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# Microbial composition and omasal flows of bacterial, protozoal, and nonmicrobial amino acids in lactating dairy cows fed fresh perennial ryegrass (*Lolium perenne* L.) not supplemented or supplemented with rolled barley

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## ABSTRACT

The objective of this study was to evaluate the effect of rolled barley supplementation on microbial composition and omasal flows of bacterial, protozoal, and nonmicrobial AA in cows fed fresh perennial ryegrass (*Lolium perenne* L.; PRG). Ten ruminally cannulated multiparous Holstein cows averaging (mean ± standard deviation) 49 ± 23 d in milk and 513 ± 36 kg of body weight were assigned to 1 of 2 treatments in a switchback design. The treatment diets were PRG only or PRG plus 3.5 kg of dry matter rolled barley (G+RB). The study consisted of three 29-d periods where each period consisted of 21 d of diet adaptation and 8 d of data and sample collection. A double-marker system was used to quantify nutrient flow entering the omasal canal along with $^{15}$N-ammonium sulfate to label and measure the microbial and nonmicrobial omasal flow of AA. Overall, rolled barley supplementation had no effect on the AA composition of the omasal liquid-associated and particle-associated bacteria. Rolled barley supplementation affected the AA concentrations of omasal protozoa; however, the differences were nutritionally minor. Particle-associated bacteria AA flow was increased for all AA, except for Trp and Pro, in cows fed the G+RB diet. Rolled barley supplementation had no effect on protozoal AA flow. On average, protozoa accounted for 23% of the microbial essential AA flow, which ranged from 17 to 28% for Trp and Lys, respectively. The flow of all AA in omasal true digesta increased in cows fed the G+RB diet compared with the PRG-only diet, resulting in a 228 g/d increase in total AA flow in cows fed the G+RB diet. This increase in total AA flow in cows fed the G+RB diet was due to an increase in microbial AA flow. Rolled barley supplementation had no effect on nonmicrobial AA flow. The nonmicrobial AA flow modestly contributed to total AA flow, accounting for 15.6% on average. These results indicated that extensive ruminal degradation of PRG AA occurred (83.5%), and we demonstrated that cows consuming PRG-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. Rolled barley supplementation can increase the omasal flow of microbial AA in cows consuming PRG-based diets. However, further research is required to elucidate if this increased AA supply can support higher milk yield under such dietary conditions.

**Key words:** microbial protein synthesis, omasal sampling, pasture

## INTRODUCTION

In many parts of the world, fresh temperate forage, particularly perennial ryegrass (*Lolium perenne* L.; **PRG**), contributes significantly to the diet of the lactating dairy cow (Humphreys et al., 2010; van den Pol-van Dasselaar et al., 2020). Although immature PRG swards are energy dense (2.75–2.9 Mcal of ME per kilogram of DM), the ME supply to cows consuming such swards is typically cited as the primary limiting factor for milk production (Kolver and Muller, 1998; Keim and Anrique, 2011). This assumption is often justified by the observation that the CP concentration of PRG exceeds the CP requirements of the lactating dairy cow (NRC, 2001; Pacheco and Waghorn, 2008). However, PRG can contain high levels of nonprotein N, soluble N, and rumen-degradable N, potentially limiting the ability of the forage to meet the AA requirement of the lactating dairy cow (Hoekstra et al., 2008; Dineen et al., 2021).

Beever and Siddons (1986) concluded that PRG that escapes ruminal fermentation had a limited contribution to MP supply (3–5% of MP), whereas a much

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larger contribution was identified when estimated using in sacco techniques (20–44% of MP; Van Vuuren et al., 1991; Valk et al., 1996). Few studies have quantified the AA flow in lactating dairy cows that consume fresh PRG (O'Mara et al., 1997; Kolver et al., 1999; Younge et al., 2004). Furthermore, the existing AA flow data were obtained using duodenal cannulas as sampling sites, which are recognized to contain considerable endogenous N contamination, and thus reduce the ability to distinguish the source of AA flows (Ørskov et al., 1986; Ahvenjärvi et al., 2000). To our knowledge, no study has previously quantified the omasal flow of AA in lactating dairy cows that consume pasture-based diets (i.e., fresh PRG cut twice daily).

