CP synthesis / g of OM digested in the rumen. Uptake
422 of AA N by cellulolytic bacteria has previously been assumed to be minimal to non-existent,
423 resulting in the assumption that NH_3-N is the sole source of N for microbial protein synthesis in
424 the rumen (Russell et al., 1992). However, more recent studies have clearly demonstrated
425 stimulatory effects of AA N on cellulolytic populations (Atasoglu et al., 2001; Yang, 2002),
426 which could possibly explain the results reported here.

427 Rumen protein degradation was decreased in cows fed the EXP diet, however rumen NH_3-N
428 pool size, concentration, and plasma urea N increased relative to cows fed the CON diet. While
429 this seems like a counter-intuitive result, one must consider that protein degradation does not
430 always proceed completely to ammonia production, thus elevating rumen ammonia
431 concentration. Production of NH_3-N is a result of complex microbial and enzymatic activities by

a community of mixed rumen microbes. Final rumen NH_3-N concentration is a dynamic balance of degradation processes, uptake by rumen microbes, passage rates and N recycling. In this study, it is possible the fermentation byproduct preferentially suppressed specific populations of proteolytic bacteria, allowing other groups with high affinity for soluble AA and peptides to benefit, such as hyper ammonia-producing bacteria (Russell et al., 1988). This group of microbes can account for disproportional amounts of ammonia production relative to their abundance in the rumen microbial population (Rychlik and Russell, 2000). Differential effects of fermentation byproduct on specific microbial populations could explain the results reported here, and may warrant further investigation.

Another possible mechanism for decreased CP degradation and increased rumen NH_3-N and could be related to the different rates of degradation of proteins vs. peptides in the rumen. Initial protein hydrolysis is rapid and occurs extracellularly, and previous in vitro work has demonstrated that subsequent peptide degradation and uptake is a rate limiting step for microbial protein synthesis (Broderick and Craig, 1989; Wallace et al., 1990). In the rumen, initial disruption of the tertiary structure of feed protein could allow peptides and AA to solubilize and flow with the liquid pool, thus escaping further degradation in the rumen. In the current experiment, it is possible that peptide hydrolysis and/or uptake by rumen microbes was decreased, resulting in increased undegraded feed N flow in the soluble phase. While the mechanism is unknown, inhibitors to peptide hydrolysis or uptake could be present in the fermentation byproduct. Commercial amino acid fermentations often utilize strains of bacteria specifically selected for increased AA production and excretion of peptides and AA. These specialized microorganisms also often have natural or artificial alterations in cellular feedback mechanisms, membrane permeability, cellular transport mechanisms, and substrate preferences

(Ikeda 2003). In a commercial fermentation, peptide degradation and AA uptake would be considered a negative trait. If signaling factors related to these traits are still present in the byproduct, it could provide a mechanism to could influence rumen microbial proteolytic activity. Of interest would be any possible inhibitors to protease or peptidase activity and changes in cell permeability. Further research into this area might provide a more specific mode of action for the results observed in this study.

While urea N recycling was not determined in this study, it is also possible that urea entry from the plasma pool allowed for elevated rumen NH_3-N levels (Marini and Van Amburgh, 2003; Valkeners et al., 2007). This may occur if post-ruminal protein metabolism, rather than rumen digestion, caused the increased ureagenesis relative to excretion. This would elevate the concentration of urea in the plasma pool and leading increased net influx into the rumen relative to the cows fed the CON diet. Without recycling information, it is unclear to the direction of N movement between these pools.

Ultimately, the sparing effect on degradable peptides and AA presents a key opportunity to utilize fermentation byproduct meal in conjunction with less expensive homegrown forages and protein feedstuffs such as alfalfa silage and untreated soybean meal. In such diets, overfeeding of degradable protein is common, as supply of metabolizable protein can be insufficient even at high levels of dietary crude protein. Future studies might investigate the ability of targeted feeding of degradable protein sources with fermentation byproducts to increase the income over feed cost and nitrogen utilization in nitrogen efficient feeding schemes.

CNCPS-Predicted vs. Observed N Flows

Total omasal N flow was well predicted by the model, while microbial N flow appeared to be under-predicted (Table 6). Alternatively, recent evaluations of CNCPS v6.5 (Van Amburgh et al., 2015) against omasal study data showed good agreement between predicted and observed microbial N flows. The difference between predicted and observed microbial flows is 16 to 28% below the measured flow and this amount of N would be similar to the protozoal contribution of the microbial flow, a microbial pool not described in this version of the CNCPS.

