THE AMINO ACID CONTENT OF RUMEN MICROBES, FEED, MILK AND TISSUE AFTER MULTIPLE HYDROLYSIS TIMES AND IMPLICATIONS FOR THE CNCPS

M. E. Van Amburgh, A. F. Ortega, S. W. Fessenden, D. A. Ross, and P. A. Lapierre
Department of Animal Science
Cornell University

Nutrient supply and requirement models such as the Cornel Net Carbohydrate and Protein System (**CNCPS**) (Higgs et al., 2015; Van Amburgh et al., 2015) and the NRC (NRC, 2001) predict post-ruminal flows of nutrients from feed proteins that escape rumen fermentation and rumen microbes. Feed and microbial protein flow has an AA content, AA profile, and a digestibility of individual AA to calculate supply of metabolizable AA. The CNCPS has been updated into a new dynamic framework (v. 7; Higgs, 2014; Higgs et al. submitted) where all amino acids and other protein components are described by their nitrogen (N) content and the model uses a more mechanistic approach then previous versions as it accounts for protozoa and endogenous AA contributions to total AA flow in addition to bacteria and feed. To improve this model, accurate representations of the AA content and digestibility of all sources of AA were needed to understand sources of error in predictions of particular AA. On an N basis, the predictions of non-ammonia nitrogen (NAN) in CNCPS v. 7 were reasonably accurate and precise. However, predictions of individual AA such as Lys, Met, Ile, Leu, and Val were biased, suggesting there was a lack of information about the true content those AA in both microbes and feeds and possibly their digestibility (Fessenden et al., 2017; Higgs, 2014; Higgs et al., submitted).

The CNCPS feed library was recently updated with new chemical composition information, especially the AA profile and content of feeds (Higgs et al., 2015). Further, the CNCPS uses the AA profile of bacteria obtained from the literature (Storm et al., 1983; Clark et al., 1992; Volden and Harstad, 1998), and few of those studies accounted for protozoal AA flows, which can contribute a substantial amount to total microbial AA flow (Dijkstra et al., 1998; Fessenden, 2016). Given the updated AA information in the feed library, the biased flow predictions of certain AA in the CNCPS v7 was surprising because the AA are described on a N basis and there was no bias in the prediction of NAN, thus to observe bias in the predictions of the AA when the total NAN was decomposed into individual AA suggests two possibilities: the AA profiles and contents of feed and microbes are not correct and or the digestibility of the fractions are misunderstood or unknown. Therefore, we needed to challenge the information that was available to us describing both AA content and digestibility of both rumen microbes and feed.

The AA content of feeds and microbes have historically been determined by single time point hydrolysis, as this represents a compromise between maximal release of AA from the matrix while minimizing the loss of acid labile AA (Rutherfurd, 2009). Determination of AA at multiple time points followed by least-squares non-linear regression appears to provide more accurate estimates of the AA profile (Darragh and Moughan, 2005). This approach has been utilized in purified protein (Darragh et al., 1996), milk protein (Rutherfurd et al., 2008) and common animal feedstuffs (Rutherfurd,

2009). Previous work in our laboratory indicated that to obtain the greatest release of branched-chain AA in forages, hydrolysis times needed to be greater than 21 hr and Ile release was greatest at 70 hr (Ross, 2004) but we did not challenge the literature at that time. It was apparent that as the hydrolysis times were extended, certain AA were destroyed, whereas others demonstrated greater release from the carbohydrate matrix which suggested the use of multiple time point hydrolysis to observe optimum recovery of all AA.

Given the data from Darragh and Moughan (2005) and Rutherfurd (2009) and the observations made from the data of Higgs et al. (submitted), the hypothesis of this work was that the standard method of determination of AA in feed, milk, tissue and ruminal bacteria and protozoa using single time point hydrolysis is not equivalent to AA determination after multiple time point hydrolysis and non-linear least-squares regression. The implications for model libraries, efficiencies of use and AA formulation is significant and requires further evaluation as we move closer to more precise and accurate predictions of AA requirements and supply.

Materials and Methods

Bacteria and protozoa included in the analysis were from the following experiments: Trial A: An omasal sampling trial with 8 cows in a 2 treatment switchback design investigating effects of a commercial byproduct feed on omasal nutrient flow (Fessenden, 2016); Trial B: An omasal sampling trial with 12 cows in a 3 treatment Latin Square design investigating the effect of rapidly degradable starch on omasal nutrient flow (Foskolos et al, unpublished data); Trial C: A ruminal N balance and recycling trial with 12 cows in a 3 treatment randomized complete block design investigating ruminal N and/or MP deficient diets (Recktenwald, 2010; Recktenwald et al., 2013). One additional protozoal sample was obtained from T. Hackmann at the University of Florida from repeated isolations from the rumen of a lactating dairy cow at the Ohio State University Columbus campus (Trial D). For trials A-C, equal parts DM were combined within microbial type, resulting in a composited sample of bacteria and protozoa from each experiment. Due to limited amount of sample for some trials (D and C) not all analysis were performed on all samples as noted throughout the text.

Bacterial isolations for trial A and C were 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. Bacterial isolation for trial B followed the same procedure as described above, however formalin was not used. Protozoa from trials A and B were isolated from whole contents using the same procedure as described by Denton et al. (2015) and modified as reported in Fessenden et al. (2017). The only difference between trials was the omission of formalin and centrifugation in Trial B.

Twenty-six feed samples, which were previously used in studies evaluating omasal flow of nutrients from lactating dairy cattle were chosen for this study, as well as six tissue samples that were collected from past experiments in the laboratory (Diaz et al., 2001; Meyer, 2005) and four milk samples that varied in MUN collected over three days. The feed samples were previously analyzed for complete chemical analysis and used in the CNCPS (v7, Higgs, 2014) to formulate diets for experiments in which AA content of the diets were known and the flow of such nutrients from the rumen was measured in the experiments.