Although many studies have investigated the effects of supplementing cereal grains to lactating dairy cows that consume pasture-based diets at a whole animal level (Bargo et al., 2003), few studies have quantified postruminal nutrient flows when compared with the more extensive literature available for conserved forage-based diets (Broderick et al., 2010; Huhtanen et al., 2010). Nutrient flow data are particularly essential in the development and evaluation of mechanistic nutritional models, with the capability to predict postruminal N and AA flows and first-limiting nutrients for milk yield. In a companion paper (Dineen et al., 2020) utilizing the omasal sampling technique developed by Huhtanen et al. (1997), it was demonstrated that rolled barley (**RB**) supplementation increased the flow of bacterial N (+50 g/d) due to a greater amount of fermentable carbohydrate (**CHO**) being digested in the rumen and greater efficiency of microbial protein synthesis when compared with a PRG-only diet. Extensive ruminal degradation of the PRG N also occurred, resulting in a negligible contribution from nonmicrobial N to the total flow of NAN. Altogether, these observations warranted further investigation into the possible effects of RB supplementation on both the quantity and source of AA supply in cows consuming PRG-based diets.

In a recent literature review, Sok et al. (2017) demonstrated that there are considerable differences among bacterial and protozoal AA composition. Hence, if the differing AA compositions and estimates regarding the flow of each microbial population could be integrated, the accuracy and precision in predicting AA supply by nutritional models could be improved (Sok et al., 2017). However, the current lack of data describing the relative contribution from microbial populations to the total AA flow limits the ability for model evaluation (Fessenden et al., 2019a). Previous experiments quantifying microbial AA flows in cows consuming pasture-based diets have not included isolation of protozoa, which likely resulted in an underestimation of microbial AA flow (Broderick et al., 2010). Furthermore, there is contrasting evidence in the literature as to the effect of dietary characteristics on microbial AA concentrations (Hvelplund, 1986; Korhonen et al., 2002). Thus, to improve the estimation of AA flows, there is a need to quantify the AA composition of both bacteria and protozoa and assess their contribution, in combination with nonmicrobial sources, to the total AA flow at the omasal canal of lactating dairy cows.

The hypothesis of this experiment was that the supplementation of RB would increase the fermentable CHO supply for ruminal microbes, and thereby increase the microbial AA flow at the omasal canal compared with a PRG-only diet. Thus, the objective of the study was to evaluate omasal flows of bacterial, protozoal, and nonmicrobial AA in cows consuming fresh PRG not supplemented or supplemented with RB. A secondary objective was to compare the effect of diet on microbial AA concentrations in cows consuming PRG-based diets. The effects of RB supplementation on ruminal metabolism, omasal flow of nutrients, and microbial dynamics have been previously described in the companion paper (Dineen et al., 2020).

## MATERIALS AND METHODS

The experiment was conducted from April to July of 2017 at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland (52°16′N; 8°25′W; 49 m above sea level). All procedures described in this experiment were approved by the Teagasc Animal Ethics Committee and conducted under experimental license (AE19132-P054) from the Health Products Regulatory Authority under European directive 2010/63/EU and S.I. no. 543 of 2012 (European Union, 2012).

### *Animals, Experimental Design, and Treatment Administration*

Ten ruminally cannulated multiparous Holstein cows averaging (mean ± SD) 49 ± 23 DIM and 513 ± 36 kg of BW were enrolled in a 3-wk prestudy acclimation period where all animals were managed and housed in a freestall barn and fed a common diet. Cows were then stratified by prestudy milk yield and randomly assigned to 1 of 2 treatment sequences in a switchback design. The study consisted of three 29-d experimental periods, where each period contained 21 d for diet adaptation and 8 d of data and sample collection. For the first 21 d of each period, the cows were housed in a freestall barn. For each 8-d period of sample collection, the cows were housed in individual tiestalls with free access to

water. Treatment diets were (1) PRG only (**G**) or (2) PRG plus 3.5 kg of DM RB (**G+RB**). In addition, both treatments were fed 40 g/d of magnesium (120 g/d of Sweetened Cal-Mag; Nutribio Ltd., Tivoli, Cork, Ireland) to reduce the risk of hypomagnesemia.

All cows received both diets throughout the 3 experimental periods according to their randomly assigned sequence of either G, G+RB, G or G+RB, G, G+RB. The swards of PRG were mechanically harvested twice daily (0800 and 1500 h) with a GrassTech Grazer GT80 (Future Grass Technology, Borris, Co. Carlow, Ireland). During the 21-d adaptation period, the cows were offered PRG twice daily at the time of harvesting. During the 8 d of data and sample collection, the cows were offered PRG 6 times daily at 0630, 0830, 1230, 1530, 1930, and 2130 h with the PRG refrigerated at 4°C between feedings to minimize respiration and nutrient loss. The RB was offered to the respective cows at the time of milking (0730 and 1530 h) as 2 equal meals. Full details of the cow and sward management are described in Dineen et al. (2020). The nutrient and AA composition of the RB supplement and experimental diets are presented in Table 1.

