Ruminal bacteria and protozoa composition, digestibility, and amino acid profile determined by multiple hydrolysis times

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ABSTRACT

Microbial samples from 4 independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA digestibility, and AA profile after multiple hydrolysis times ranging from 2 to 168 h. Similar bacterial and protozoal isolation techniques were used for all isolations. Omasal bacteria and protozoa samples were analyzed for AA digestibility using a new in vitro technique. Multiple time point hydrolysis and least squares nonlinear regression were used to determine the AA content of omasal bacteria and protozoa, and equivalency comparisons were made against single time point hydrolysis. Formalin was used in 1 experiment, which negatively affected AA digestibility and likely limited the complete release of AA during acid hydrolysis. The mean AA digestibility was 87.8 and 81.6% for non-formalin-treated bacteria and protozoa, respectively. Preservation of microbe samples in formalin likely decreased recovery of several individual AA. Results from the multiple time point hydrolysis indicated that Ile, Val, and Met hydrolyzed at a slower rate compared with other essential AA. Single time point hydrolysis was found to be nonequivalent to multiple time point hydrolysis when considering biologically important changes in estimated microbial AA profiles. Several AA, including Met, Ile, and Val, were underpredicted using AA determination after a single 24-h hydrolysis. Models for predicting postruminal supply of AA might need to consider potential bias present in postruminal AA flow literature when AA determinations are performed after single time point hydrolysis and when using formalin as a preservative for microbial samples.

Key words: amino acid, digestibility, hydrolysis, ruminal bacteria, ruminal protozoa

INTRODUCTION

Nutrient supply and requirement models, such as the Cornell Net Carbohydrate and Protein System (CNCPS; Higgs et al., 2015; Van Amburgh et al., 2015) and the NRC (2001), along with their derivative models predict postruminal flows of bacterial biomass. Bacterial protein flow is assigned an AA content, AA profile, and a digestibility of individual AA to calculate supply of metabolizable AA. These field-applicable models typically use the AA profile of bacteria obtained from the literature (Storm et al., 1983; Clark et al., 1992; Volden and Harstad, 1998), and few account for protozoal AA flows, which can contribute a substantial amount to total microbial AA flow (Dijkstra et al., 1998; Fessenden, 2016). A new, dynamic version of the CNCPS (v. 7; Higgs, 2014; R. J. Higgs, Cornell University, current address Ohaupo, New Zealand, unpublished data) uses a similar approach in a N-based model and mechanistically 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 bacterial and protozoal AA were needed to understand sources of error in predictions of particular AA. On an N basis, the predictions of NAN in CNCPS version 7 were reasonably accurate and precise. However, predictions of individual AA, such as Lys, Met, Ile, and Val, were biased, likely due to a lack of information about the true content those AA in both microbes and feeds (Higgs, 2014; R. J. Higgs, Cornell University, current address Ohaupo, New Zealand, unpublished data).

The methods used for isolation of microbial fractions and analysis of AA vary widely across the literature, and much of the data used for nutrition models still rely on older methods where more robust alternatives now exist. An example can be found in the isolation of protozoa, where differential centrifugation has historically been used to isolate microbial cells with significant contamination from bacteria and feed particles. A procedure developed for isolation of cultivatable mixed ruminal protozoa for quantitative PCR and competi-

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tion studies (Sylvester et al., 2004, 2005; Denton et al., 2015) can be used to isolate protozoa for more accurate nutrient analysis. In addition, several of the methods employ the use of formalin in the isolation and storage of microbial and specifically protozoal samples (Martin et al., 1994; Rossi et al., 2013); this might be problematic, as formaldehyde is known to react with AA (Barry, 1976).

Intestinal digestibility of individual microbial AA has been estimated by several different methods, including regression approaches (Tas et al., 1981; Hvelplund and Hesselholt, 1987), in vitro assays such as the modified 3-step assay (Gargallo et al., 2006), and in vivo assays utilizing the mobile bag technique. In vivo and in vitro procedures relying on retention in bags are largely inadequate, as both indigestible and digestible protein and AA can pass through the pores in the bags, resulting in inflated digestibility values. In vivo assays have clear advantages in providing realistic and applicable enzymatic hydrolysis conditions; however, they also require surgically altered animal models. The precision-fed cecectomized rooster bioassay was recently applied to ruminal bacteria (Fonseca et al., 2014); however, data are still lacking on protozoa AA digestibility. Additionally, avian species have different enzymes and pH conditions that might reduce the ability to make direct comparisons to ruminant digestion (Keller, 1968; Guerino and Baumrucker, 1987). Ross et al. (2013) developed an in vitro assay with ruminal fluid to determine intestinally unavailable N (uN) in ruminant feeds that addressed some issues of nonphysiologic or species-specific enzyme activities, poor retention of small particles, and extensive cannulation procedures. This assay might provide an adequate assessment of intestinal digestibility of ruminal bacteria and protozoa. Considering the ability of formaldehyde to reduce feed protein degradation, it is also possible that the use of formalin in preservation of microbial samples, as reported in Reynal et al. (2003) and others, might reduce the measured intestinal digestibility of microbial AA. An evaluation of the digestibility of unpreserved and formalin-preserved samples using the uN assay might simultaneously provide more information on formalin effects on individual microbial AA while testing the ability of the uN assay to detect differences in samples treated with a compound known to decreased digestibility.