Rumen undegraded protein flow was overpredicted by 50% and 18% in CON and EXP diets, respectively. The gram amount of predicted RUP were fairly similar between diets, indicating that the model is not accounting for protein sparing effect of fermentation byproducts. Within the structure of the model, microbial populations are stimulated when peptide balance is positive (Russell et al., 1992), however the assigned rates of degradation of many feedstuffs results in high peptide balance in most simulations. Updates to the feed library (Higgs et al., 2015) and model (Van Amburgh et al., 2010; Van Amburgh et al., 2015) have sought to correct this; however the current structure of the rumen sub-model in CNCPS v. 6.5 has limited the ability to describe microbial N dynamics in a more mechanistic way, especially the interactions and associative affects between microbial populations and substrate. Endogenous N contributions to RUP flow are not differentiated in this study and are not mechanistically described in the CNCPS. This would lead to additional differences between model predictions and observations in this experiment.

CONCLUSIONS

In this study, the inclusion of a fermentation byproduct vs. urea and wheat midds resulted in changes in omasal N flows. Previous in vitro studies utilizing the same product have observed

498 increases in apparent microbial N flows; which was attributed to stimulation of rumen microbial
499 growth by soluble AA and peptides. In this study, it is unlikely that differences in flow were due
500 to the stimulation of rumen microbes. Total NAN and Microbial N flow was not different
501 between diets; however, we did observe a tendency for increased in NANMN flow at the omasal
502 canal. Rumen undegraded protein (% of DM intake) was significantly increased in cows fed the
503 fermentation byproduct. The 65 g difference in NANMN flow was unlikely to be caused by the
504 hypothesized stimulation of microbes by soluble AA and peptides, since the treatments only
505 provided an additional 18 grams of soluble AA N. It is more likely that a different factor present
506 in the fermentation byproduct altered microbial degradation and/or microbial uptake of N
507 through an unknown mechanism, resulting in a 15% decrease in apparent ruminal protein
508 degradation. This result may be beneficial in feeding applications where excess rumen
509 degradable protein is fed; as is typical in many feeding applications using fermented forages and
510 byproducts from human food and fiber production.

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Table 1. Chemical composition (mean ± SD)[1] of select feeds used in the experiment

Item	Corn silage	Alfalfa silage	Fermenten[2]
DM, %	32.6 ± 0.7	33.7 ± 0.9	90.1
CP, % of DM	7.3 ± 0.4	21.8 ± 0.6	51.1
Soluble protein, % of CP	57.2 ± 2.7	61.3 ± 3.7	77.1
NDICP, % of CP	14.3 ± 1.2	10.7 ± 1.2	1.3
ADICP, % of CP	11.4 ± 0.3	8.8 ± 1.0	4.0
aNDFom, % of DM	40.0 ± 2.6	40.3 ± 2.0	23.6
30h uNDFom, % of aNDFom	46.2 ± 2.1	52.4 ± 3.0	-
120h uNDFom, % of aNDFom	29.6 ± 1.0	46.5 ± 2.7	-
240h uNDFom, % of aNDFom	25.1 ± 1.8	42.3 ± 2.6	-
ADF, % of DM	26.2 ± 2.2	34.2 ± 2.2	23.8
ADL, % of DM	3.2 ± 0.2	7.9 ± 0.6	2.5
Starch, % of DM	33.6 ± 1.8	1.0 ± 0.5	14.8
Ether extract, % of DM	3.5 ± 0.1	4.0 ± 0.3	2.9
Ash, % of DM	3.1 ± 0.1	11.0 ± 0.4	5.9

[1]Analyzed values from 3 period composite samples.

