Development of an Assay to Predict Intestinal Nitrogen Indigestibility and Application of the Assay in High Producing Lactating Cattle: One Step Closer to Feeding a Cow like a Pig?

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Summary

1. An up-dated method to estimate intestinal nitrogen indigestibility of feeds for ruminants was developed from a combination of current methods and then refined to reduce particle and N loss.
2. The assay is comprised of a 16 hr in-vitro incubation in rumen fluid and buffer and then a 24 hr in-vitro incubation in a specific intestinal enzyme cocktail in a shaking water bath.
3. The assay was developed primarily for non-forage feeds and represents a departure from the detergent system used to fractionate most feeds.
4. For most feeds the results from the assay differ significantly from acid detergent insoluble protein demonstrating differences between feed chemistry versus the bio-assay.
5. To investigate the accuracy and precision of the assay predictions, a study was conducted with high producing lactating cattle to evaluate the sensitivity to differences in predicted indigestibility o two different blood meal products.
6. In the cattle study milk yield and overall performance of lactating dairy cattle was reduced in cattle fed the lower digestibility protein source and the difference in the amount of available N supplied was 32 grams, less than 5% of total N intake.

Introduction

Current cattle diet formulation models rely on library estimates of intestinal digestibility of proteins and carbohydrates to predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008). As models become more accurate and precise in the prediction of nutrient supply and evaluation of requirements and nutrient balance, greater scrutiny will be placed on inputs currently relegated to static library values. Although CP is not a functional dietary nutrient for cattle, many diets are still formulated on this metric, creating confusion due to inadequate information provided by the value, especially with regard to MP supply and amino acid availability. As diets are formulated to be closer to MP requirements and rumen ammonia balance, they will, under most circumstances, be lower in CP, thus, accurate estimates of intestinal digestibility (ID) of protein and amino acids are increasingly important to ensure an adequate supply of those nutrients. Application of outdated feed library values to all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production. This paper describes the redevelopment of an in-vitro intestinal digestion (IVID) assay for protein containing feeds used in ruminant nutrition, including intact commercially available feeds designed to resist rumen degradation. The methods used were developed to provide adequate sample size, minimize sample loss, and to allow for standardization of enzyme activity and concentration. The assay contains positive and negative controls to evaluate standardization among and within laboratories.

The feed library of the Dairy NRC (National Research Council, 2001) and the Cornell Net Carbohydrate and Protein System (CNCPS) (Tylutki et al., 2008; Higgs et al., 2015) has static values for intestinal protein digestibility values for various protein fractions, and acid detergent insoluble protein (ADIP) is used to define the unavailable protein. The committee that developed the 2001 Dairy NRC adjusted available MP from feed by assigning a digestibility of 5% to the ADIP fraction based on data indicating that some amino acids could be liberated and absorbed from this fraction (NRC, 2001). The results from the assay described in this paper can be compared to both the ADIP and the adjusted ADIP value from the NRC calculation as an unavailable protein fraction.

Further, current cattle diet formulation models rely on library estimates of intestinal digestibility of proteins and carbohydrates to predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008). As models become more accurate and precise in the prediction of nutrient supply and nutrient balance, there is a greater need to evaluate and be able to adapt the inputs currently used as static library values. Although CP is not a functional dietary nutrient for cattle, many diets are still formulated on this metric, creating confu-

sion due to inadequate information provided by the value, especially with regard to MP supply and amino acid availability. As diets are formulated closer to the MP requirements of cattle and subsequently lower in CP, accurate estimates of intestinal digestibility (ID) or indigestibility of protein and amino acids are increasingly important to ensure an adequate supply of those nutrients. Use of outdated feed library values to all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production.

Since the inception of the Cornell Net Carbohydrate and Protein System (Fox et al., 2004; Tylutki et al., 2008), the detergent system of fractionation has been applied to both the carbohydrate and protein components of feeds (Sniffen et al., 1992). More recent work suggests this approach, especially for feeds not containing NDF, might not be appropriate to accurately characterize how protein is partitioned and digests in the rumen and post-ruminally. Several approaches have been developed to predict the intestinal digestibility of protein in feeds and are a departure from the detergent system of feed chemical composition (Calsamiglia and Stern, 1995; Gargallo et al., 2006; Ross et al., 2013). The N assay was developed to predict N indigestibility, and will be briefly described in that manner throughout the paper. The cattle study described in this paper was conducted by formulating two different diets in high producing cattle using two different blood meals with different predicted intestinal protein indigestibility to test the accuracy and precision of both the assay (Ross et al., 2013) and our ability to apply those values in the CNCPS for diet formulation.