Feed, tissue and milk samples were analyzed for dry matter (DM) after 16 h at 105°C (AOAC, 2016). Milk samples were analyzed for dry matter after freeze drying for 24h. Total feed and tissue N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). The AA content of all feed samples was determined by HPLC following hydrolysis at 110°C in a block heater (Gehrke et al., 1985) for 2, 4, 6, 12, 18, 21, 24, 30, 48, 72, 120 and 168 h. For tissues and milk only 21, 72 and 168 h time points were used. The time points chosen were based on publications by Rutherfurd et al. (2008) and Rutherfurd (2009) where long-term hydrolysis was shown to release certain AA from the sample, thus increasing the estimated AA content of various substrates that were used in each individual study. In addition, the time points were similar to a previous study performed in our laboratory on rumen and omasal microbial composition, which demonstrated similar AA outcomes as hydrolysis time was extended (Fessenden et al., 2017). For Trp in tissue and milk, only 16 h and 24 h time points were used as it has been shown that there is no significant change in Trp concentration after these time points. The entire time course was performed twice for each sample using acid-washed (50% nitric acid) glassware, and the reported values are the mean of the two determinations.

Standard Acid Hydrolysis

Samples containing 2 mg of N was weighed into Teflon-lined screw top hydrolysis tubes and 50 µL of 125 mM norleucine was added as an internal standard, as well as 5 mL of high-purity 6 M HCl. The tubes were then flushed with N_2 gas for 10 seconds and placed in boiling water for 10 minutes to remove oxygen. The samples were hydrolyzed as described above for the different time points. After hydrolysis, the tubes were cooled slightly and the tube contents were filtered through Whatman 541 filter paper and the filtrate was diluted to 50 mL in a volumetric flask with HPLC grade H_2O. Aliquots of 0.3 mL were frozen at -20°C to prevent loss of various AA, such as Ser, Thr and Tyr (Gehrke et al., 1985). Aliquots were evaporated at 65°C under constant N_2 flushing, with 3 rinses and re-evaporations with HPLC grade H_2O to remove acid residues, as indicated by the smell of chlorine. After final evaporation, the hydrolysate was dissolved in 0.6 mL of Na

diluent and analyzed by the HPLC (Na220, Pickering Laboratories, Mountain View, CA).

Sulfur AA Acid Hydrolysis

Analyzing the sulfur-containing AA, Met and Cys, samples containing 2 mg of N and the internal standard, norleucine, were pre-oxidized with 1 mL performic acid to be analyzed as cysteic acid and methionine sulfone. Performic acid was prepared by combining 0.9 mL of 88 % formic acid, 0.1 mL of 30% H_2O_2 and 5 mg phenol, incubated for 40 minutes at room temperature and constant stirring, and moved to an ice bath at 4°C for 20 minutes. The tubes were then sonicated in slushy water for 15 minutes and transferred to an ice bath at 4°C for 16 h (Mason et al., 1980). The oxidizing reaction was stopped and the excess performic acid reduced by adding 0.2 mL of concentrated HCl and allowing the tubes to stand for 15 minutes. For tubes being used for the 120h and 168h time points, the tubes were placed under vacuum using a water aspirator to remove residual PA and HCl (Elkin and Griffith, 1985). All tubes were then hydrolyzed and filtered as described above for standard acid hydrolysis.

AA Analysis by HPLC

Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no 1154110T, Pickering Laboratories, Mountain View, CA) using a 4-buffer step gradient and column temperature gradient. Detection of separated AA was performed at 560 nm following post-column ninhydrin derivation. Standards (250 nM/mL) for the individual AA were prepared by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto the column was 10 µL.

Tryptophan Hydrolysis and Analysis

For Trp determination, a separate aliquot of sample containing 2 mg N was added to a Teflon-lined screw top hydrolysis tube with 1.2 g of $Ba(OH)_2$, 0.125 ml of 5-methyl-trp (5MT), and about two mL of HPLC grade H_2O. The tubes were flushed with N_2 gas for 10 seconds and placed in boiling water for 15 minutes to remove oxygen. The tubes were hydrolyzed at 110°C for the same time course as used for the other AA on a block heater (Landry and Delhaye, 1992; Ross, 2004). Included in the hydrolysis was 125µL of 5-Methyl- Trp (4m*M*) as an internal standard. Tubes were removed quickly and the contents were transferred to an Eppendorf tube and placed in ice. After cooling to precipitate barium ions, an aliquot (3 µL) of the hydrolysate was added to 1 mL of acetate buffer (0.07 *M* sodium acetate) and analyzed by HPLC in which AA were detected by fluorescence (excitation = 285 nm, emission = 345 nm).

Calculations and Statistical Analysis

The AA concentrations were corrected for norleucine, the internal standard, using Equation 1 and calculated as mg AA g DM^{-1} with Equation 2 (Ross, 2004).

Equation 1. Norleucine correction

$$Corrected\ nM\frac{AA}{ml} = \frac{\left(nM\frac{AA}{ml}from\ chromatogram\right) \times (nM\frac{Norl}{ml}added)}{(nM\ Norl\ from\ chromatogram)}$$

Equation 2. Amino acid content of residue, mg AA g DM^{-1}

$$mg\ AA\ g\ DM^{-1} = \frac{(Corrected\ nM\frac{AA}{ml} \times AA\ MW \times hydrolyzed\ sample\ volume, ml)}{(sample\ wt, g \times 10^6 \times residue\ DM, \frac{g}{g})}$$

Determination of the true AA concentration of feed was performed using a method similar to that of Fessenden et al. (In press, 2017). Each AA concentration (mg/g of DM) was plotted against hydrolysis time using the following a non-linear equation:

$$B(t) = \frac{A_0 h(e^{-lt} - e^{-ht})}{h - l}$$

where $B(t)$ is the AA concentration at time t, h is the hydrolysis rate (proportion of bound AA hydrolyzed per hour), l is the loss rate (proportion of bound AA destroyed per hour) and A_O is the actual AA content of the protein within the sample (Rutherfurd 2008; Rutherford et al. (2009). The variables, A_O, h and l for each sample were derived from each AA using least-squares non-linear regression with the constraints that $A_O > 0$, and $h > 0$, using SAS version 9.4 (SAS institute, Cary, NC). The 24h (21h) and 168h AA concentrations were compared for each EAA and feed, milk, or tissue and a T-test was performed to measure significance between concentrations. Significance was declared when $P < 0.05$ and a trend was identified at $P < 0.10$.