[2]Church & Dwight, Inc., Princeton, NJ. Single batch/lot used for entire experiment

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Table 2. Ingredient and nutrient composition (mean ± SD)[1] of experimental diets

	Diet	
Item	CON	EXP
Ingredient composition, % DM		
Corn silage	44.6	44.6
Alfalfa silage	12.0	12.0
Corn meal	12.0	12.0
Expeller soybean meal[2]	8.0	8.0
Soybean hulls	5.8	5.8
Citrus pulp, dry	3.3	3.3
Chocolate meal	2.4	2.4
Saturated fatty acid[3]	1.2	1.2
Molasses	0.9	0.9
Blood meal	1.7	1.7
Wheat middlings	4.8	3.2
Fermentation byproduct[4]	–	3.0
Calcium carbonate	–	0.7
Urea	0.4	–
Calcium sulfate, dihydrate	1.7	–
Sodium bicarbonate	0.33	0.40
Salt white	0.30	0.32
Magnesium oxide	0.17	0.17
Dicalcium phosphate	0.16	0.16
Supplemental methionine[5]	0.06	0.06
Vitamin and mineral mix[6]	0.18	0.18
Nutrient composition		
DM, %	44.5 ± 0.7	44.2 ± 0.8
OM, % of DM	93.9 ± 0.3	93.8 ± 0.6
CP, % of DM	15.9 ± 0.6	16.1 ± 0.5
RDP, % of DM[7]	8.4 ± 0.1	8.0 ± 0.1
Starch, % of DM	27.5 ± 1.1	27.8 ± 0.5
Sugars, % of DM	5.4 ± 0.4	5.3 ± 0.3
NFC, % of DM[7]	41.7 ± 0.2	41.8 ± 1.3
aNDFom, % of DM	30.9 ± 0.2	31.2 ± 0.2
ADF, % of DM	19.9 ± 1.5	19.7 ± 0.6
ADL, % of NDF	10.0 ± 0.9	10.0 ± 1.4
Ether extract, % of DM	5.0 ± 0.2	4.9 ± 0.2
ME, Mcal/kg[7]	2.5 ± 0.1	2.5 ± 0.1

[1]Analyzed values from 3 period composite samples.
[2]SOYPLUS (West Central Cooperative, Ralston, IA).
[3]ENERGY BOOSTER 100 (MSC Company, Dundee, IL).
[4]FERMENTEN (Church & Dwight, Inc., Princeton, NJ).
[5]SMARTAMINE M (Bluestar Adisseo Nutrition Group, Alpharetta, GA).
[6]Provided (per kg of diet DM): 44 mg of Zn, 32 mg of Mn, 10 mg of Cu, 1 mg of Co, 1 mg of I, 0.3 mg of Se, 5000 IU of vitamin A, 980 IU of vitamin B, and 25 IU of vitamin E.
[7]Calculated by the Cornell Net Carbohydrate and Protein System v. 6.5.

Table 3. Effect of rumen available nitrogen source on dry matter intake, milk production, and animal performance

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
Dry matter intake, kg/d	25.5	26.4	0.9	0.34
Milk yield, kg/d	41.7	43.1	1.4	0.36
ECM, kg/d	41.7	43.1	1.9	0.48
Milk fat, %	3.53	3.50	0.11	0.77
Milk fat, kg/d	1.47	1.51	0.08	0.60
Milk true protein, %	2.85	2.86	0.07	0.86
Milk true protein, kg/d	1.19	1.22	0.06	0.55
Milk urea N, mg/dL	10.5	13.0	0.4	<0.01
Plasma urea N, mg/dL	8.7	11.0	0.7	0.01
Urine urea N, mg/dL	30.4	48.1	19.2	0.37
Feed efficiency[3]	1.64	1.64	0.06	0.97
Body weight change, kg/d	0.29	0.39	0.12	0.58

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Values calculated from data collected on d 19-21 of each experimental period.
[3]ECM/dry matter intake.

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Table 4. Effect of rumen available nitrogen source on rumen concentration and pool size[1] of ammonia N and volatile fatty acids (VFA)

	Diet[2]			
Item	CON	EXP	SEM	*P*
Ammonia N pool size, g	4.50	5.24	0.45	0.02
Ammonia N concentration, mg/dL	5.41	6.41	0.39	0.01
VFA pool size, mol				
Total VFA	8.05	8.12	0.31	0.81
Acetate (A)	5.23	5.30	0.16	0.71
Propionate (P)	1.87	1.87	0.14	0.95
Butyrate	0.73	0.73	0.03	0.97
Isobutyrate	0.02	0.02	0.00	0.87
Valerate	0.10	0.11	0.01	0.45
Isovalerate	0.09	0.10	0.01	0.56
Branched-chain VFA	0.12	0.12	0.01	0.76
A:P ratio, mol/mol	2.96	2.88	0.16	0.62
VFA concentration, m*M*				
Total VFA	97.3	99.3	3.0	0.48
Acetate	63.6	64.8	2.2	0.55
Propionate	22.1	23.0	1.0	0.55
Butyrate	8.9	9.0	0.4	0.69
Isobutyrate	0.3	0.3	0.1	0.77
Valerate	1.2	1.3	0.1	0.30
Isovalerate	1.1	1.2	0.1	0.53
Branched-chain VFA	1.4	1.5	0.2	0.78

[1]Nutrient concentration x rumen liquid volume measured from total rumen evacuation.