Assay Development Considerations

The following discussion points are provided to highlight potential problems or concerns with current methods and to provide evidence for the need to develop alternative approaches.

Use of bags:

- Created a microbial barrier to feed access and microbial attachment which artificially prolongs the lag phase of digestion.
- Demonstrated loss of highly soluble components of feeds from the bag prior to digestion and loss of particles as digestion progresses. Measured losses of up to 30% of the initial sample prior to any analyses have been reported.

Use of enzymes:

- Profiles and activities are not properly described and characterized.
- The digestive process of the ruminant is a continuous flow of digesta with continuous secretion of enzymes and digestive juices (Hill, 1965).

Abomasal digestion:

- Pepsin, an endopeptidase, hydrolyzes approximately 15-20 % of dietary protein to AA and small peptides (Kutchai, 1998). Bovine pepsin has approximately ~60-70 % of the activity of porcine pepsin with hemoglobin as substrate (Lang and Kassell, 1971). Porcine pepsin is generally used in the first step of IV intestinal digestion assays to measure ruminant intestinal digestion (Calsamiglia and Stern, 1995; Gargallo et al., 2006).
- One mg of porcine pepsin contains 200 to 625 units with pH between 1.5 and 2.5, for optimum pepsin activity.
- Lysozymes which aid in digestion of microbes are also secreted in the digestive tract. Bovine digestive lysozyme has a lower optimum pH than chicken lysozyme (7.65 vs. 10.7, respectively) with a pH optimum 5, not 7, making it resistant to pepsin hydrolysis. Furthermore, bovine lysozymes lyse gram-negative and gram-positive bacteria, while chicken lysozyme acts only on gram positive bacteria (Dobson et al., 1984; Protection of plants against plant pathogens: http://www.patentstorm.us/patents/5422108/description.html; accessed Nov 1, 2010). However, bovine digestive lysozyme is commercially unavailable.

Small intestine digestion:

- Species differences exist in the activities of proteases in the pancreas. In rats, trypsin activity represents ~80 % while in ruminants it represents only 15 % and chymotrypsin makes up 43 % (Keller et al., 1958).
- The calculated activities of trypsin and chymotrypsin in intestinal contents from 5 month old calves (Gorrill et al., 1968) were 19.48 and 15.9 U/ml, respectively using p-toluene-sulfonyl-L-arginine methyl ester (TAME) and benzoyl-L- tyrosine-ethyl ester (BTEE), as substrates.
- In sheep, the activities of trypsin, chymotrypsin and carboxypeptidase A increased from the pylorous to 7 m beyond with maximum specific activities of 24, 150, and 35 µM of respective substrates (benzoyl-L-arginine-ethyl ester (BAEE), acetyl- L- tyrosine-ethyl ester (ATEE), hippuryl-DL-phenyl-lactic acid) per minute per ml digesta, and then decreased (Ben-Ghedalia et al.,1974).
- Sklan and Halevy (1985) found maximal activities of pancreatic enzymes in the proximal segments of the ovine SI at 1 m distal to the pylorous and then relatively constant ratios of enzyme levels

(trypsin, chymotrypsin, elastase, carboxypeptidases A & B) to cerium-141, an unabsorbed reference, of 0.065, 0.053, 0.015, 0.05 and 0.045, respectively, 1.5 to 9 m distal to the pylorous. No other in vivo activities for bovine pancreatic proteolytic enzymes were measured.
- Units of enzyme activity are dependent upon substrate (a protein or ester) hydrolyzed in addition to the wavelength used. Among the studies reviewed, this data varies considerably and is not standardized.
- The current three step assays (Calsamiglia and Stern, 1995; Gargallo et al., 2006; Borucki Castro et al., 2007; Boucher et al., 2009a,b,c) use 3 g of pancreatin per L after an IV abomasal digestion with 1 g L-1 of porcine pepsin in 0.1 N HCl N at pH 1.9 or 2. However, the pancreatin concentration in the assay of Calsamiglia and Stern (1995) was 1.69 mg ml-1 based on the conditions described for the assay as published.
- Pancreatin always contains amylase and lipase but over time the proteolytic enzyme has changed from trypsin to many enzymes, including trypsin, ribonuclease and protease (specifications for P7545; (www.sigmaaldrich.com/catalog/product/sigma/p7545?lang=en. accessed, Nov 10, 2010) and specific units of enzymatic activity are not provided.
- Further, lipase activity is essentially nonexistent in bovine pancreatic juice (Keller, 1958) but is high in saliva. Calsamiglia and Stern (1995) attributed the increase in digestion of their proteins over those obtained using the multi-enzyme system of Hsu et al. (1977) to the presence of amylase and lipase in pancreatin.
- Bovine bile salts were added to the enzyme system to improve the emulsification of samples, especially those containing fat.