**Table 1.** Nutrient composition of supplement and diet[1]

| Item[2] | Diet or supplement | | |
|---|---|---|---|
| | RB | G | G+RB |
| DM,[3] % | 86.9 | 20.2 | 34.2 |
| N,[3] % of DM | 1.9 | 2.6 | 2.5 |
| Soluble N,[3] % of N | 17.1 | 35.3 | 31.7 |
| NDIN,[3] % of N | 8.0 | 14.9 | 13.5 |
| ADIN,[3] % of N | 3.0 | 2.5 | 2.6 |
| Starch,[3] % of DM | 60.7 | 2.5 | 14.7 |
| WSC,[3] % of DM | 7.1 | 22.4 | 19.2 |
| aNDFom,[3] % of DM | 19.2 | 36.1 | 32.6 |
| uNDFom,[3] % of aNDFom | 32.9 | 9.8 | 14.7 |
| Ether extract,[3] % of DM | 1.7 | 3.1 | 2.8 |
| Ash,[3] % of DM | 2.6 | 7.0 | 6.1 |
| EAA N, % of TN | | | |
| Arg | 9.4 | 11.1 | — |
| His | 3.3 | 3.0 | — |
| Ile | 2.3 | 3.0 | — |
| Leu | 4.2 | 5.4 | — |
| Lys | 3.5 | 5.5 | — |
| Met | 0.9 | 1.2 | — |
| Phe | 2.3 | 2.8 | — |
| Thr | 2.3 | 3.4 | — |
| Trp | 1.1 | 1.7 | — |
| Val | 3.3 | 4.0 | — |
| Total EAA | 32.6 | 41.2 | — |
| NEAA N, % of TN | | | |
| Ala | 3.7 | 6.6 | — |
| Asp | 3.6 | 6.4 | — |
| Cys | 1.2 | 0.7 | — |
| Glu | 14.8 | 7.2 | — |
| Gly | 4.5 | 6.5 | — |
| Pro | 6.7 | 3.2 | — |
| Ser | 3.5 | 3.7 | — |
| Tyr | 1.4 | 1.6 | — |
| Total NEAA | 39.4 | 36.0 | — |
| TAA N, % of TN | 72.0 | 77.2 | — |

[1]RB = rolled barley; G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% hulled rolled barley.

[2]aNDFom = amylase- and sodium sulfite–treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite–treated NDF corrected for ash residue; TN = total N; TAA = total AA.

[3]Previously reported in Dineen et al. (2020).

### Sample Collection and Processing

Spot samples of omasal digesta were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). The double-marker method utilizing CoEDTA (Udén et al., 1980) and undigested amylase- and sodium sulfite–treated NDF corrected for ash residue after 240 h of in vitro fermentation (Raffrenato et al., 2018) were used to quantify liquid and particle flow entering the omasal canal, respectively. To estimate microbial flow at the omasal canal, 8.8 g/d of ammonium sulfate (Cambridge Isotope Laboratories Inc., Andover, MA) with a 10% enrichment of $^{15}N$ (187 mg/d of $^{15}N$) was added to the CoEDTA infusate. Before starting the infusion, samples of ruminal contents (liquid and solids) were taken for later determination of $^{15}N$ background. Omasal sampling began approximately 74 h after the beginning of marker infusion to allow uniform marker distribution. Further details on marker preparation and infusion are reported in Dineen et al. (2020).

Samples of omasal contents were collected from the omasal canal during three 8-h intervals as follows: at 1600, 1800, 2000, and 2200 h on d 24; at 0000, 0200, 0400, and 0600 h on d 26; and at 0800, 1000, 1200, and 1400 h on d 27. A 425-mL spot sample was obtained during the first 3 sampling time points, and a 675-mL spot sample was obtained during the last sampling time points of each interval. Each spot sample was split into subsamples of 50 mL (×2), 125 mL, and 200 mL, with an additional 250-mL subsample at the last time point. The 50-mL subsamples were used for a separate study of nutrient flows (Dineen et al., 2020). The 125-mL subsample was placed on ice and combined within the interval, yielding a 500-mL sample for bacterial isolation. The 200-mL subsamples were combined within the period and stored at −20°C, generating a 2.4-L composite for digestion phase separation. The additional 250-mL subsample obtained on the final time point of each interval was used to isolate omasal protozoa.