Microbial AA Content and Profiles

The release of individual AA in trial B bacteria and protozoa are in Tables 1 and 2. Extraction of Ile, Met, and Val demonstrated greater release over time and thus positive slopes at time points greater than 24 h and hydrolysis rate were lowest for these AA. Of the NEAA of the protozoa, Ala, Cys and Pro demonstrated increasing concentrations of AA as hydrolysis time increased. Overall, total AA were hydrolyzed from the sample matrix at a rate of 0.415 and 0.357 mg/h for bacteria and protozoa, respectively. The same least-squares non-linear regression approach has been previously employed in the analysis of other AA containing compounds, including lysozyme (Darragh et al., 1996), cat hair (Hendriks et al., 1998), human milk (Darragh and Moughan, 1998) and some common feedstuffs (Rutherfurd, 2009). Rutherfurd (2009) reported similarly low h for Ile and Val, while Ser was reported to have the highest l of any AA.

The use of multiple hydrolysis times provides some insight into the appropriateness of single time point hydrolysis for AA in rumen microbial samples. While both techniques are simply estimates of the theoretical unknown true AA composition, the regression method has been shown to more accurately estimate the true AA profile in purified proteins (Darragh et al., 1996). The AA profile determined from the regression compared with the value determined at 24 h was used to establish the equivalency of the two methods in relation to biologically relevant ranges (Table 6). This alternative framework of hypothesis testing requires thoughtful interpretation of the results. While some AA may exhibit negligible mean differences between analysis method, such as His and Thr, the interpretation of the 90% CI indicates that they are not equivalent, as the CI lies outside the pre-determined rage of biologically relevant differences. Of the bacterial AA, the 24 h time point method was determined to be not equivalent to the multiple time point hydrolysis method for every AA except Gly. The 90 % CI of the mean difference was greater than ± 1 g/100g AA for Ile, Leu, Met, and Val. The relatively large underestimation of Ile, Met, and Val results in an overestimation of approximately 5% for the rapidly hydrolyzed AA such as Arg, Leu, and Lys. This is similar to the results of Rutherfurd (2009), where soybean meal Ile content was underestimated by 8.4 %, followed by Val (7.0%), Ser (4.6%), and Thr (4.3%). The relatively low range in acceptable equivalence (mean difference of -0.4 to 0.4 g/g100 AA for bacteria) serves to emphasize the importance of the AA profile of bacteria on AA supply determinations.

Table 1. Comparison of the AA composition (g/100 g AA) of omasal bacteria from trial B[1] determined using multiple hydrolysis time point or single hydrolysis time point methods (Fessenden et al., 2017).

	Method				90 % CI		
AA	Single	Multiple	S - M	SED[2]	Lower	Upper	EQ[3]
Essential AA							
Arg	5.00	4.73	0.27	0.03	0.06	0.48	No
His	2.12	2.11	0.01	0.14	-0.85	0.86	No
Ile	4.05	4.62	-0.58	0.46	-3.46	2.31	No
Leu	5.60	5.32	0.28	0.26	-1.35	1.91	No
Lys	7.54	7.17	0.37	0.04	0.11	0.63	No
Met	4.49	4.63	-0.14	0.36	-2.41	2.13	No
Phe	6.00	5.77	0.23	0.09	-0.31	0.77	No
Thr	5.49	5.53	-0.04	0.10	-0.69	0.60	No
Trp	5.97	5.77	0.20	0.03	-0.01	0.41	No
Val	5.92	6.32	-0.41	0.32	-2.41	1.60	No

[1]Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.
[2]Standard error of the difference.
[3]Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -0.4 to 0.4 g/100g of AA.

Protozoa AA determinations between methods showed more general agreement between hydrolysis methods, largely due to the greater range in equivalence limits (mean difference of -1.5 to 1.5 g/100g AA for protozoa). Six of the 10 EAA were deemed

equivalent between methods (Table 2). Similar to the bacterial results, Ile and Met were underestimated (13.4 and 16.5 %, respectively) when determined with a single time point hydrolysis, resulting in over estimation of several other AA, namely Lys.

Table 2. Comparison of the AA composition (g/100 g AA) of omasal protozoa from trial B[1] determined using multiple vs. single time point hydrolysis methods (Fesssenden et al., 2017).

	Method				90 % CI		
AA	Single	Multiple	S - M	SED[2]	Lower	Upper	EQ[3]
Essential AA							
Arg	5.35	5.26	0.09	0.15	-0.84	1.03	Yes
His	2.53	2.52	0.01	0.01	-0.03	0.05	Yes
Ile	3.80	4.39	-0.59	0.06	-0.94	-0.24	Yes
Leu	6.11	6.25	-0.14	0.41	-2.70	2.42	No
Lys	8.81	8.55	0.26	0.06	-0.10	0.62	Yes
Met	3.14	3.77	-0.62	0.47	-3.58	2.34	No
Phe	6.49	6.58	-0.08	0.24	-1.61	1.45	No
Thr	5.41	5.34	0.07	0.03	-0.13	0.26	Yes
Trp	4.76	4.95	-0.19	0.27	-1.90	1.52	No
Val	4.65	4.75	-0.10	0.04	-0.38	0.18	Yes

[1]Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.
[2]Standard error of the difference.
[3]Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -1.5 to 1.5 g/100g of AA.