Thus, the enzymes used in the assay for the abomasal and intestinal digestion step and their respective activities were based on the data described and were adopted and run in parallel with pancreatin.

Assay Methods Evaluated

A description of the assay development follows in a sequential manner with statements about sources of variation and decisions made to optimize the assay while minimizing or eliminating irrelevant sample loss.

General procedures:

- Unless specified otherwise, all analyses were conducted on duplicate samples.
- Dry matter was determined at 105ºC in a forced-air oven overnight.
- Nitrogen (N) content of original feeds and residues was measured by block digestion and steam distillation with automatic titration (Application Note, AN300; AOAC Official method 2001.11; Foss, 2003; Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11).

Exposure to rumen microbes:

This step in the assay was evaluated in three stages to evaluate variation and sample loss.
- Three bag materials with different pore sizes (15 µm, mesh; 25 µm, fiber (Ankom) and 50 µm, in situ (Ankom)) were evaluated for in vitro intestinal digestion following in vitro vs. in situ fermentation (Ross, et al., 2010). After many attempts at developing conditions that minimized loss of material prior to assay or during the assay, it was difficult to distinguish digestion from bag loss, thus the use of any bags was abandoned.

From this point forward 16-h fermentation was performed via IV methods in Erlenmeyer flasks.
- Plastic centrifuge tubes were evaluated as a fermentation vessel and found to be unfavorable for rumen bacterial growth and sample size had to be reduced to work appropriately in 50 mL tubes.
- Glass Erlenmeyer flasks provided the greatest digestibility values, and had lower variability and superior repeatability compared to plastic centrifuge tubes. For this reason, flasks were chosen as the vessel for the fermentation step. Commercial protein sources (0.5 g) were included in their unground form, while forages, byproducts and non-commercial protein sources were ground through a 2 mm screen in a Wiley Mill (Thomas Scientific, Swedesboro, NJ).

Enzymatic hydrolysis

- Pepsin: Porcine pepsin used but added at 60 % of previous methods in pH 2 HCl (~0.013 M) to contain ~282 U ml-1 in flask.
- Intestinal (ID) enzymes: Initially, enzymes and activities described by Ben-Ghedalia et al. (1974) were used in the enzyme mix until carboxypeptidase A became unavailable. Different combinations of elastase and carboxypeptidase Y in addition to trypsin and chymotrypsin were then evaluated for intestinal digestion. Amylase and lipase were added along with trypsin and chymotrypsin (50 and 4; 24 and 20 U ml-1, respectively) which yielded digestion approximately similar to levels observed with carboxypepetidases A & B. Pancreatin at a level similar to Calsamiglia and Stern (1995; 1.72 mg ml-1, difference due to ini-

tial dilution so maintained throughout) was also analyzed concurrently with the mixture of individual enzymes. Bovine bile salts were also added to ensure adequate emulsification of fat to provide realistic digestibility of fat encapsulated proteins.
- Assay termination for both IV fermentation and enzymatic digestion was accomplished by quantitative filtration under vacuum though 9 cm glass microfiber filter (pore size of 1.5 µm; Whatman 934-AH; GE Healthcare Bio-Sciences Corp., Piscataway, NY) using hot (not boiling) water to transfer. Hot water was necessary to help dissolve away viscous residues from the in vitro step.

Discussion

Use of positive and negative controls to evaluate IV and intestinal digestibility:

Positive and negative controls for both fermentation and intestinal digestibility steps were included. To evaluate the fermentation phase, NDF digestion of corn silage ND residue sample was run concurrently. A heat damaged blood meal with near zero ruminal and intestinal digestibility was included throughout as a negative control. A feed with similar digestibility as samples, i.e., a soy product or blood meal, was also included. A blood meal with known high intestinal digestibility was included as a positive control for the ID assay.