Amino acid 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 (Rutherford, 2009). Determination of AA at multiple time points followed by least squares nonlinear regression provides more accurate estimates of the AA profile (Darragh and Moughan, 2005). This

approach has been used in purified protein (Darragh et al., 1996), milk protein (Rutherford et al., 2008), and common animal feedstuffs (Rutherford, 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 h, whereas Ile release was greatest at 70 h (Ross, 2004). To our knowledge, AA determination after multiple hydrolyses times has not been performed on rumen microbial biomass.

Given the data from Darragh and Moughan (2005), Rutherford (2009), and the observations made in the data from R. J. Higgs (Cornell University, current address Ohaupo, New Zealand, unpublished), the hypothesis of the current study was that the standard method of determination of AA in ruminal bacteria and protozoa using single time point hydrolysis is not equivalent to AA determination after multiple time point hydrolysis and nonlinear least squares regression. Additional objectives of our study were to summarize the chemical composition and AA profile of ruminal bacteria and protozoa from high-producing lactating dairy cows and to evaluate intestinal digestibility of microbial AA using a newly developed in vitro assay.

MATERIALS AND METHODS

All cannulated cows used as rumen or omasal fluid donors for the microbial isolations in this experiment were cared for according to the guidelines of the Institutional Animal Care and Use committee appropriate for the university responsible for their care. The committees reviewed and approved the experiment and all procedures carried out in the study.

Microbial Isolation Procedures

Microbial samples from several independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA content, and intestinal nutrient and AA digestibility. Bacteria and protozoa included in the analysis were from the following experiments. Trial A was an omasal sampling trial with 8 cows in a 2-treatment switchback design investigating effects of a commercial by-product feed on omasal nutrient flow (Fessenden, 2016). Trial B was 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 (A. Foskolos, unpublished data). Trial C was a ruminal N balance and recycling trial with 12 cows in a 3-treatment randomized complete block design investigating ruminal N- or MP-deficient diets (Recktenwald, 2010; Recktenwald et al., 2014). 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 to C, equal parts DM were combined within microbial type, resulting in a composited sample of bacteria and protozoa from each experiment. Therefore, the possible effects of treatments from trials A to C are not represented in this data set. Information regarding the chemical composition of the average diet fed to cows in each experiment, along with the number of individual collections and isolations represented by each composited sample, are reported in Table 1. 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.

For trials A and B, microbial samples were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). Samples of whole omasal contents were collected from the omasal canal every 2 h during three 8-h intervals. Details of sampling for trial A are described in Fessenden (2016). Trial B sampling occurred per a very similar sampling schedule as trial A by the same researchers (A. Foskolos and S. Fessenden). Trial C collection methods are described in Recktenwald (2010) and Recktenwald et al. (2014).

Table 1. Donor cow diet ingredient and chemical composition, intake, and milk production

Item	Trial ¹			
	A	B	C	D
Number of isolations in each sample	72	108	NA ²	8
Diet ingredient composition, % of DM				
Corn silage	44.0	28.6	45.6	45.3
Haycrop silage	12.0	22.9	—	13.8
Wheat straw	—	—	2.1	—
Corn meal	12.0	28.6	11.1	12.5
Barley grain, ground	—	—	6.2	—
Soybean meal	—	—	4.5	8.6
Canola meal	—	9.2	—	—
Rumen-protected soybean meal	8.2	—	3.8	2.1
Commercial fermentation byproduct	1.5	—	—	—
Corn distillers	—	—	—	3.8
Cottonseed	—	—	8.4	6.4
Wheat middlings	3.4	—	—	2.8
Soybean hulls	5.8	5.5	—	—
Citrus pulp	3.3	1.0	7.3	—
Sugar	—	—	2.2	—
Molasses	0.9	—	—	—
Fatty acid supplement	1.2	0.6	—	1.0
Blood meal	1.7	1.4	1.1	—
Minerals, vitamins, and additives	6.2	2.2	7.7	3.7
Diet chemical composition				
OM, % of DM	93.9	92.6	92.5	91.8
CP, % of DM	16.0	16.9	14.8	16.3
Soluble protein, % of CP	35.7	43.7	31.9	34.1
RDP, ³ % of CP	51.9	59.8	54.3	62.0
aNDFom, ⁴ % of DM	31.1	30.6	33.6	32.1
ADF, % of DM	19.8	20.5	NA	20.2
ADL, % of DM	3.0	3.4	1.9	3.1
Sugars, % of DM	5.4	3.5	5.4	2.8
Starch, % of DM	27.6	27.9	25.4	26.7
Ether extract, % of DM	4.9	3.9	4.5	5.6
ME, ⁵ Mcal/kg	2.5	2.6	2.7	2.6
Cattle intake and production				
DMI, kg/d	27.3	26.1	23.8	NA
Milk production, kg/d	41.7	41.6	30.9	NA

¹Trial A: Fessenden (2016); Trial B: A. Foskolos (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2014); Trial D: T. Hackmann, unpublished data.

²NA = not available.

³Chemical composition estimated using CNCPS v. 6.55 (Van Amburgh et al., 2015) using diet ingredient composition.