[2] CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.

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Table 5. Effect of rumen available nitrogen source on digestibility of DM, OM, and NDF

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
DM				
Intake, kg/d	23.8	23.9	0.7	0.91
Flow at omasal canal, kg/d	16.7	16.1	0.6	0.41
Apparently digested in the rumen, kg/d	7.1	7.9	0.4	0.15
Truly digested in the rumen, kg/d[3]	14.3	14.2	0.4	0.90
% of DM intake	60.3	59.6	1.4	0.72
Total tract apparent digestibility, %	68.6	68.2	0.5	0.47
OM				
Intake, kg/d	22.1	22.0	0.6	0.95
Flow at omasal canal, kg/d	13.4	12.8	0.5	0.39
Apparently digested in the rumen, kg/d	8.7	9.3	0.4	0.30
Truly digested in the rumen, kg/d[3]	15.0	14.9	0.4	0.77
% of OM intake	68.2	67.4	1.6	0.73
Total tract apparent digestibility, %	70.9	69.2	1.0	0.07
NDF				
Intake, kg/d	7.3	7.5	0.2	0.72
Flow at omasal canal, kg/d	5.1	5.0	0.2	0.70
Apparently digested in the rumen, kg/d	2.3	2.5	0.1	0.18
% of NDF intake	31.2	33.4	1.3	0.24
% of pdNDF intake	44.9	47.4	1.9	0.36
Total tract apparent digestibility, %				
% of NDF intake	41.0	40.8	1.0	0.89
% of pdNDF intake	59.0	57.8	1.3	0.49

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]Values calculated from data collected on d 24-27 of each experimental period.
[3]Corrected for microbial and volatile fatty acid contribution to flows.

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Table 6. Effect of rumen available nitrogen source on omasal nitrogen flow and digestibility

	Diet[1]			
Item[2]	CON	EXP	SEM	*P*
N intake, g/d	603	613	18	0.70
CNCPS fraction PA1	61	43	-	-
CNCPS fraction PA2	171	183	-	-
CNCPS fraction PB1	304	310	-	-
RDP Supply[3]				
g/d	2578	2230	117	0.05
% of DMI	10.9	9.4	0.6	0.07
Flow at omasal canal				
Total N, g/d	664	693	25	0.37
Total N flow predicted by CNCPS v. 6.5, g/d	664	674	-	-
Ammonia N, g/d	21.5	22.4	1.5	0.67
NAN				
g/d	642	670	25	0.38
% of N intake	106.6	109.1	3.4	0.58
NANMN				
g/d	191	256	26	0.09
% of N intake	31.3	41.7	3.5	0.05
RUP[4]				
g/d	1192	1601	159	0.09
% of DMI	5.0	6.7	0.6	0.04
RUP flow predicted by CNCPS v. 6.5, g/d	1784	1887	-	-
Microbial NAN				
g/d	450	409	28	0.31
% of total NAN	69.9	61.5	3.5	0.11
Microbial N flow predicted by CNCPS v. 6.5, g/d	351	352	-	-
Microbial efficiency				
g of microbial CP/kg of OTDR	28.9	26.1	1.7	0.26
True ruminal N digestibility, %	68.7	58.3	3.5	0.05
aNDFom digested/g of dietary CP degraded	0.97	1.23	0.1	0.02

[1]CON = 3% of diet DM as urea control mix; EXP = 3% of diet DM as fermentation byproduct.
[2]NANMN = non-ammonia non-microbial N, OTDR = organic matter truly digested in the rumen.
[3]Rumen degradable protein (RDP) supply = CP intake – RUP flow.
[4]Rumen undegradable protein (RUP) = NANMN x 6.25.