Comparison of modified TSP with Cornell assay

Digestibility of two blood meals (from Boucher et al., 2011) were evaluated using the new method with the enzyme mix and pancreatin (Table 1) and compared with the modified TSP. Rumen N digestibility of BM4 was 18 % higher using bags but 6 % lower for BM5. The implication from this comparison is that material was solubilized or lost from the bag prior to being analyzed which provided higher rumen degradability in the TSP. Total N digestibility for BM5 was similar between both procedures and the enzyme mix and pancreatin. However pancreatin digestion of BM4 in the modified TSP was lower than either ID digestion using the Cornell procedure - using the Cornell method, BM4 had higher intestinal digestion.

Comparison of intestinal digestion with the acid detergent insoluble protein

Within the current structure of many contemporary nutrition models, acid detergent insoluble nitrogen (ADIN) represents the unavailable N component of feed (NRC, 2001; Tylutki et al., 2008) however, the NRC for Dairy Cattle (2001) provides for 5% digestibility of the ADIN fraction. The implication is that the ADIN fraction is not completely unavailable to the animal. Accordingly, the ID assay as outlined was utilized to ascertain whether ADIN is indigestible (Table 2). The ADIN of solvent extracted soybean meal and Soy1 were very similar to undigested feed N following IV fermentation, abomasal and intestinal digestion with either the enzyme mix or pancreatin; however, the ADIN of heat damaged blood meal was roughly 2 % while undegraded N from both intestinal digestion treatments was 95 %. Undegraded N of corn silage following digestion and after correction for microbial contamination was roughly 3 times higher than ADIN content.

This approach for determining the unavailable N from feeds departs from the traditional detergent partitioning system established by Van Soest and others, and implementation within nutrition models like the CNCPS will create a fraction that crosses the fractions described by detergent chemistry and has a different behavior. We believe this to be more appropriate approach for describing available protein for cattle. For forages, a longer in vitro step might be necessary to make the assay relevant for estimating protein availability since forage particle retention is usually great than 16-18 hr and closer to 30 hr so more work needs to be conducted to fully evaluate the assay for those feeds.

Dairy Cattle Evaluation Study

Treatments, Animals and Experimental Design

Treatments were established from a quantity of two blood meals secured through the marketplace that would allow an inclusion level of approximately 1 kg per head per day for the entire experimental period. The two blood meals were analyzed for unavailable N (uN) prior to the start of the study using the in-vitro assay described by Ross et al. (2013). Briefly, 0.5g of sample are placed into a 125ml Erlenmeyer flask. 40ml of rumen buffer and 10ml of rumen fluid are added to each flask. Flasks are incubated in a water bath at 39°C for 16h under continuous CO2. Samples are then acidified with 3M HCL to bring the pH down to 2. Samples are incubated on a shaking bath for one hour after the addition of 2ml of pepsin and pH 2 HCl. Samples are then neutralized with 2ml of 2M NaOH to stop the pepsin reaction. An enzyme mix containing trypsin, chymotrypsin, lipase and amylase is added to the flask and incubated for 24h in the shaking bath at 39°C. Samples are then filtered with a 1.5 µm glass filter and boiling water. Nitrogen content of the residue is determined by Kjeldahl and expressed as a % of total N in the sample. The blood meals are characterized by their predicted intestinal N indigestibility (INID) since that is the

outcome of the assay. The predicted uN of the low (LOW treatment) INID blood meal was 9%, whereas that of the other treatment (HIGH) was 33.8%. Thus, the two dietary treatments were established by inclusion of these blood meals in two different diets on an iso-N basis. The rest of the diets were formulated to be identical. The low uN blood meal was 15.04% N and the higher uN blood meal was 14.6% N, thus at approximately 1 kg inclusion level, the maximum difference in intestinal N availability was 38.5g N. The composition of the two diets fed to cattle is in Table 1.

Due to potential changes in milk yield in both treatments due to stage of lactation, the protein content of both diets was adjusted down at approximately 5 weeks of treatment by reducing the canola meal inclusion level by 50% to be more consistent with the ME allowable milk and to maintain the N supply to a level the cattle should remain sensitive to the treatment differences in N availability created by the inclusion of the two different blood meals.