⁴aNDFom = the NDF content after the addition of alpha-amylase, sodium sulfite, and ash correction.

⁵Metabolizable energy predicted using CNCPS v. 6.55 (Van Amburgh et al., 2015).

Trial D protozoa were collected on 4 separate days with 2 separate aliquots filtered per day for 8 aliquots total (T. Hackmann, unpublished data).

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, solids were rinsed once with saline, and the filtrate (I) was treated with formalin (0.1% vol/vol 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% vol/vol in final solution). Filtrates I and II were then combined and centrifuged at $1,000 \times g$ for 5 min at 4°C to remove small feed particles and protozoa. The supernatant was centrifuged at $15,000 \times g$ 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, but 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 (2016; Figure 1). The only difference between trials was the omission of formalin and centrifugation in trial B. The isolations were performed by the same researcher for both trials. Strained omasal fluid (250 mL) was combined 1:1 with prewarmed, anaerobically prepared with an N-free buffer (Simplex type, pH 6.8; Williams and Coleman, 1992) and added to a prewarmed separatory funnel. Plant particles were removed by aspiration after 1 h of incubation at 39°C. Funnel contents were then preserved with formalin (0.1% vol/vol in final solution) and stored for <4 d at 4°C. Preserved contents were centrifuged at $1,000 \times g$ for 5 min at 4°C, the pellet was resuspended in saline, and protozoa were isolated on a nylon cloth with a 20 µm pore size (14% open area, Sefar, Buffalo, NY). The protozoa isolate was washed several times with saline (500 mL) to reduce bacterial contamination. After isolation, protozoa were stored at -20°C before lyophilization. Protozoa from trial C were isolated from strained ruminal fluid by flocculation to remove feed particles followed by preservation with formalin and centrifugation at $500 \times g$ for 5 min at 10°C. The pellet obtained after centrifugation was assumed to be representative of the ruminal protozoa (Recktenwald, 2010). Protozoa isolation from trial D was performed at the Ohio State University according

to Denton et al. (2015), except 25 mL instead of 30 mL of clarified fluid was filtered per isolation.

Chemical Analysis and Hydrolysis Times

All samples were analyzed for DM after 6 h at 105°C and ash according to AOAC International (2005). Total N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). Amino acid content of all samples was determined by HPLC following hydrolysis for 24 h at 110°C in a block heater (Gehrke et al., 1985). Trial B bacteria and protozoa were also hydrolyzed for 2, 4, 6, 12, 18, 21, 24, 30, 48, 72, 120, and 168 h to evaluate the rate of release of each AA. The time points chosen were based on a similar analysis performed on milk proteins (Rutherford et al., 2008). The entire time course was performed twice for

Figure 1. Flowchart for preparation of protozoa isolates in trials A and B. Fractions discarded are crossed out. Trial B isolation differed only in the omission of the formalin preservation step.

each sample, and the reported values are the mean of the 2 determinations. Insufficient sample amount from trials A, C, and D precluded the multiple time point hydrolysis of samples for AA determination.

For all AA, excluding Met, Cys, and Trp, a sample containing 2 mg of N was weighed into hydrolysis tubes with 25 μ L of 250 mM norleucine as an internal standard. Samples were then hydrolyzed as described above with high-purity 6 M HCl (5 mL) after flushing with N₂ gas (Mason et al., 1980). For Met and Cys, additional aliquots containing 2 mg of N and the internal standard were preoxidized with 1 mL of performic acid (0.9 mL of 88% formic acid, 0.1 mL of 30% H₂O₂, and 5 mg of phenol) for 16 h at 4°C before acid hydrolysis (Elkin and Griffith, 1985). After hydrolysis, tube contents were filtered through Whatman 541 filter paper (GE Healthcare Life Sciences, Marlborough, MA) and filtrate was diluted to 50 mL in a volumetric flask with HPLC-grade H₂O. Aliquots (0.5 mL) were evaporated at 60°C under constant N₂ flushing, with 3 rinses and re-evaporations with HPLC-grade H₂O to remove acid residues. After final evaporation, the hydrolysate was dissolved in 1 mL of Na diluent (Na220, Pickering Laboratories, Mountain View, CA).

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) using a 4-buffer step gradient and column temperature gradient. Detection of separated AA was performed at 560 nm following postcolumn ninhydrin derivation. Standards (250 nmol/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 μ L.

For Trp determination, a separate aliquot of sample containing 2 mg of N was hydrolyzed with 1.2 g of Ba(OH)₂ at 110°C for the same time course as other AA on a block heater according to the method of Landry and Delhay (1992). Included in the hydrolysis was 125 μ L of 5-Methyl-Trp (5 mM) as an internal standard. After cooling to precipitate barium ions, an aliquot (3 μ L) of the hydrolysate was added to 1 mL of acetate buffer (0.07 M sodium acetate) and analyzed using fluorescence detection (excitation = 285 nm, emission = 345 nm) after HPLC separation.