Implications for AA Predictions in Mathematical Nutritional Models

The non-equivalence of the determination methods are important to consider when developing models that rely on AA profiles of microbial protein and feedstuffs. The results from this study and the Rutherfurd (2009) data indicate that specific AA, especially Ile, Leu, Met, and Val could be underestimated in many post-ruminal AA flow studies when utilizing single time point hydrolysis between 21 and 24 h. This consideration should recognized when literature values for AA are used in development and evaluation of nutritional models that seek to accurately predict AA supply, especially those that utilize mechanistic post-absorptive sub-models. For example, in this analysis Met was determined to contribute more to total AA than has previously been reported. Currently, the CNCPS v.6.55 uses a profile that corresponds to approximately 1.2% of microbial AA as Met (Higgs et al, 2015; Van Amburgh et al., 2015). Compared with the current analysis (4.7 % of total AA), predictions of AA supply from the model would be expected to increase more than 2 fold (assuming microbial AA accounts for 50% of total AA). Adoption of these values will likely result in a re-evaluation of many common ratios and relationships currently used to balance essential AA for lactating cattle. Given the data presented here and by Rutherfurd (2009), this might also be true for many of the EAA. The current data, especially regarding the branched-chain AA, would help explain the prediction bias for those AA observed in CNCPS v.7 despite the relatively good prediction

of NAN (Higgs, 2014; Higgs et al. submitted). Overall, this analysis illustrates how sensitive nutritional models that rely on microbial AA profiles could be to errors in AA analysis, especially when a single profile accounts for a large portion of the predicted AA supply. Additionally, future studies should evaluate the use of formalin as a microbial preservative if AA analysis or digestibility is considered as an outcome. Model developers should not include any data from procedures that utilize formalin as a microbial preservative, as it will likely lead to biases and poor model evaluation.

Feed AA content

The sum of the concentrations of EAA released from the feeds after multiple hydrolysis times from 24 h to 168 h are presented as least-squares non-linear regression lines, with each data point representing the amount of AA released at each hydrolysis time. The two concentrates and two forages, out of the 26 feeds analyzed, were chosen because they are the most widely used feeds in dairy cattle diets. Overall, the EAA show an increase in release after 24 hr, which resulted in the positive slope observed in the regressions (Figure 1) ($P < 0.05$). The hydrolysis rate for the sum of the EAA ranged from 0.3 to 0.5 mg/h. Of the EAA, the BCAA (Ile, Leu, Val) and Lys are also presented as least-squares non-linear regression lines because of they are the EAA with the greatest increase in release after the 24 hours (Figures 2, 3, and 4). The hydrolysis rate of Leu averaged 0.3 mg/h, except for corn silage that averaged 0.7 mg/h (Figure 2). The hydrolysis rate for Ile averaged 0.3 mg/h for the four feeds in Figure 3. And the hydrolysis rate of Lys averaged 0.45 mg/h, except for corn silage that averaged 1.0 mg/h (Figure 4). For the four feeds represented and the selected AA, it is apparent that there is a continued release of AA from the feed matrix over time and that maximum recoveries of the BCAA and Lys are occurring at very long hydrolysis times with no apparent degradation of the AA.

For the five feeds highlighted in Table 3, the BCAA content increased when measured at the 168 hr hydrolysis endpoint. A similar observation can be seen for Lys for four of the feeds except the corn grain, which remained stable (Table 3). Also, when measured at the longer hydrolysis time point, His content was significantly greater in the blood meal suggesting that blood meal of high digestibility could be a better source of His than currently recognized. The AA content of the analyzed feeds follows the same pattern as that observed in the microbial data, suggesting that the AA content currently being used in the CNCPS feed library needs to be revised again to better reflect the true AA content of feeds. Another update would be a significant undertaking as no database currently exists describing these observations, so many feeds need to be analyzed to fully describe the AA content using the updated approach. Given the uniform response in AA content, either up or down from 21 to 168 hr, it seems reasonable to consider analyzing the AA content at the two time points to characterize feed or other substrate AA content. The least square, non-linear regression takes a considerable amount of time points to use and if the results are uniform, then running simple t-tests or equivalency tests as done in the microbial data might allow us to evaluate more feeds and substrates in a more time and cost efficient manner. Also, if the release of AA from particular substrates is uniform, then it might be possible to apply simple coefficients for specific AA for forages or

concentrates for example and this would be very efficient if found to be precise and accurate.

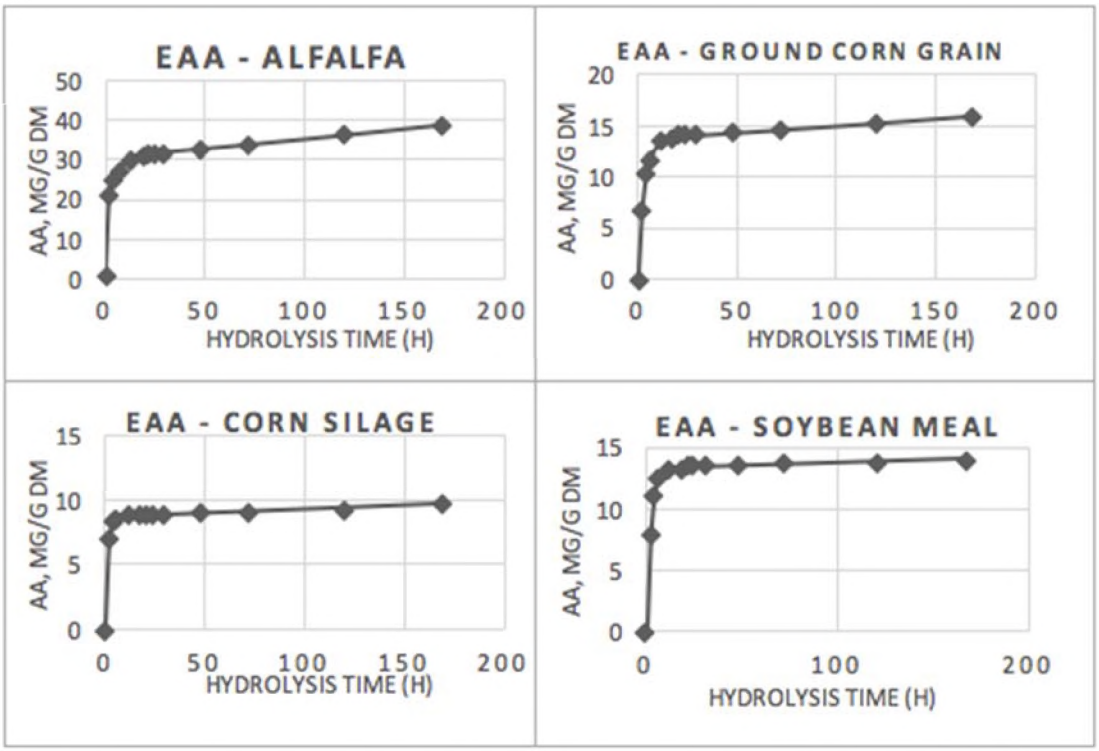

Figure 1. Effect of hydrolysis time (h) on release of EAA (mg/g DM) from two concentrates and two forages.