Ninety-six multiparous cows (726 ± 14.2 kg BW; 147 ± 64 DIM) and thirty-two primiparous cows (607 kg ± 29.5kg BW; 97 ± 20 DIM) were distributed by DIM and BW into 8 pens of 16 cows (12 multiparous and 4 primiparous). Pens were stratified into four levels of milk production, and each stratum randomly allocated to treatments. Diets were formulated using Cornell Net Carbohydrate and Protein System (CNCPS v6.1; Van Amburgh et al., 2013) using the chemical composition of the ingredients used in the experimental diets (Table 3).

The lactation trial consisted of a two week adaptation period, one week covariate period and 9 week experimental period, between March 30 and June 21, 2014 at Cornell University Ruminant Center (Harford, NY). All cows were fed the LOW uN diet during adaptation and covariate periods. Cows were housed in pens under a four row barn design with one bed and more than one headlock per cow and free access to water. All cows received rBST (Posilac, Elanco Animal Health, Indianapolis, IN) on a 14 day schedule throughout the length of the trial.

Cattle were fed once per day for approximately 5% refusal and milked 3 times per day at 6:00, 14:00 and 22:00 and data from all milkings was recorded using Alpro herd management system (DeLaval International AB, SG). Individual milk samples were collected weekly during three consecutive milkings, and preserved with 2-bromo-2-nitropane-1, 3-diol at 4°C until analyzed. Milk yield was expressed as 3.5% energy corrected milk (ECM) according to the equation of Tyrell and Reid (1965): ECM (kg) = (12.82 * kg fat) + (7.13 * kg protein) + (0.0323 * kg milk).

Cattle were weighed once per week using a walk scale XR3000 (Tru-test, TX) after the morning milking. Further, BCS on a scale of 1 to 5 was determined every two weeks by the same two evaluators. An average of the two evaluators was used for calculation of the mean BCS.

Results and Discussion

Animal Performance

Overall DMI and N intake for the treatments were similar and milk yield was significantly different for cattle fed the two treatments (Table 4). Milk yield was 1.6 kg/d lower for cattle fed the HIGH uN diet and energy corrected milk (ECM) was 1.9 kg/d lower on the same diet. Further, cattle fed the HIGH uN diet had significantly lower MUN levels that cattle fed the LOW uN diet (Table 2). From this information, it is apparent that the cattle fed the different blood meals had significantly different MP supply, consistent with the predicted values from the uN assay. The predicted difference described earlier (38.5 g N) is equal to approximately 240 g MP, about the amount required to produce 5 kg of milk under the conditions of this study.

However, the observed difference on an ECM basis was 1.9 kg, thus the difference between the absolute levels measured in the assay and the observed ECM yield are either due to differences in digestibility within the cow, the amount of the blood meal arriving at the small intestine or the amount of nutrients partitioned to body reserves, or a combination of all of those factors. Although the change in BW and BCS were not significant, the changes are still biologically relevant given the partitioning of nutrients to reserves and away from milk.

To evaluate the outcome of the study, CNCPS v6.55 (Van Amburgh et al., 2015) with the updated feed library rates and pool sizes was used to evaluate the predictions. The chemical composition of the feeds used in the study was inputted into the model. To evaluate the assay within the structure of the model and against the study data, the blood meal values for the uN and ADIN were the only values changed. For the two blood meals, the uN values were inputted in place of the ADIN value, and intestinal digestibility left at zero. Further, the intestinal digestibility of the NDIN value were set to 100% although after being analyzed for aNDFom, the blood meals do not contain any ND residue, so that pool is zero. With this

approach, all of the protein in blood meals is in the A2, B1 and C fractions.

The current intestinal digestibility of the NDIN fraction for all feeds is 80% and it appears that the assay of Ross et al. captures that portion of the indigestible protein, therefore by difference; the remaining fractions should be set at 100% digestibility. Thus, with continued testing and implementation of the uN assay for all feeds, the NDIN fraction ID will be set to 100% because it appears that in NDF containing feeds, the uN assay spans both the ADIN and NDIN fractions.

For the cattle inputs, the expected BW change based on the target growth approach was used and the BCS change was also inputted over the period of the study (9 wks), thus this accounted for the distribution of nutrients to other productive uses and not just milk output. With all of the inputs accounted for, the prediction of ME and MP allowable milk with the uN assay information is in Table 5.