In Vitro Digestibility of N and AA

Microbial samples from trials A and B were analyzed for intestinal digestibility of N and AA according to the assay described by Ross (2013) with minor modifications. The ruminal incubation step was omitted because microbial samples were isolated from the oma-

sum. For each sample, 150 mg of DM was weighed in duplicate into 125-mL Erlenmeyer flasks and 40 mL of prewarmed ruminal buffer was added (Van Soest, 2015). Samples were then acidified to a pH of 2 with 3 M HCl followed by addition of 2 mL of pepsin solution (282 U/mL). After 1 h of incubation at 39°C in a shaking water bath, contents of the flask were neutralized with 2 mL of 2 M NaOH. Ten milliliters of enzyme mixture containing trypsin (24 mg/mL), chymotrypsin (20 mg/mL), amylase (50 mg/mL), and lipase (4 mg/mL) was then added to the flasks, followed by 24 h of incubation at 39°C in a shaking water bath. After incubation, flask contents were filtered on previously tared Whatman 934AH filters under vacuum. Samples were allowed to air dry, followed by drying and storage in a desiccator. Filter plus residue weight was then recorded, and DM remaining on the filter was corrected for a blank carried throughout the process. Each filter was cut in half and weighed; with one half used for determination of residual N, whereas the other half was used for AA analysis of the residual material. Determination of residual AA, except Trp, was performed after 24 h of hydrolysis with preoxidation of Met and Cys, as described previously. Insufficient sample N on the filters precluded the determination of Trp on the residues.

Calculations and Statistical Analysis

Digestibility of DM, N, and individual AA was calculated as the disappearance of DM, OM, N, or AA after enzymatic hydrolysis, corrected for the procedure blank. Determination of the AA concentration of microbes after multiple hydrolysis times was performed using a method similar to Rutherford et al. (2008) and Rutherford (2009). Amino acid concentration (mg/g of DM) was plotted against hydrolysis time and a nonlinear equation was used to fit the curves to each plot:

$$B(t) = \frac{A_o h (e^{-lh} - e^{-ht})}{h - l},$$

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; $A_o h$ and l for each sample were derived from each AA using least squares nonlinear regression with the constraints that $A_o > 0$, and $h > 0$, and e is a mathematical constant whose natural logarithm is equal to 1 and is approximately equal to 2.71828. Rutherford (2009) used an additional term to account for free AA content (analyzed as AA

determined before hydrolysis); this was not included in the current model, as free AA in bacterial and protozoal samples was considered to be negligible.

The AA profile of trial B bacteria and protozoa determined using the different hydrolysis methods was compared using 2 one-sided paired *t*-tests (TOST option in the TTEST procedure of SAS version 9.3; SAS Institute, Cary, NC). This procedure, common in bio-equivalence testing, allows the researcher to specify biologically relevant differences in means determined by competing methods. In our study, an AA profile difference that would theoretically alter the calculated flow of any individual AA by ≥ 6 g/d was considered biologically relevant because (1) commercial AA products fed at 10 g/d (a common minimum feeding rate) provide approximately 6 g of metabolizable AA (Whitehouse, 2016), and (2) with a commonly limiting AA, such as Met, a 6-g shift represents a 10% difference in the total supply of 60 g/d. To convert this 6 g/d of metabolizable AA value into a relevant change in bacterial or protozoal AA profile (measured in g/100 g of AA) the following calculations were made. The microbial AA flowing out of the rumen of a lactating dairy cow was assumed to be approximately 1,900 g/d (Reynal et al., 2007; Fessenden, 2016). Assuming 80% of the flow is bacteria AA and 20% is protozoa AA (Dijkstra et al., 1998; Fessenden, 2016), this corresponds to 1,520 and 380 g/d of AA for bacteria and protozoa, respectively. Therefore, a 6-g/d change in supply corresponds to 0.4 (6 g/1,520 g) and 1.5 percentage units (6 g/380 g) change in any individual AA in the AA profile of bacteria and protozoa, respectively. In a nutrition modeling context, Fessenden (2016) saw significant improvements in CNCPS predictions of AA flow when similar magnitude changes in AA profile of microbial fractions were evaluated. The bio-equivalency testing framework requires thoughtful interpretation of the results. Emphasis is placed on the comparison of the 90% confidence interval of the mean difference and its comparison the predetermined biologically relevant ranges defined previously in this section.

For AA digestibility, a 2-sample *t*-test was used to compare the digestibility estimates between trials A and B. Only means from trials A and B were analyzed, as a limited amount of sample precluded digestibility and multiple hydrolysis time analysis for trials C and D. For all analysis, $n = 2$ for each comparison.

RESULTS AND DISCUSSION

Microbial Chemical Composition and Digestibility

All donor cows were fed diets with similar chemical and nutrient composition (Table 1). Diets were typi-