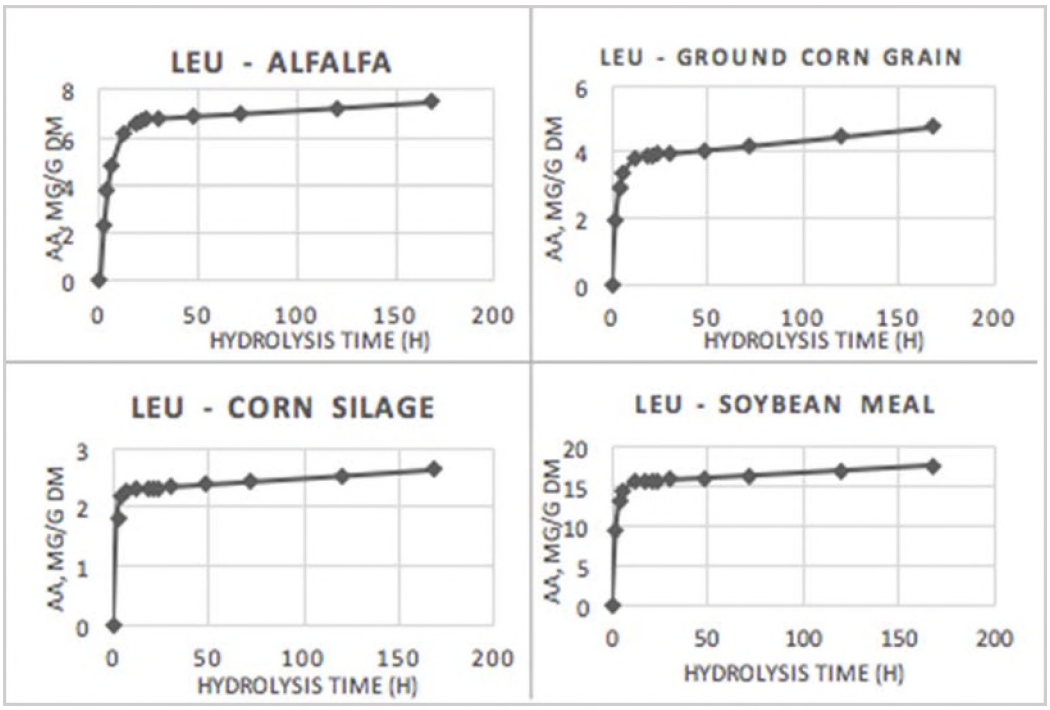

Figure 2. Effect of hydrolysis time (h) on release of leucine (mg/g DM) from two concentrates and two forages.

Table 3. The amino acid composition (mg/g DM) of five feeds analyzed at 24 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues.

	Alfalfa			Canola meal			Ground Corn Grain			Corn Silage			Bloodmeal		
AA[1]	24h	168h	SEM[2]	24h	168h	SEM[2]	24h	168h	SEM[2]	24h	168h	SEM[2]	24h	168h	SEM[2]
Arg	2.84	3.17	0.17	10.92[a]	10.59[b]	0.16	1.77	1.75	0.01	0.54	0.52	0.01	18.13	19.60	0.74
His	1.99	1.94	0.03	6.07[a]	5.63[b]	0.22	1.26	1.33	0.03	0.41[a]	0.33[b]	0.04	25.20[a]	30.79[b]	2.80
Ile	3.45[a]	4.44[b]	0.49	5.88[a]	7.37[b]	0.75	1.08	1.39	0.15	0.92[a]	1.07[b]	0.08	2.75	3.02	0.14
Leu	6.74	7.48	0.37	11.26[a]	12.69[b]	0.72	3.91[a]	4.75[b]	0.42	2.33[a]	2.63[b]	0.15	48.44[a]	59.99[b]	5.77
Lys	3.74[a]	4.56[b]	0.41	8.66[a]	9.83[b]	0.58	1.10	1.40	0.15	0.55[a]	0.71[b]	0.08	35.88[a]	43.55[b]	3.83
Phe	5.91[a]	7.25[b]	0.67	8.76[a]	9.20[b]	0.22	2.01	2.36	0.17	1.86[a]	2.07[b]	0.10	34.65	35.45	0.40
Thr	3.45[a]	4.44[b]	0.49	5.88[a]	7.37[b]	0.75	1.08	1.39	0.15	0.92[a]	1.07[b]	0.08	2.75	3.02	0.14
Val	4.73[a]	5.54[b]	0.41	8.22[a]	9.39[b]	0.59	1.66	1.85	0.09	1.35[a]	1.51[b]	0.08	29.76[a]	41.54[b]	5.89
Met	2.20	2.41	0.11	5.49	5.40	0.04	0.94	0.92	0.01	0.81	0.83	0.01	8.78	8.75	0.02
Trp	2.87	3.59	0.36	8.39	9.22	0.42	1.25	0.89	0.18	0.53	0.53	0.00	25.14[a]	56.32[b]	15.6

[a, b] Different superscripts for a given feed at 24h vs 168h signifies $p < 0.05$