In the CNCPS evaluation (Table 5), it is apparent that the feed chemistry described through the detergent system is not appropriate to allow the model to predict the most limiting nutrient in this comparison using blood meal as the treatment. When the uN data are used to describe the chemistry of the blood meals, the model provides an acceptable and realistic prediction of the most limiting nutrient. It is also important to recognize that an accurate and complete description of the animal characteristics was important to make this evaluation and in the absence of that information, the model would predict over 4 kg of MP allowable milk difference. The sensitivity of the model predictions to complete and accurate animal characterization cannot be overstated and helps explain why literature data to evaluate any model rarely allows for robust predictions of most limiting nutrients due the lack of complete information.

In summary, the uN assay appears to provide protein indigestibility predictions that are consistent with cattle responses and serves as a platform for modifying the approach to predict protein digestibility within the CNCPS and will improve the model's ability to identify the most limiting nutrient. The data also demonstrate we are ready to move beyond the detergent system of fractionation for protein and move to a system that fractionates proteins based on solubility and indigestibility. This approach should allow us to develop a prediction model to more effectively estimate rates of protein degradation because we now have what appears to be a more robust method to predict the indigestible protein pool, consistent with the approach for NDF (Raffrenato et al., 2009) and this fraction is important for accurate calculations of the rate of digestion of the available protein.

References

AOAC International. 2003. Nitrogen. AOAC Official Methods of Analysis. Arlington, VA.

Ben-Ghedalia, D., H.Tagari, and A.Bondi, 1974. Protein digestion in the intestine of sheep. Br J Nutr 31:128-142.

Borucki Castro, S. I., L. E., Phillip, H. Lapierre, P. W., Jardon, and R. Berthiaume,. 2007. Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. J Dairy Sci. 90:810-822.

Boucher, S. E., Calsamiglia, S., Parsons, C. M., Stein, H. H., Stern, M. D., Erickson, P. S., Utterback, P. L., and Schwab, C. G. 2009. Intestinal digestibility of amino acids in rumen undegradable protein estimated using a precisio-fed cecectomized rooster bioassay: I. Soybean meal and SoyPlus. J Dairy Sci. 92:4489-4498.

Boucher, S. E., Calsamiglia, S., Parsons, C. M., Stein, H. H., Stern, M. D., Erickson, P. S., Utterback, P. L., and Schwab, C. G. 2009. Intestinal digestibility of aino acids in rumen-undegraded protein estimated using a precision-fed cecectomized rooster bioassay: II. distillers dried grains with solubles and fish meal. J Dairy Sci. 92:6056-6067.

Boucher, S. E., S. Calsamiglia, C. M. Parsons, M. D. Stern, M. Ruiz Moreno, , M. Vázquez-Añón , and C. G. Schwab. 2009. In vitro digestibility of individual amino acids in rumen-undegraded protein: The modified three-step procedure and the immobilized digestive enzyme assay. J Dairy Sci. 92:3939-3950.

Boucher, S. E., S. Calsamiglia, C. M. Parsons, M. D. Stern, C. G. Schwab, K. W. Cotanch, J. W. Darrah and J. K. Bernard. 2011. Method evaluation for determining digestibility of rumen undegraded amino acids in blood meal. J Dairy Sci. 94:388. E-Suppl. 1.

Calsamiglia, S. and Stern, M. D. 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. J Anim. Sci. 73:1459-1465.

Dobson, D. E., E. M. Prager and A. C., Wilson. 1984. Stomach lysozymes of ruminants. I. distribution and catalytic properties. J. Biological Chem. 259:11607-11616.

Fox, D. G., L. O. Tedeschi, T. P. Tylutki, J. B. Russell, M. E. Van Amburgh, L. E. Chase, A. N. Pell and T. R. Overton. 2004. The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. Animal Feed Science Tech 112:29-78.

Gargallo, S., S. Calsamiglia and A. Ferret. 2006.

Technical note: A modified three-step in vitro procedure to determine intestinal digestion of proteins. J Anim Sci 84:1-5.

Gorrill, A. D. L., D. J. Schingoethe and J. W. Thomas. 1968. Proteolytic activity and in vitro enzyme stability in small intestinal contents from ruminants and nonruminants at different ages. J Nutr 96:342-348.

Higgs R.J., L.E. Chase, D.A. Ross, 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. 98:6340-60. doi: 10.3168/jds.2015-9379.