cal of the northeastern and Midwestern United States, with corn silage and alfalfa silage as the principal forages. Organic matter content in bacteria and protozoa was similar to values obtained previously from ruminal and omasal isolates (Brito et al., 2006, 2007), although OM content is strongly influenced by the isolation procedures used (Martin et al., 1994). Trial A bacteria and protozoa had numerically decreased DM, OM, and N digestibility compared with trial B (Table 2); this is likely due to the use of formalin in trial A versus trial B, as formaldehyde readily reacts with proteins to form products resistant to digestion (Barry, 1976). Whereas diet and animal differences between trials might also have contributed to observed differences, the direction and magnitude of the difference between trial A and B for OM, DM, and N digestibility suggests formalin treatment is a likely cause of the differences observed. Bacteria isolates had similar AA as a percent of DM, whereas the lower N content of bacteria from trial C (7.5%) resulted in AA N contributing more to total N compared with bacteria from trials A and B. Protozoa from trial D had the highest AA N as a percent of N. Bacterial AA N values for trials A and B were on the low end of the range (54.9–86.7% of total N) reported by Clark et al. (1992). This could be related to the site of sampling, as microbes in trials A and B were isolated from omasal contents whereas trials C and D were isolated from ruminal contents, which may contribute to different amounts of NAN. Volden et al. (1999b) reported diaminopimelic acid and purines were affected by diet and differed between protozoa and bacterial fractions in the rumen; similarly, Illg and Stern (1994), noted wide ranges in nonamino N concentration between duodenal and ruminal samples.

Microbial isolates averaged 50.6 and 49% EAA as a percent of total AA for bacteria and protozoa, respectively (Table 3). Bacterial isolates from trial B had numerically increased concentrations of Lys and Met, whereas trials B and D protozoa also demonstrated increased Lys and Met concentrations. For NEAA, bacteria and protozoa isolations were similar among trials, with the exception of Tyr, which was reduced in trials A and C. Again, the differences in microbial isolates from trial A and C for individual AA are likely due to formalin treatment. Volden et al. (1999b) reported decreased recoveries of Lys, Met, and Tyr with versus without formaldehyde treatment in solid- and liquid-associated bacteria, and Whitehouse et al. (1994) reported approximately 20% less Tyr in microbial samples after treatment with formaldehyde. Beyond these differences, likely due to formaldehyde treatment, the AA profile of bacteria and protozoa agreed fairly well with literature reports, with some exceptions. Methionine averaged 3.2% of total AA among all samples and was at the

Table 2. Chemical composition and intestinal digestibility of bacteria and protozoa isolates

Item	Trial ¹			
	A	B	C	D
Bacteria				
OM, % of DM	83.9	92.1	90.1	—
N, % of DM	8.1	8.9	7.5	—
uN, ² % of total N	36.4	15.1	—	—
AA, % of DM	28.2	34.3	32.5	—
AA N, % of N	47.3	51.8	57.8	—
DM digestibility, %	53.6	69.0	—	—
OM digestibility, %	68.2	81.9	—	—
N digestibility, %	63.6	84.9	—	—
Protozoa				
OM, % of DM	87.0	85.0	90.8	93.8
N, % of DM	8.3	8.2	8.1	8.4
uN, ² % total N	46.7	24.7	—	—
AA, % of DM	28.4	31.7	37.4	45.3
AA N, % of N	53.2	53.7	65.9	71.3
DM digestibility, %	48.7	61.9	—	—
OM digestibility, %	65.9	88.4	—	—
N digestibility, %	53.3	75.3	—	—

¹Trial A: Fessenden (2016); Trial B: A. Foskolos (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2014); Trial D: T. Hackmann, unpublished data.

²Intestinally unavailable N (uN) as determined by the procedure of Ross et al. (2013).

high end of the range reported by Clark et al. (1992). The variability of reported AA composition is likely related more to the isolation techniques rather than true differences among microbial populations. Protozoa AA

composition has been shown to remain fairly constant among sampling times (Martin et al., 1996; Volden et al., 1999a). Differences among microbial fractions (solid-associated bacteria, liquid-associated bacteria, and protozoa) have been well documented (Chiquette and Benchaar, 1998; Korhonen et al., 2002); however, reasons for the differences are not clear. Procedures used to detach microbes report recoveries ranging from 20 (Martín-Orúe et al., 1998) to 80% (Whitehouse et al., 1994); this might call into question the true ability of recovered bacteria to represent the particle associated bacteria (Korhonen et al., 2002). Ultimately, it is likely that differences in isolation methods are responsible for much of the reported ranges of AA composition, whereas a smaller portion of the variation can be considered a true difference in AA composition (Fonseca et al., 2014).

Bacteria and protozoa from trial A demonstrated decreased digestibility (Table 4) for most AA, which again is likely related directly to formalin treatment. Total bacterial EAA digestibility averaged 74.9 and 88.0% for trials A and B, respectively ($P = 0.01$). Protozoa AA digestibility was also likely affected by formalin treatment in trial A; however, not all AA were significantly different between trials. Arginine, Leu, Val, and Glu all demonstrated decreased digestibility with formalin treatment. The ability of the uN assay to detect differences in digestibility due to formalin treatment indicates the assay might be a useful evaluation tool for

Table 3. Amino acid profile (% of total AA) of microbial isolates using a 24-h hydrolysis time