[1] AA = Amino acid

[2] SEM = Standard error of the mean

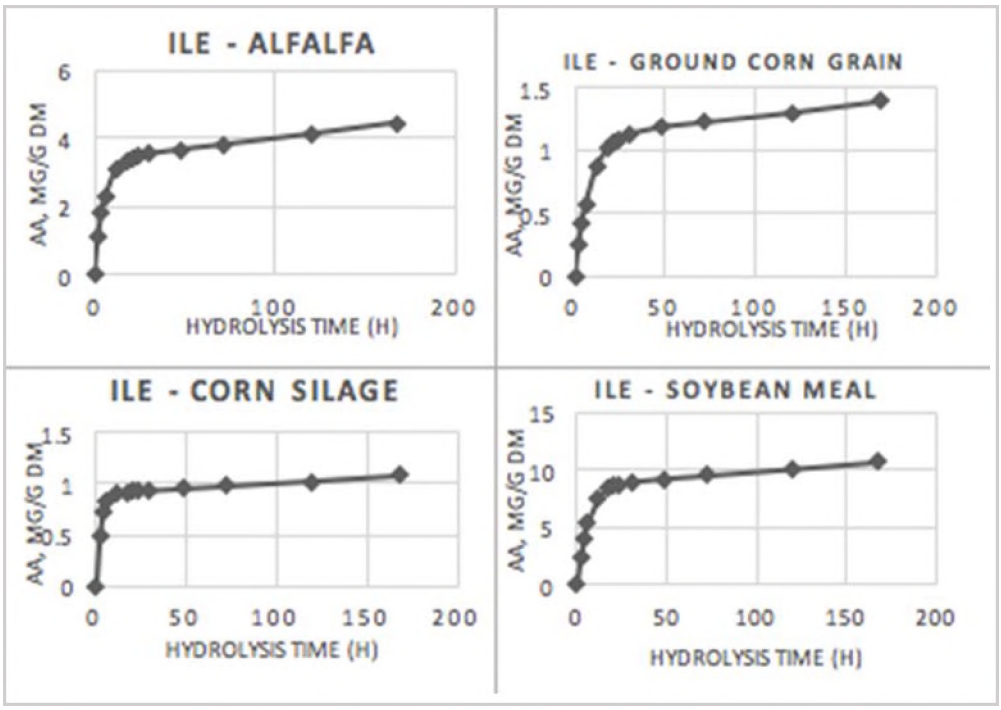

Figure 3. Effect of hydrolysis time (h) on release of isoleucine (mg/g DM) from two concentrates and two forages.

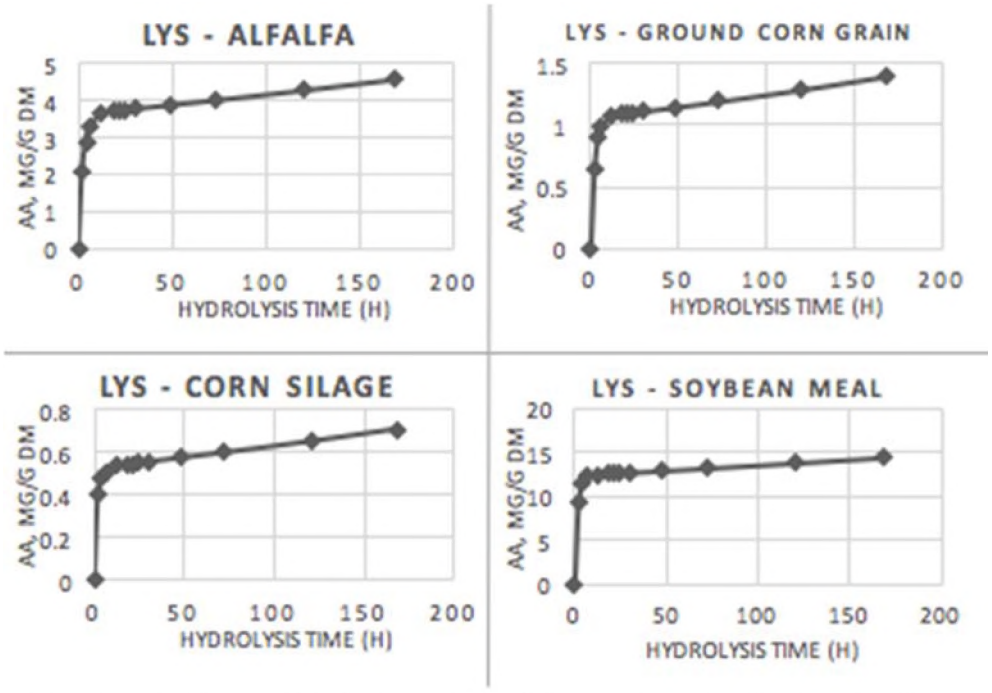

Figure 4. Effect of hydrolysis time (h) on release of lysine (mg/g DM) from two concentrates and two forages.

Milk and Tissue AA Content

After analyzing the microbial and feed AA content, it made sense to re-evaluate both milk and tissue, recognizing that the increased AA content of substrates could not be isolated to the supply side of the model. Again, consistent with the previous data, the whole milk from the CURC Dairy demonstrates there is some variability in the AA content of milk when evaluated at the two hydrolysis times. This data has not been fully analyzed and is included for review, as the hydrolysis and integration off the HPLC was finishing as the deadline was approaching. Overall, the data on milk AA content shows some variability and modest increase at 168 hr hydrolysis compared to the 21 hr time point, but differences are smaller than those reported for milk by Rutherfurd (2008) and much smaller than what was demonstrated for microbes and feeds.