Hill, K. J. 1965. Abomasal secretory function in the sheep. In: R. W. Dougherty, R. S. Allen, and A. D. McGillard (Eds.) Physiology of Digestion in the Ruminant. pp. 221-230. Buttersworths, Washington.

Hsu, H. W., D. L. Vavak, L. D. Satterlee and G. A. Miller. 1977. A multienzyme technique for estimating protein digestibility. J Food Sci 42:1269-1273.

Keller, P. J., E. Cohen and H. Neurath. 1958. The proteins of bovine pancreatic juice. J. Biological Chem. 233:344-349.

Kutchai, H. C. 1998. Chapter 39. Digestion and Absorption. In: R. M. Berne and M. N. Levy (Ed.). Physiology, 4th ed. Pp. 651. Mpsby. Inc. St. Louis.

Lang, H. M. and B. Kassell. 1971. Bovine pepsinogens and pepsins. III. composition and specificity of the pepsins. Biochem 12:2296-2301.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. National Academy of Science. Washington, DC.

Raffrenato, E., R. Fievisohn, K. W. Cotanch, R. J. Grant, P. J. Van Soest, L. E. Chase and M. E. Van Amburgh. 2009. aNDF, NDFd, iNDF, ADL and kd: what have we learned? Pp. 81-97. Proc. Cornell Nutr. Conf. Dept. of Animal Sci. Syracuse, NY

Ross, D. A., M. Gutierrez-Botero, and M. E. Van Amburgh. 2013. Development of an in-vitro intestinal digestibility assay for ruminant feeds. Pp. 190-202. Proc. Cornell Nutr. Conf. Dept. of Animal Sci. Syracuse, NY

Ross, D. A., M. M. McCullouch, and M. E. Van Amburgh. 2010. A comparison of methods to evaluate in vitro intestinal digestibility. J Dairy Sci. 93:164. E-suppl. 1.

Sklan, D. and O. Halevy. 1985. Digestion and absorption of protein along ovine gastrointestinal tract. J Dairy Sci 68:1676-1681.

Sniffen, C. J., J. D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70:3562-3577.

Steg, A., W. M. Van Straalen, V. A. Hindle, W. A. Wensink, F. M. H. Dooper and R. L. M. Schils. 1994. Rumen degradation and intestinal digestion of grass and clover at two maturity levels during the season in dairy cows. Grass Forage Sci 49:378-390.

Tylutki, T. P., D. G. Fox, V. M. Durbal, L. O.Tedeschi, J. B. Russell, M. E. Van Amburgh, T. R. Overton, L. E. Chase and A. N. Pell. 2008. Cornell net carbohydrate and protein system: a model for precision feeding of dairy cattle. An Feed Sci Tech 143:174-202.

Tyrrell, H. F., and J. T. Reid. 1965. Prediction of the energy value of cow's milk. J. Dairy Sci. 48: 1215-1223.

Van Amburgh M.E., Collao-Saenz EA, Higgs RJ, Ross DA, Recktenwald EB, Raffrenato E, Chase LE, Overton TR, Mills JK, Foskolos A.2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J Dairy Sci.98:6361-80. doi: 10.3168/jds.2015-9378.

Van Soest, P. J., J. B. Robertson and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583-3597.

Table 1. Comparison of the percent N digested in two blood meals using the modified three step procedure (from Boucher et al., 2011) with Cornell procedure.

	Modified TSP*			Cornell		
	Rumen	Pancreatin		Rumen	Enzyme Mix	Pancreatin
	----% N digested----			-------% N digested----------		
BM4	19.9	89		1.0	96.6	97.1
BM5	42.3	94		48.7	97.4	97.0

*Boucher

Table 2. Comparison of percent feed N and acid detergent insoluble N versus undigested feed N after 16-h IV ruminal fermentation followed by 1-h abomasal digestion with pepsin in HCl and 24-h intestinal digestion using either a mix of trypsin, chymotrypsin, amylase and lipase or pancreatin (n=2).