Item	Bacteria AA ¹				Protozoa AA ¹			
	A	B	C	D	A	B	C	D
EAA								
Arg	5.4	5.0	5.0	—	5.5	5.4	5.4	4.7
His	2.1	2.1	2.0	—	2.9	2.5	2.3	2.0
Ile	5.0	4.0	4.9	—	4.7	3.8	5.7	5.5
Leu	4.8	5.6	6.8	—	5.5	6.1	4.6	4.2
Lys	4.7	7.5	4.8	—	5.7	8.8	5.3	10.2
Met	3.3	4.5	2.6	—	2.7	3.1	2.1	3.8
Phe	6.6	6.0	6.8	—	7.4	6.5	7.3	7.6
Thr	6.3	5.7	5.4	—	5.4	4.8	6.1	4.7
Trp	5.7	5.5	5.3	—	4.6	4.5	3.1	1.4
Val	6.7	5.9	5.6	—	5.7	4.7	4.7	4.7
Total EAA	50.8	51.9	49.3	—	50.1	50.2	46.5	48.8
NEAA								
Ala	7.9	6.9	6.7	—	5.9	5.1	4.7	4.0
Asp	12.2	11.1	8.8	—	11.8	10.8	12.1	11.6
Cys	1.4	1.5	1.1	—	2.0	2.1	1.7	2.2
Glu	12.4	11.3	13.0	—	14.0	13.6	15.2	13.6
Gly	5.7	4.9	5.2	—	4.7	4.5	4.3	4.0
Pro	2.1	2.0	6.9	—	2.7	3.0	7.9	5.6
Ser	5.5	4.6	5.4	—	5.3	5.4	5.5	4.2
Tyr	2.0	6.0	3.5	—	3.5	5.4	2.0	6.1
Total NEAA	49.2	48.1	50.7	—	49.9	49.8	53.5	51.2

¹Trial A: Fessenden (2016); Trial B: A. Foskolos (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2014); Trial D: T. Hackmann, unpublished data.

other protein-containing feedstuffs, especially rumen-protected protein supplements. Other techniques, such as the mobile bag technique (Hvelplund et al., 1992) and the modified 3-step assay (Gargallo et al., 2006), rely on retention of all undigested proteins in bags, and, as such, estimates of microbial digestibility of AA from those assays are of limited value due to potential for loss from the bag. The in vivo nature of the mobile bag technique does ensure exposure to idealized enzymatic hydrolysis conditions; however, this requires extensively cannulated animals and a significant amount of sample to use the method, something not easily done with isolated omasal microbial samples. Heat-damaged blood meal, although relatively soluble and able to pass through pores, can have a very low digestible fraction (Ross et al., 2013). Alternative in vivo techniques, such as the precision-fed cecectomized rooster bioassay, have been used in ruminant feeds (Titgemeyer et al., 1990, Boucher et al., 2009), and Fonseca et al. (2014) recently applied the technique to ruminal bacteria isolates. Total AA digestibility reported by Fonseca et al. (2014) averaged 76%, with a range of 62 (Cys) to 82% (Met). The mean AA digestibility in non-formalin-treated bacteria in the current trial was 88%, with a range of 84 (Tyr) to 95% (Cys). Differences between the studies are likely related to the different enzyme activities and

digestive processes between these 2 methods and the enzyme and pH differences between avian and ruminant digestion (Guerino and Baumrucker, 1987; Ross et al., 2013). Storm et al., (1983) calculated a mean intestinal digestibility of 85% (range of 80 to 88%) in sheep maintained on VFA, minerals, and isolated ruminal microorganisms. Mathematical techniques have also been used to estimate digestibility, and Tas et al. (1981) used a regression approach to estimate true digestibility of rumen bacteria at 87%, whereas Hvelplund and Hesselholt (1987) reported true AA digestibilities between 80 and 91% for most AA using a similar approach. General agreement between the previously used techniques in ruminants and the current application of the assay developed by Ross et al. (2013) suggest that in vitro uN determination might be useful for future studies of AA digestibility in diverse supplemental protein sources of metabolizable AA.

AA Determination from Multiple Hydrolysis Times

The release of individual AA in trial B bacteria and protozoa are in Figures 2, 3, and 4. 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 (Table 5) were lowest for

Table 4. Intestinal digestibility (% of AA) of AA of omasal bacteria and protozoa

Item	Bacteria				Protozoa			
	Trial ¹		SEM	P-value	Trial ¹		SEM	P-value
	A	B			A	B		
EAA								
Arg	74.0	88.2	0.9	0.03	69.0	89.0	0.4	0.01
His	78.4	90.7	1.9	0.03	69.7	70.9	11.9	0.93
Ile	76.8	88.9	0.6	0.00	75.3	85.7	3.0	0.08
Leu	78.5	92.2	1.5	0.01	74.3	92.5	1.1	0.01
Lys	75.5	91.0	1.8	0.02	68.2	77.5	12.6	0.54
Met	80.7	88.8	1.0	0.07	78.4	90.1	0.9	0.17
Phe	69.0	83.2	1.2	0.01	67.5	79.7	2.7	0.06
Thr	76.3	89.8	1.1	0.01	73.5	79.2	9.3	0.61
Trp	—	—	—	—	—	—	—	—
Val	69.9	88.0	4.0	0.05	63.2	83.3	3.8	0.04
Total EAA	74.9	88.0	1.4	0.01	70.6	82.8	4.4	0.11
NEAA								
Ala	72.5	87.2	2.2	0.02	67.5	80.1	6.6	0.20
Asp	77.5	90.8	1.2	0.01	75.8	86.3	5.0	0.18
Cys	82.1	94.8	0.7	0.05	89.3	93.6	1.7	0.15
Glu	68.5	85.2	3.6	0.04	67.5	87.7	0.8	0.01
Gly	69.4	85.8	3.7	0.05	63.5	72.8	13.4	0.56
Pro	77.9	88.4	1.4	0.10	78.1	80.7	4.7	0.66
Ser	75.0	89.3	1.3	0.01	71.9	56.7	33.6	0.70
Tyr	34.0	84.3	0.4	0.00	55.9	73.8	4.5	0.06
Total NEAA	71.6	87.5	1.9	0.02	70.2	80.2	7.6	0.32
Total AA	73.3	87.8	1.7	0.01	70.4	81.6	6.0	0.21

¹Trial A: Fessenden (2016); Trial B: A. Foskolos (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2014); Trial D: T. Hackmann, unpublished data. n = 2 for each comparison.