The 2.4-L pooled omasal composite was subsequently thawed and separated into the omasal large-particle, small particle, and liquid phases. The fractions were separated as described by (Reynal and Broderick, 2005). Samples were squeezed through a single layer

of large-pore polyethylene cheesecloth (Graytec, GD Textile, Manchester, UK), and the retained solids were defined as the omasal large-particle phase. The filtrate was centrifuged at 1,000 × *g* for 5 min at 4°C, and the supernatant was decanted from the pellet. The supernatant was defined as the omasal liquid phase, and the pellet was defined as the omasal small particle phase. All omasal phase samples were lyophilized and either ground through a 1-mm screen on a Cyclotec mill (large particle; Foss, Hillerød, Denmark) or homogenized with a mortar and pestle (small particle and liquid phase) before analysis. As the digesta was fractionated into 3 phases, the small particle was considered to be part of the particulate matter in the double-marker system. Concentrations of Co and undigested amylase- and sodium sulfite–treated NDF corrected for ash residue were then used to calculate the concentration of each nutrient in a sample, theoretically representing omasal true digesta (**OTD**; France and Siddons, 1986).

Bacterial isolation was performed according to Whitehouse et al. (1994) with modifications. Briefly, omasal samples were squeezed through a single layer cheesecloth, and the retained solids were washed once with saline solution and squeezed again through single layer cheesecloth. The resulting filtrate (A) was stored at 4°C for further centrifugation. The solids retained were placed in a shaking incubator for 1 h at 39°C in a 0.1% methylcellulose solution to detach particle-associated bacteria, and transferred to a 4°C cooler for 24 h. After 24 h, the sample was blended for 1 min. The blended sample was squeezed through single layer cheesecloth, and the retained solids were washed once with saline solution and squeezed again through cheesecloth. The resulting filtrate (B) was stored at 4°C for further centrifugation. Filtrates A and B were centrifuged at 1,000 × *g* for 5 min at 4°C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 × *g* for 20 min at 4°C, and the bacterial pellet was collected and stored at −20°C until lyophilization and later analysis. The bacterial pellets recovered from filtrates A and B represented the omasal liquid-associated bacteria (**LAB**) and omasal particle-associated bacteria (**PAB**), respectively. The bacterial isolations from each 8-h interval were subsequently combined within period to generate a LAB and a PAB sample per cow per period.

The 250-mL subsample obtained on the final time point of each interval was immediately processed to isolate omasal protozoa. The subsample was squeezed through a single layer of polyethylene cheesecloth and protozoa were subsequently isolated as described by Denton et al. (2015) using flocculation and filtration techniques. After isolation, protozoa were stored at −20°C, followed by lyophilization and measurement of OM to calculate the yield of protozoal OM per liter of omasal liquid (Ahvenjärvi et al., 2002).

### Laboratory Analysis

Lyophilized samples of bacteria, protozoa, and omasal phases were analyzed for DM at 105°C for 16 h 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). Samples were analyzed for NAN and $^{15}N$ using a Carlo Erba NC2500 elemental analyzer interfaced with an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY). Sample preparation and ammonia volatilization were carried out as described by Fessenden et al. (2019b). Ammonia N concentration was determined in omasal fluid using an ABHoriba Pentra 400 chemistry analyzer (Horiba-ABXDiagnostics, Kyoto, Japan).

Amino acid concentrations in feed, omasal phases, bacteria, and protozoa were analyzed by HPLC. For all AA, except Trp, 2 mg of N with 50 μL of 125 m*M* norleucine as an internal standard were hydrolyzed at 110°C for 21 h in a block heater (Gehrke et al., 1985) with high-purity 6 *M* HCl (5 mL) after flushing with $N_2$ gas. For Met and Cys, before acid hydrolysis (as described above), 2 mg of N and internal standard were preoxidized with 1 mL of performic acid (0.9 mL of 88% formic acid, 0.1 mL of 30% $H_2O_2$, and 5 mg of phenol) for 16 h at 4°C (Mason et al., 1980; Elkin and Griffith, 1985). After hydrolysis, tube contents were filtered through Whatman 541 filter paper (GE Healthcare UK Ltd., Buckinghamshire, UK), and the filtrate was diluted to 50 mL in a volumetric flask with HPLC-grade $H_2O$. Aliquots (0.3 mL) 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. After final evaporation, the hydrolysate was dissolved in 0.6 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 post-column ninhydrin derivatization. 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 μL. To determine concentrations of Trp, a separate aliquot of the sample containing 2 mg of N was hydrolyzed with 1.2 g of $Ba(OH)_2$ at 110°C for 16 h on a block heater (Landry and Delhaye, 1992). Included

in the hydrolysis was 125 μL of 5-methyl-Trp (5 m*M*) as an internal standard. 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 using fluorescence detection (excitation = 285 nm, emission = 345 nm) after HPLC separation.