	Feed N	ADIN	% Undigested Feed N	
	% DM	% N	Enzyme Mix*	Pancreatin
Anchovy meal	11.50	1.3	25.5	20.1
Bakery waste	1.80	3.3	20.6	23.6
Blood meal 1	16.20	4.7	22.9[a]	8.0[b]
Blood meal 285	16.89	1.1	0.0	na
Blood meal 300	16.20	7.5	4.6	na
Blood meal 350	15.13	0.9	23.6	na
Blood meal 800	16.50	1.8	2.8	na
Canola 1	6.50	6.3	16.2	12.5
Canola 2	6.60	5.8	14.0	14.0
Citrus pulp	1.04	15.8	55.0	45.4
Corn germ	4.27	11.2	18.5	9.4
Corn gluten	3.13	16.9	28.7	18.9
Corn gluten feed	3.08	11.2	20.7	16.2
Distillers grains 1	4.90	13.1	11.7	9.5
Distillers grains 2	6.40	32.7	27.9[a]	13.6[b]
Solv. extract. soybean meal	7.60	6.7	7.8	7.6
Soy product 1	7.70	6.5	9.0	4.3
Soy product 2	7.30	7.9	11.1[a]	6.6[b]
Wheat midds	3.30	3.1	9.3	7.2
Heat damaged blood meal	16.10	1.8	95.0	95.0

[abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncans Multiple Range test. Not all samples were statistically evaluated for this manuscript. NA – not available.

Table 3. The ingredient content and chemical composition of two diets containing blood meals with Low and High indigestible intestinal N digestibility.

	Treatment	
Ingredient, % DM	LOW uN	HIGH uN
Alfalfa haylage	11.5	11.5
BMR corn silage	49.3	49.3
Bakery	1.8	1.8
Blood meal High	3.7	---
Blood meal Low	---	4.0
Canola meal	3.0	3.0
Corn grain	16.1	16.1
Energy Booster 100	1.8	1.8
Molasses	1.8	1.8
Smartamine M	0.1	0.1
Sodium bicarbonate	0.6	0.5
Soybean hulls	4.6	4.5
Urea	0.2	0.2
Wheat midds	4.6	4.5
Min/vit mix	1.0	1.0
Chemical composition		
DM, % as fed	50.0	50.5
CP, % DM	15.2	15.2
NDF, % DM	31.9	32.3
ADF, % DM	21.3	20.5
Ether extract, % DM	4.3	3.9
Starch, % DM	30.4	31.2
Sugar, % DM	3.6	3.3
Ca, % DM	0.65	0.60
P, % DM	0.43	0.43
ME[1], Mcal/kg DM	1.8	1.7
Lys:Met[1], % MP	3.21	3.19

[1]CNCPS predicted

Table 4. Effect of N availability on intake, milk production, milk composition and body weight gain of dairy cows fed diets with low and high unavailable N

	Treatment			
Item[1]	LOW uN	HIGH uN	SEM	P-value
DMI, kg	27.4	27.1	0.61	0.75
N Intake, kg DM	671.1	664.4	14.8	0.77
Milk production				
Milk, kg	42.0	40.4	0.31	<0.01
ECM, kg	41.9	40.0	0.32	<0.01
Fat, kg	1.51	1.42	0.02	<0.01
Protein, kg	1.26	1.23	0.01	0.03
Milk composition				
Fat, %	3.6	3.5	0.03	<0.03
Protein, %	3.03	3.06	0.02	0.20
Lactose, %	4.90	4.86	0.02	0.18
MUN, mg/dl	9.4	8.0	0.18	<0.01
SCC (log1000/ml)	3.9	4.0	0.05	0.13
BW and BCS				
BWinitial, kg	684.1	692.1	10.1	0.58
BWchange, kg	34.7	29.7	2.25	0.12
BCSchange, (1-5)	0.20	0.16	0.03	0.29
Efficiency				
Feed efficiency[2]	1.56	1.50	0.03	0.34
Milk N efficiency[3]	30.0	29.7	0.70	0.76

[2] calculated as kg milk / kg DMI
[3] calculated as milk N/N intake*100

Table 5. The actual and energy corrected milk and the metabolizable energy (ME) and protein (MP) allowable milk for both treatments predicted by the CNCPS using the assay data of Ross et al., (2013) to estimate intestinal digestibility of blood meal, or using the original fractionation approach using acid detergent insoluble nitrogen as the unavailable fraction

	Treatment	
Item	LOW uN	HIGH uN
Actual milk, kg	42.0	40.4
Energy corrected milk, kg	41.9	40.0
Using uN assay inputs		
ME allowable milk, kg	45.0	46.0
MP allowable milk, kg	42.6	39.3
Using NDIN and ADIN		
MP allowable milk, kg	44.9	44.6