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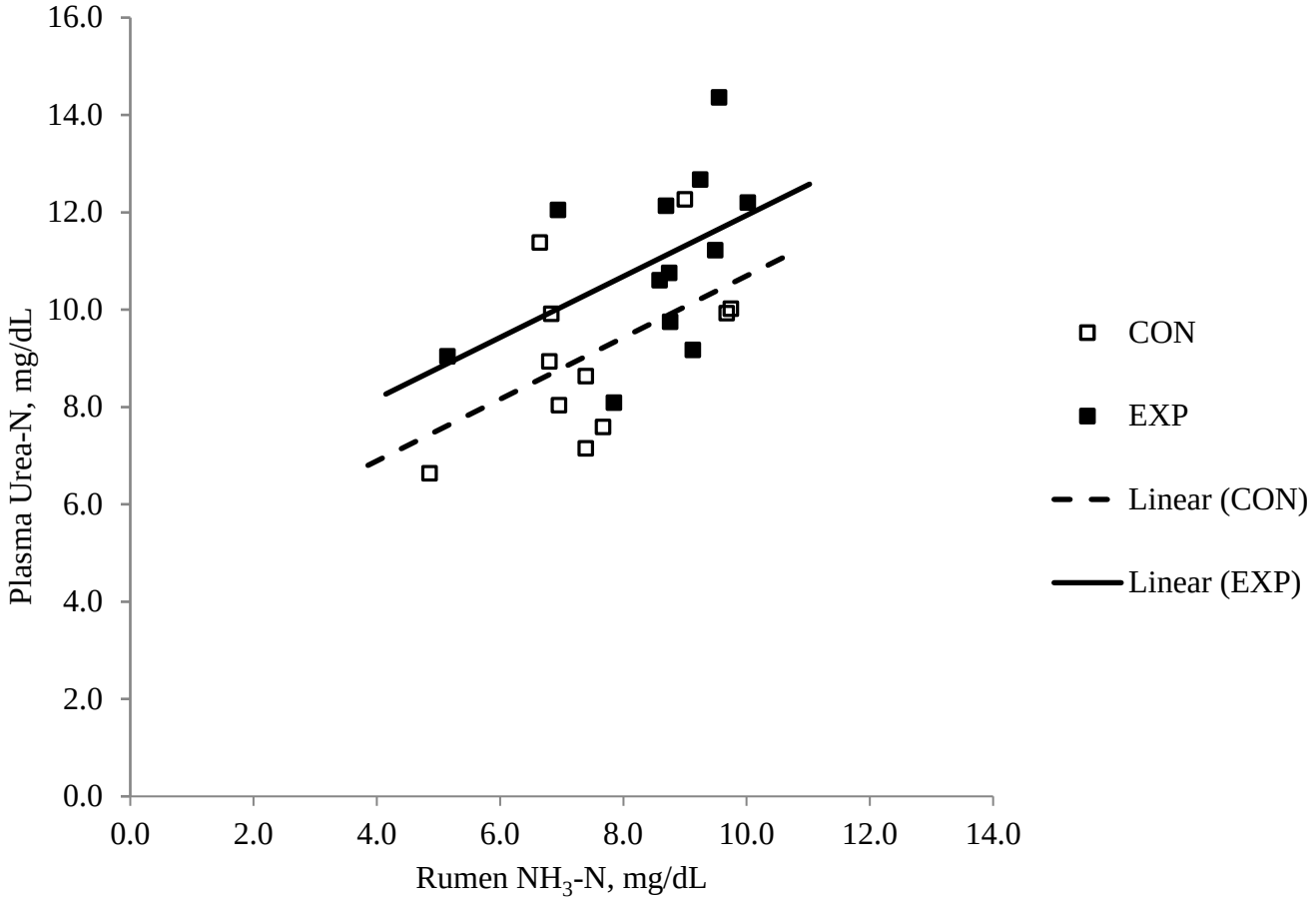

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671 **Figure 1.** Relationship between rumen NH_3-N and plasma urea N in lactating dairy cows fed two
672 different sources of rumen available N, where CON (□) = 3% of diet DM as urea control mix;
673 EXP (■) = 3% of diet DM as fermentation byproduct meal. The equation representing
674 relationship in cattle fed diet CON is $y = 0.6338x + 4.356$, $R^2 = 0.27$; the equation describing the
675 relationship in cattle fed diet EXP is $y = 0.6274x + 5.663$, $R^2 = 0.22$.

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