### Calculations

Total N omasal flow and the partitioning of total N flow are described in the companion paper (Dineen et al., 2020). Briefly, the concentration of ammonia N in an omasal fluid sample, in combination with the flow of liquid determined by the double-marker system, were used to calculate the omasal flow of ammonia N. This was subtracted from the total N flow to determine NAN flow. The NAN flow was partitioned into 4 fractions that consisted of PAB N, LAB N, protozoal N, and nonmicrobial N. Non-ammonia nonmicrobial N (**NANMN**) was assumed to contain primarily undigested feed N and a smaller contribution of endogenous N (Ørskow et al., 1986; Lapierre et al., 2008). Microbial NAN flow was determined from $^{15}$N atom percent excess (**APE**) as described in Dineen et al. (2020). Protozoal OM (g/L) was calculated using gravimetric determinations in a known quantity of omasal liquid, as described by Fessenden et al. (2019a), assuming that protozoa only leave the rumen in the liquid phase (Ahvenjärvi et al., 2002; Karnati et al., 2007). To calculate protozoal OM flow (g/d), the quantity of protozoal OM (g/L) was multiplied by the daily volume of liquid flow (L/d) at the omasal canal. To calculate protozoal NAN flow (g/d), the protozoal OM flow (g/d) was multiplied by the protozoal NAN concentration (g/g of OM). Accounting for $^{15}$N APE in protozoa, the LAB NAN (g/d) flow was calculated as follows:

$$\text{LAB NAN flow (g/d)} = \{[\text{Liquid NAN flow (g/d)} \times \text{Liquid } ^{15}\text{N APE (g/g of NAN)}] - [\text{Protozoal NAN flow (g/d)} \times \text{Protozoal } ^{15}\text{N APE (g/g of NAN)}]\}/\text{LAB } ^{15}\text{N APE (g/g of NAN)}.$$

Particle-associated bacterial NAN flow was calculated as follows:

$$\text{PAB NAN flow (g/d)} = [\text{Particle NAN flow (g/d)} \times \text{Particle } ^{15}\text{N APE (g/g of NAN)}]/\text{PAB } ^{15}\text{N APE (g/g of NAN)}.$$

From this, total bacterial and total microbial N flow were calculated as follows:

$$\text{Bacterial NAN flow (g/d)} = \text{LAB NAN flow (g/d)} + \text{PAB NAN flow (g/d)};$$

$$\text{Microbial NAN flow (g/d)} = \text{Protozoal NAN flow (g/d)} + \text{Bacterial NAN flow (g/d)}.$$

The isolated LAB and PAB were assumed to be representative of the bacterial biomass flowing with the liquid and particulate phases, respectively (Reynal and Broderick, 2005). The NAN concentration (g/g of OM) of the LAB, PAB, and protozoal samples was used to calculate the flow of total microbial biomass. The flow of NANMN was calculated as the difference between total NAN flow and microbial NAN flow.

The concentrations of AA in feed, omasal phases, bacteria, and protozoa were corrected for incomplete recovery of AA using correction factors published by Lapierre et al. (2019). Data demonstrates that complete recovery of AA is not accomplished by 21 to 24 h of acid hydrolysis (Rutherfurd, 2009; Lapierre et al., 2019). In the current study, the AA concentrations obtained after 21 h of hydrolysis were multiplied by an average correction factor specific to each AA, determined by nonlinear regression ($A_0$) as described by Lapierre et al. (2019). The flows of individual AA in OTD were then calculated using the corrected concentration of AA in each omasal phase and the double-marker system described in Dineen et al. (2020). Liquid-associated bacterial, PAB, protozoal, total microbial, and nonmicrobial AA flow were calculated as follows:

$$\text{Liquid-associated bacterial AA flow (g/d)} = \text{LAB N flow (g/d)} \times \text{LAB AA (g/g of N)};$$

$$\text{Particle-associated bacterial AA flow (g/d)} = \text{PAB N flow (g/d)} \times \text{PAB AA (g/g of N)};$$

$$\text{Protozoal AA flow (g/d)} = \text{protozoal N flow (g/d)} \times \text{protozoal AA (g/g of N)};$$

$$\text{Microbial AA flow (g/d)} = \text{LAB AA flow (g/d)} + \text{PAB AA flow (g/d)} + \text{protozoal AA flow (g/d)};$$

$$\text{Nonmicrobial AA flow (g/d)} = \text{OTD AA flow (g/d)} - \text{microbial AA flow (g/d)}.$$