Similarly, the differences in tissue AA content between 21 and 168 hr have not been analyzed, so the data are presented for review and comparison with the supply side information. As with the previous substrates, the tissue BCAA content at 168 hr hydrolysis is generally higher than that observed at 21 hr, whereas the Lys appears to be destroyed at longer hydrolysis times. Thus, for AA like Lys and Met, the 21 hr time point appears to be a reasonable endpoint to ensure optimum and maximum recovery of those AA, especially from tissue, consistent with the original AA methods. This differs greatly from the forages and microbes where Lys release continues without destruction in many of those substratesTable 4. The amino acid composition (mg/g DM) of four whole milk samples taken from the bulk tank at the Cornell University Ruminant Center and analyzed at 21 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

Table 4. The amino acid composition (mg/g DM) of four whole milk samples taken from the bulk tank at the Cornell University Ruminant Center and analyzed at 21 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

	Milk (11.70% MUN)			**Milk (8.80% MUN)**			**Milk (10.10% MUN)**			**Milk (9.90% MUN)**		
AA[1]	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**
Arg	5.65	6.05	0.43	3.27	4.35	0.36	4.08	4.15	0.48	4.31	5.48	0.31
His	3.22	3.23	0.02	9.87	1.75	3.73	2.77	2.94	0.22	3.46	3.95	0.11
Ile	5.50	5.09	0.09	3.78	5.79	0.59	3.90	6.04	0.62	4.94	7.25	0.45
Leu	11.49	10.97	0.11	8.72	9.24	0.63	9.39	10.69	0.49	10.99	13.59	0.52
Lys	10.02	9.68	0.17	8.65	6.97	0.49	8.02	6.63	0.38	9.66	8.02	0.33
Phe	8.45	8.52	0.07	5.91	6.37	0.18	5.35	7.17	0.81	6.62	7.84	0.25
Thr	5.20	5.08	0.03	3.72	4.60	0.27	3.77	4.37	0.33	4.77	5.66	0.20
Val	7.09	6.64	0.10	4.97	7.12	0.68	5.79	7.44	0.49	6.10	8.63	0.49
Met	5.81	5.78	0.06	4.72	4.72	0.14	4.66	5.05	0.25	6.01	4.68	0.26

[1] AA = Amino Acid

[2]SEM = standard error of the mea

Table 5. The amino acid composition (mg/g DM) of bovine tissues from Diaz et al., (2001) and Meyer (2005) analyzed at 21 and 168 hr of hydrolysis and calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

	Carcass -1			Carcass -2			Head/Hide/Feet/Tail-1			Head/Hide/Feet/Tail-2			Blood/Organs -1			Blood/Organs -2		
AA[1]	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**	**21h**	**168h**	**SEM[2]**
Arg	11.74	16.20	0.86	15.52	14.96	2.32	20.47	24.55	0.90	17.70	21.89	0.94	8.95	10.19	0.28	8.35	9.74	0.29
His	6.12	7.10	0.22	11.32	8.97	2.64	6.41	6.15	0.09	5.52	5.41	0.17	5.50	6.16	0.15	5.29	6.01	0.14
Ile	4.38	8.06	0.75	11.60	12.11	3.18	4.35	5.91	0.37	3.61	6.10	0.79	3.10	4.31	0.23	3.15	4.53	0.26
Leu	11.75	15.84	0.83	21.42	18.34	4.91	12.35	15.30	0.66	10.82	12.67	0.48	12.96	15.68	0.54	12.23	15.36	0.59
Lys	12.66	11.47	0.33	18.63	12.90	2.86	12.83	10.57	0.46	11.11	9.00	0.46	11.43	9.31	0.42	11.74	9.74	0.51
Phe	9.13	11.22	0.43	11.24	9.90	1.28	11.70	13.37	0.48	9.30	15.80	1.88	9.19	11.49	0.57	8.83	10.54	0.32
Thr	5.74	7.68	0.40	8.32	7.31	1.16	6.48	7.51	0.27	5.63	6.03	0.23	5.85	6.20	0.10	5.56	6.24	0.14
Val	6.89	10.48	0.76	9.93	9.68	1.51	7.94	10.30	0.48	7.02	8.65	0.40	8.48	10.56	0.41	7.76	10.37	0.50
Met	8.24	8.38	0.19	8.78	8.17	0.31	5.17	6.23	0.54	6.29	5.18	0.55	4.41	5.58	0.57	4.46	5.82	0.50

[1] AA = Amino Acid
[2] SEM = standard error of the mean

Overall, this data strongly suggests there are more AA available or contained in the feed, microbes, tissues and milk than the standard AOAC AA method would yield. The implications for model development is significant because the AA content of all feeds in the feed library need to be evaluated against this information. Further, this calls into question the availability of these AA and whether the animal can realize these AA as the feed moves through the post-ruminal gastrointestinal system and this creates more need to ensure intestinal digestibility is well characterized.

The use of 6N HCl as a hydrolyzing agent was developed many years ago and the AOAC standard hydrolysis time of 21 to 24 hr (Gerkhe et al. 1985) is a compromise where the release of all the AA from the protein is maximized while the degradation of the more acid labile AA is minimized (Rutherfurd, 2009). When comparing this laboratory hydrolysis of protein to that developed evolutionarily by the mammalian gastrointestinal system, it is not surprising that it takes longer to extract the AA from the complex matrix of carbohydrates, proteins, lipids and mineral. The true stomach of most mammals has multiple enzyme systems available to aid in the extraction and breakdown of proteins such as pepsin, trypsin, chymotrypsin, lipase, lysozyme and amylase among many others. Furthermore, different animals have different gastrointestinal tract conditions for digestion and some are more acidic in nature whereas others are more basic to optimize enzyme activity and digestion. For example, the pH optimum for many of the enzymes in the ruminant intestine is more acidic (~pH 5) than the pH required for optimum function in the chicken (pH ~7). Thus, depending upon the species, not only are there enzymes available, but the pH of the system varies in order to improve the efficiency of digestion, something not considered in the laboratory procedures for AA analysis.

REFERENCES

Association of Official Analytical Chemists International. 2016. Official methods of analysis. 20th ed. AOAC International, Gaithersburg, MD.

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304–2323. https://doi.org/10.3168/ jds.S0022-0302(92)77992-2.

Darragh, A. J., D. J. Garrick, P. J. Moughan, and W. H. Hendriks. 1996. Correction for amino acid loss during acid hydrolysis of a purified protein. Anal. Biochem. 236:199–207. https://doi. org/10.1006/abio.1996.0157.