Figure 2. Effect of hydrolysis time (h) on content of EAA (mg/g of DM) from freeze-dried isolations of omasal bacteria (●) and protozoa (○) from trial B. The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective data set.

Figure 3. Effect of hydrolysis time (h) on content of NEAA (mg/g of DM) from freeze-dried isolations of omasal bacteria (●) and protozoa (○) from trial B. The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective data set.

these AA. Of the NEAA of the protozoa, Ala, Cys, and Pro demonstrated increasing concentrations of AA as hydrolysis time increased. Serine concentrations of bacteria and protozoa decreased markedly after 24 h of hydrolysis as indicated by a relatively high loss rate (Table 5). 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 nonlinear 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 (Rutherford, 2009). To our knowledge, no previous work has reported rumen microbial AA content after

multiple hydrolysis times. Rutherford (2009) reported similarly low hydrolysis rate for Ile and Val, whereas Ser was reported to have the highest loss rate 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. Although 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 2 methods in relation to biologically relevant ranges (Table 6). This alterna-

tive framework of hypothesis testing requires thoughtful interpretation of the results. Whereas some AA may exhibit negligible mean differences between analysis method, such as His and Thr, the interpretation of the 90% confidence interval indicates that they are not equivalent, as the confidence interval lies outside the predetermined range 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% confidence interval of the mean difference was greater than ± 1 g/100 g of AA for Ile, Leu, Met, and Val (Figure 5). 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 Rutherford (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/100 g of AA for bacteria) serves to emphasize the importance of the AA profile of bacteria on AA supply determinations.

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/100 g of AA for protozoa). Six of the 10 EAA and 6 of the 8 NEAA were deemed equivalent between methods (Table 7). Similar to the bacterial results, Ile and Met were underestimated (13.4 and 16.5%, respectively; Figure 6) when determined with a single time point hydrolysis, resulting in over estimation of several other AA, namely Lys and Asp.

Figure 4. Effect of hydrolysis time (h) on content of EAA, NEAA, and total AA (mg/g of DM) from freeze-dried isolations of omasal bacteria (●) and protozoa (○) from trial B. The mean of each time point (2 replicates each) is plotted against the least squared regression line for each respective data set.

Implications for AA Predictions in Mathematical Nutritional Models

The nonequivalence of the determination methods are important to consider when developing models that rely on AA profiles of microbial protein and feedstuffs. The results from our study and the Rutherford (2009) data indicate that specific AA, especially Ile, Leu, Met, and Val, could be underestimated in many postruminal AA flow studies when utilizing single time point hydrolysis between 21 and 24 h. This consideration should be 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 use mechanistic postabsorptive submodels. 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 EAA for lactating cattle. Given the data presented here and by Rutherford (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; R. J. Higgs, Cornell University, current address Ohaupo, New Zealand, unpublished data). Overall, this analysis illustrates how sensitive nutritional models that rely

Table 5. Rate of hydrolysis (h)¹ and loss (l)² for individual and total AA from omasal bacteria and protozoa isolates from trial B [values are presented as mean (SEM)]

Item	Bacteria		Protozoa	
	h (h ⁻¹)	l (h ⁻¹)	h (h ⁻¹)	l (h ⁻¹)
EAA				
Arg	0.414 (0.045)	0.00039 (0.00078)	0.303 (0.027)	-0.00025 (0.00001)
His	0.577 (0.222)	-0.00027 (0.00048)	0.409 (0.110)	0.00026 (0.00038)
Ile	0.107 (0.037)	-0.00054 (0.00080)	0.106 (0.007)	-0.00122 (0.00034)
Leu	0.323 (0.019)	-0.00037 (0.00086)	0.217 (0.002)	-0.00162 (0.00060)
Lys	0.421 (0.017)	-0.00012 (0.00034)	0.333 (0.026)	-0.00016 (0.00051)
Met	0.234 (0.079)	-0.00097 (0.00008)	0.198 (0.100)	-0.00112 (0.00041)
Phe	0.782 (0.193)	-0.00050 (0.00108)	0.529 (0.286)	-0.00052 (0.00086)
Thr	0.323 (0.056)	0.00077 (0.00007)	0.318 (0.030)	0.00015 (0.00042)
Trp	0.283 (0.196)	0.00054 (0.00090)	0.332 (0.303)	-0.00072 (0.00122)
Val	0.312 (0.054)	-0.00054 (0.00055)	0.169 (0.021)	-0.00153 (0.00003)
Total EAA	0.303 (0.009)	-0.00038 (0.00022)	0.287 (0.002)	-0.00084 (0.00033)
NEAA				
Ala	0.571 (0.183)	0.00001 (0.00063)	0.658 (0.132)	-0.00156 (0.00100)
Asp	0.523 (0.057)	0.00014 (0.00039)	0.548 (0.168)	-0.00044 (0.00077)
Cys	0.377 (0.035)	-0.00035 (0.00005)	0.429 (0.125)	-0.00070 (0.00023)
Glu	0.550 (0.027)	-0.00019 (0.00005)	0.397 (0.060)	-0.00048 (0.00118)
Gly	0.599 (0.174)	-0.00049 (0.00020)	0.541 (0.030)	-0.00081 (0.00009)
Pro	0.324 (0.115)	0.00018 (0.00021)	0.304 (0.014)	-0.00081 (0.00045)
Ser	0.622 (0.097)	0.00218 (0.00049)	0.418 (0.011)	0.00198 (0.00079)
Tyr	0.804 (0.254)	0.00036 (0.00024)	0.328 (0.476)	-0.00046 (0.00038)
Total NEAA	0.568 (0.112)	0.00014 (0.00024)	0.447 (0.107)	-0.00045 (0.00075)
Total AA	0.415 (0.048)	-0.00018 (0.00021)	0.357 (0.037)	-0.00067 (0.00054)