### Statistical Analysis

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc. Cary, NC). The

same model as described in Dineen et al. (2020) was used as follows:

$$Y_{ijkl} = \mu + S_i + C_{j:i} + P_k + T_l + PT_{kl} + \varepsilon_{ijkl},$$

where $Y_{ijkl}$ = dependent variable, $\mu$ = overall mean, $S_i$ = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, $P_k$ = fixed effect of period $k$, $T_l$ = fixed effect of treatment l, $PT_{kl}$ = fixed interaction effect of period $k$ and treatment $l$, and $\varepsilon_{ijkl}$ = residual error. Sequence effects and the interaction term including period and treatment were removed from the model when $P > 0.1$. Degrees of freedom were determined using the Kenward-Roger option. Means were determined using the least squares means statement. Statistical significance was considered at $P \leq 0.05$, and trends were considered at $0.05 < P \leq 0.10$.

## RESULTS AND DISCUSSION

### Microbial Composition

The N concentrations of omasal LAB, PAB, and protozoa were not affected by RB supplementation (Table 2). On average, the N concentration of PAB and protozoa were similar (8.9% and 9.0% of OM, respectively) and lower than that of LAB (10.2% of OM). Greater N concentrations in LAB than in PAB and protozoa were reported (Martin et al., 1994). Reports from the literature on protozoal N concentrations are extremely variable, which is likely due to differing levels of feed particle contamination or washing procedures during isolation (Ahvenjärvi et al., 2002; Reynal et al., 2005). In the current study, $^{15}$N enrichment was not affected by diet and was similar among all 3 microbial populations. Generally, the $^{15}$N enrichment of protozoa is reported to be lower than that of bacteria (Hristov and Broderick, 1996; Fessenden et al., 2019a). Lower $^{15}$N enrichment of protozoa has been attributed to the engulfment of unenriched dietary protein due to the limited capacity of protozoa to utilize ammonia N for growth (Williams and Coleman, 1997; Reynal et al., 2005). However, when investigating ruminal N metabolism, Ahvenjärvi et al. (2018) noted that the direct incorporation of free AA or peptides by protozoa was negligible and that protozoa selectively ingest bacterial N. Hence, similar $^{15}$N enrichment among protozoa and bacteria is plausible because the predominant source of N for protozoa is bacteria. In agreement with the current study, similar enrichment levels among protozoa and bacteria were observed when forage was the principal true protein source in the diet (Brito et al., 2006, 2007). Furthermore, the length of $^{15}NH_3$ infusion has been implicated as a factor affecting the relative enrichment of protozoa (Brito et al., 2006). In the current study, $^{15}NH_3$ was infused for 192 h, which might have contributed to the greater relative enrichment of protozoa compared with previous literature (Ahvenjärvi et al., 2002).

Total AA concentration of omasal LAB, PAB, and protozoa were not affected by RB supplementation. The total AA concentration of PAB and protozoa were similar, averaging 72.1% and 72.8% of N, respectively, and were higher than that of LAB (58.6% of N; Table 2); this relationship was previously reported (Volden et al., 1999a). Both Isaacson et al. (1975) and Volden et al. (1999b) suggest that differences in total AA concentration between LAB and PAB could be attributed to higher RNA content or higher nucleic acid concentration in LAB. The AA composition of omasal LAB and PAB were unaffected by RB supplementation (Table 3). Rolled barley supplementation had an effect on some, but not all, protozoal AA concentrations (Table 3). Of interest, the concentration of His was greater among all 3 microbial populations when cows consumed the G+RB diet. Dietary characteristics have been previously demonstrated to affect some AA concentrations of microbes (Martin et al., 1996); however, although some microbial AA concentrations were different in the current study, the biological implications of the differences were small. Currently, a lack of data and ambiguous reporting of the AA composition of bacteria limit our ability to evaluate the overall effect of dietary characteristics on microbial AA concentrations (Sok et al., 2017).