Darragh, A. J., and P. J. Moughan. 1998. The amino acid composition of human milk corrected for amino acid digestibility. Br. J. Nutr. 80:25–34. https://doi.org/10.1017/S0007114598001731.

Darragh, A. J., and P. J. Moughan. 2005. The effect of hydrolysis time on amino acid analysis. J. AOAC Int. 88:888–893.

Denton, B. L., L. E. Diese, J. L. Firkins, and T. J. Hackmann. 2015. Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. Appl. Environ. Microbiol. 81:1832–1838. https://doi.org/10.1128/AEM.03736-14.

Diaz, M.C., M.E. Van Amburgh, J.M. Smith, J.M. Kelsey, E.L. Hutten. 2001. Composition of growth Holstein calved fed milk replacer from birth to 105-kilogram body weight. J. Dairy Sci. 84:830-842

Dijkstra, J., J. France, and S. Tamminga. 1998. Quantification of the recycling of microbial nitrogen in the rumen using a mechanistic model of rumen fermentation processes. J. Agric. Sci. 130:81–94.
Elkin, R. and J. Griffith. 1984. Hydrolysate preparation for analysis of amino acids in sorghum grains: effect of oxidative pretreatment. J. of AOAC Intl. 68:1117-1121.
Fessenden, S. W. 2016. Amino acid supply in dairy cattle. PhD Dissertation. Animal Science Department, Cornell Univ., Ithaca, NY. https://ecommons.cornell.edu/handle/1813/45365.
Fessenden, S., W., T. J. Hackmann, D.A. Ross, A. Foskolos, and M.E. Van Amburgh. 2017. Ruminal bacteria and protozoa composition, digestibility and amino acid profile determined by multiple hydrolysis times. J. Dairy Sci. 2017 100:7211-7226. doi: 10.3168/jds.2016-12531.
Gehrke, C.W., L. Wall Sr, J. Absheer, F. Kaiser, and R. Zumwalt. 1985. Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. Journal of AOAC International. 68:811-821.
Hendriks, W., M. Tarttelin, and P. Moughan. 1998. The amino acid composition of cat (*Felis catus*) hair. Anim. Sci. 67:165–170. https://doi.org/10.1017/S1357729800009905.
Higgs, R.J. 2014. Development of a dynamic rumen and gastro-intestinal model in the Cornell Net Carbohydrate and Protein System to predict the nutrient supply and requirements of dairy cattle. Ph.D. Dissertation. Cornell University, Ithaca, NY.
Higgs, R.J., L. Chase, D.A. Ross, and M.E. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98:6340-6360. http://dx.doi.org/10.3168/jds.2015-9379.
Higgs, R. J., D. R. Ouellet, H. Lapierre, E. A. Collao-Saenz, H. R. Kingi, and M. E. VanAmburgh. 2017. Version 7 of the Cornell Net Carbohydrate and Protein System:III. Endogenous nitrogen and amino acid requirements. Submitted J. Dairy Sci.
Landry, J., and S. Delhaye. 1992. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. Journal Agricultural and Food Chemistry. 40:776-779.
Mason, V.C., S. Bech-Andersen, and M. Rudemo. 1980. Hydrolysate preparation for amino acid determinations in feed constituents. Z Tierphysiol Tierernahr Futtermittelkd. 43:146-164.
Meyer, M.J. 2005. Developmental, nutritional, and hormonal regulation of mammary growth, steroid receptor gene expression, and chemical composition of retained tissues in prepubertal bovine. PhD Dissertation. Animal Science Department, Cornell Univ., Ithaca, NY.
National Research Council. (2001). Nutrient requirements of dairy cattle: 2001. National Academies Press.
Recktenwald, E. B. 2010. Urea N recycling and its utilization by ruminal microbial populations in lactating dairy cattle. PhD Dissertation. Department of Animal Science, Cornell Univ., Ithaca, NY. http://hdl.handle.net/1813/17757.
Recktenwald, E. B., D. A. Ross, S. W. Fessenden, C. J. Wall, and M. E. Van Amburgh. 2014. Urea-N recycling in lactating dairy cows fed diets with 2 different levels of

dietary crude protein and starch with or without monensin. J. Dairy Sci. 97:1611–1622. https://doi.org/10.3168/jds.2013-7162.

Ross, D. A. 2004. Amino acid composition of ruminant feeds and feed fractions and evaluation of the methods used to obtain the insoluble and true precipitable protein fractions of feedstuffs. M.S. Thesis, Cornell Univ., Ithaca, NY.

Rutherfurd, S. M., P.J. Moughan, D. Lowry, and C.G Prosser. 2008. Amino acid composition determined using multiple hydrolysis times for three goat milk formulations. International Journal of Food Sciences and Nutrition. 59:679-690. http://dx.doi.org/10.1080/09637480701705424

Rutherfurd, S. M. 2009. Accurate determination of the amino acid content of selected feedstuffs. International Journal of Food Sciences and Nutrition. 60:53-62.

Storm, E., D. S. Brown, and E. R. Ørskov. 1983. The nutritive value of rumen micro-organisms in ruminants 3. The digestion of microbial amino and nucleic acids in, and losses of endogenous nitrogen from, the small intestine of sheep. Br. J. Nutr. 50:479–485.

Van Amburgh, M.E., E. Collao-Saenz, R. Higgs, D.A. Ross, E. Recktenwald, E. Raffrenato, L. Chase, T. Overton, J. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. Journal of Dairy Science. 98:6361-6380. http://dx.doi.org/10.3168/jds.2015-9378.

Volden, H., and O. M. Harstad. 1998. Amino acid composition of bacteria harvested from the rumen of dairy cows fed three diets differing in protein content and rumen protein degradability at two levels of intake. Acta Agric. Scand. Anim. Sci. 48:210–215. https://doi.org/10.1080/09064709809362422.

Whitehouse, N. L., V. Olson, C. Schwab, W. Chesbrot, K. Cun- ningham, and T. Lykos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. J. Anim. Sci. 72:1335–1343. https://doi. org/10.2527/1994.7251335x.