¹Proportion of bound AA hydrolyzed per hour.²Proportion of bound AA destroyed per hour.**Table 6.** Comparison of the AA composition (g/100 g of AA) of omasal bacteria from trial B¹ determined using multiple hydrolysis time point or single hydrolysis time point methods

AA	Method		Single – multiple	SED ²	90% CI		EQ ³
	Single	Multiple			Lower	Upper	
EAA							
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
NEAA							
Ala	6.88	6.94	-0.06	0.21	-1.36	1.24	No
Asp	11.06	10.79	0.28	0.03	0.05	0.50	No
Cys	1.45	1.40	0.05	0.07	-0.40	0.51	No
Glu	11.26	11.06	0.21	0.58	-3.45	3.86	No
Gly	4.86	4.83	0.02	0.02	-0.10	0.15	Yes
Pro	2.03	1.91	0.12	0.16	-0.89	1.12	No
Ser	4.58	4.89	-0.31	0.44	-3.06	2.44	No
Tyr	5.71	5.65	0.06	0.13	-0.76	0.89	No

¹Trial B: A. Foskolos (unpublished data). n = 2 for all comparisons.²SED = standard error of the difference.³Equivalence 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/100 g of AA.

Table 7. Comparison of the AA composition (g/100 g of AA) of omasal protozoa from trial B¹ determined using multiple versus single time point hydrolysis methods

AA	Method		Single – multiple	SED ²	90% CI		EQ ³
	Single	Multiple			Lower	Upper	
EAA							
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
NEAA							
Ala	5.14	5.03	0.11	0.07	−0.31	0.53	Yes
Asp	10.85	10.14	0.71	0.47	−2.27	3.69	No
Cys	2.06	2.16	−0.09	0.01	−0.15	−0.04	Yes
Glu	13.56	13.03	0.52	0.04	0.30	0.74	Yes
Gly	4.48	4.41	0.07	0.01	0.03	0.12	Yes
Pro	2.99	2.89	0.10	0.10	−0.52	0.71	Yes
Ser	5.36	5.28	0.08	0.03	−0.14	0.29	Yes
Tyr	4.50	4.70	−0.20	0.34	−2.32	1.92	No

¹Trial B: A. Foskolos (unpublished data). n = 2 for all comparisons.²SED = standard error of the difference.³Equivalence 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/100 g of AA. **Figure 5.** Equivalency chart for difference in AA composition (g/100 g of AA) of trial B bacteria determined using multiple versus single time point hydrolysis methods. Open circles (O) represent the mean difference and error bars represent the 90% CI around the mean difference. Shaded region represents equivalency defined as −0.4 to 0.4 g/100 g of bacterial AA.

Figure 6. Equivalency chart for difference in AA composition (g/100 g of AA) of trial B protozoa determined using multiple versus single time point hydrolysis methods. Open circles (O) represent the mean difference and error bars represent the 90% CI around the mean difference. Shaded region represents equivalency defined as -1.5 to 1.5 g/100 g of protozoal AA.

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 use formalin as a microbial preservative, as it will likely lead to biases and poor model evaluation.

CONCLUSIONS

Microbial composition and digestibility of individual AA are very important for the accurate predictions in many nutrition models used to feed dairy cattle. Previous literature reports have used techniques that have known limitations, whereas new procedures developed to address these limitations might provide better estimations of key parameters needed to properly characterize metabolizable AA supply. Multiple time point hydrolysis has been shown to improve the determination of AA in feeds, and the technique has been applied here. Digestibility of AA in microbial isolations were measured using a new in vitro technique. Although in vivo techniques might better represent the bio-physical

condition of the ruminant animal, they can be expensive and rely on surgically altered animals. In vitro digestibility estimates were similar to those reported using previous techniques, and the assay was sensitive to formalin treatment, a process known to reduce bio-availability of proteins. Methionine and branched-chain AA concentration of microbial isolates was higher than previously reported, and this might have implications for predicting AA supply in cattle.

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