**Table 2.** Nitrogen concentration, $^{15}$N enrichment, and AA concentration of omasal microbes in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item[3] | G | G+RB | SEM | *P*-value |
| LAB | | | | |
| N, % of OM | 10.26 | 10.16 | 0.09 | 0.44 |
| $^{15}$N atom % excess | 0.038 | 0.039 | 0.002 | 0.73 |
| Total AA, % of OM | 44.9 | 44.4 | 0.6 | 0.51 |
| Total AA N, % of N | 58.6 | 58.5 | 0.7 | 0.90 |
| PAB | | | | |
| N, % of OM | 8.98 | 8.86 | 0.11 | 0.42 |
| $^{15}$N atom % excess | 0.040 | 0.041 | 0.002 | 0.78 |
| Total AA, % of OM | 49.5 | 47.7 | 0.8 | 0.13 |
| Total AA N, % of N | 72.7 | 71.5 | 1.2 | 0.47 |
| Protozoa | | | | |
| N, % of OM | 9.12 | 8.78 | 0.28 | 0.22 |
| $^{15}$N atom % excess | 0.039 | 0.039 | 0.001 | 0.80 |
| Total AA, % of OM | 50.3 | 48.1 | 1.7 | 0.19 |
| Total AA N, % of N | 72.9 | 72.6 | 1.4 | 0.89 |

[1]PRG = perennial ryegrass.

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

[3]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

**Table 3.** Amino acid composition of omasal bacteria and protozoa in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | LAB[2] AA, g/100 g of AA | | | | PAB[2] AA, g/100 g of AA | | | | Protozoal AA, g/100 g of AA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment[3] | | | | Treatment | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-value | G | G+RB | SEM | *P*-value | G | G+RB | SEM | *P*-value |
| EAA | | | | | | | | | | | | |
| Arg | 5.02 | 5.04 | 0.03 | 0.69 | 4.92 | 4.99 | 0.03 | 0.08 | 4.62 | 4.70 | 0.05 | 0.06 |
| His | 1.71 | 1.77 | 0.02 | <0.05 | 1.68 | 1.72 | 0.01 | <0.05 | 1.66 | 1.70 | 0.02 | <0.05 |
| Ile | 5.61 | 5.54 | 0.04 | 0.26 | 5.84 | 5.84 | 0.03 | 0.93 | 6.50 | 6.38 | 0.07 | <0.05 |
| Leu | 7.38 | 7.43 | 0.04 | 0.38 | 7.63 | 7.63 | 0.02 | 0.91 | 7.62 | 7.70 | 0.05 | 0.12 |
| Lys | 7.45 | 7.52 | 0.07 | 0.37 | 7.55 | 7.52 | 0.05 | 0.55 | 9.81 | 9.46 | 0.21 | <0.05 |
| Met | 3.25 | 3.29 | 0.05 | 0.57 | 2.97 | 2.96 | 0.05 | 0.85 | 2.39 | 2.30 | 0.05 | 0.20 |
| Phe | 4.24 | 4.29 | 0.03 | 0.29 | 4.46 | 4.44 | 0.01 | 0.15 | 4.65 | 4.65 | 0.02 | 0.76 |
| Thr | 5.59 | 5.52 | 0.04 | 0.16 | 5.48 | 5.47 | 0.02 | 0.56 | 4.76 | 4.76 | 0.02 | 0.96 |
| Trp | 2.02 | 2.02 | 0.05 | 0.91 | 2.13 | 2.02 | 0.03 | <0.05 | 1.39 | 1.46 | 0.05 | 0.34 |
| Val | 5.77 | 5.74 | 0.03 | 0.44 | 5.71 | 5.67 | 0.03 | 0.20 | 4.68 | 4.74 | 0.04 | 0.08 |
| Total EAA | 48.0 | 48.2 | 0.08 | 0.26 | 48.4 | 48.3 | 0.07 | 0.21 | 48.1 | 47.8 | 0.13 | <0.05 |
| NEAA | | | | | | | | | | | | |
| Ala | 7.33 | 7.21 | 0.04 | <0.05 | 6.86 | 6.81 | 0.04 | 0.22 | 4.49 | 4.58 | 0.08 | 0.08 |
| Asp | 11.63 | 11.55 | 0.08 | 0.50 | 11.62 | 11.60 | 0.05 | 0.68 | 13.03 | 12.72 | 0.15 | <0.01 |
| Cys | 1.15 | 1.12 | 0.02 | 0.19 | 1.22 | 1.24 | 0.02 | 0.44 | 1.72 | 1.64 | 0.05 | 0.23 |
| Glu | 13.94 | 13.93 | 0.05 | 0.82 | 13.75 | 13.96 | 0.04 | <0.01 | 15.97 | 16.16 | 0.07 | 0.07 |
| Gly | 5.31 | 5.27 | 0.02 | 0.29 | 5.32 | 5.29 | 0.02 | 0.28 | 4.34 | 4.43 | 0.05 | <0.05 |
| Pro | 3.50 | 3.57 | 0.08 | 0.43 | 3.72 | 3.68 | 0.04 | 0.46 | 3.57 | 3.83 | 0.08 | <0.01 |
| Ser | 4.45 | 4.47 | 0.02 | 0.33 | 4.53 | 4.54 | 0.02 | 0.57 | 4.29 | 4.34 | 0.03 | 0.06 |
| Tyr | 4.66 | 4.70 | 0.04 | 0.49 | 4.63 | 4.63 | 0.02 | 0.92 | 4.49 | 4.47 | 0.03 | 0.61 |
| Total NEAA | 52.0 | 51.8 | 0.08 | 0.26 | 51.6 | 51.7 | 0.07 | 0.21 | 51.9 | 52.2 | 0.13 | <0.05 |

[1]PRG = perennial ryegrass.

[2]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

[3]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

The omasal microbial AA composition in the current study agrees with a recent review of the literature (Sok et al., 2017). The Lys concentration of protozoa was higher (+ 28%) compared with bacteria, which is consistent with other findings (Volden et al., 1999b; Korhonen et al., 2002; Sok et al., 2017). For other AA, the concentration of Ala was 56% higher, Thr was 16% higher, Val was 22% higher, and Ile was 13% lower in bacteria compared with protozoa in the current study. Furthermore, the concentration of Met was 33% higher in bacteria compared with protozoa, in agreement with Korhonen et al. (2002). When investigating AA flows in lactating dairy cows, these results demonstrate the importance of quantifying the contribution from both bacteria and protozoa in prediction models.

### *Omasal Flows of OM, NAN, and AA in Bacteria and Protozoa*

Supplementation of PRG with RB increased the flow of PAB OM and PAB NAN at the omasal canal compared with the G diet (Table 4). In addition, supplementation of PRG with RB increased the flows of PAB AA, with the exception of Trp and Pro (Table 5). The increased flow of PAB in cows supplemented with RB was likely due to a greater amount of fermentable CHO digested in the rumen and greater efficiency of microbial protein synthesis when compared with the G diet (Dineen et al., 2020). This would suggest that nonstructural CHO-degrading bacteria were primarily associated with the particle phase. This is in contrast with Ahvenjärvi et al. (2002), who observed increased flow of LAB when a grass silage based-diet was supplemented with barley. In the current study, PAB N was considered to be all bacterial N flowing in the particle phase, whereas Ahvenjärvi et al. (2002) considered only bacterial N in the large-particle phase to be PAB N.

The flows of LAB OM, LAB NAN, and LAB AA numerically increased with the supplementation of RB; however, significant differences were not detected. This might be related to the calculation method used in the current study. The LAB flow was calculated by difference using $^{15}$N APE in the liquid phase after accounting for $^{15}$N APE in omasal protozoa. Previous investigations have observed large animal-to-animal variation in the measurement of protozoa (Sylvester et al., 2005; Yáñez-Ruiz et al., 2006), which was also observed in the current study. Due to the calculation methods used, this variability likely increased the variation observed in the measurement of LAB flow.

The contribution of LAB and PAB N to the total microbial N omasal flow were unaffected by RB

**Table 4.** Omasal microbial OM and NAN flows in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| Item | G | G+RB | SEM | *P*-value |
| DMI,[3] kg/d | 16.1 | 17.1 | 0.4 | <0.01 |
| NAN flow,[3] g/d | 373 | 422 | 18 | <0.01 |
| LAB[4] nutrient flow, g/d | | | | |
| OM flow | 1,340 | 1,601 | 169 | 0.16 |
| NAN flow | 137 | 164 | 18 | 0.17 |
| % of microbial NAN flow | 42.5 | 44.0 | 4.0 | 0.70 |
| PAB[4] nutrient flow, g/d | | | | |
| OM flow | 1,264 | 1,523 | 57 | <0.01 |
| NAN flow | 111 | 134 | 4 | <0.01 |
| % of microbial NAN flow | 34.0 | 36.1 | 1.3 | 0.28 |
| Protozoal nutrient flow, g/d | | | | |
| OM flow | 847 | 814 | 103 | 0.75 |
| NAN flow[3] | 79 | 73 | 11 | 0.55 |
| % of microbial NAN flow[3] | 23.5 | 20.0 | 3.2 | 0.24 |

[1]PRG = perennial ryegrass.

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

[3]Previously reported in Dineen et al. (2020).

[4]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

supplementation (Table 4). Liquid-associated bacteria accounted for a higher proportion of the microbial N flow than PAB, averaging 43% and 35%, respectively. Although previous investigations have reported that a large proportion of the bacteria in the rumen are associated with particulate matter (Craig et al., 1987),

**Table 5.** Omasal flow of bacterial and protozoal AA in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | LAB[2] AA flow, g/d | | | | PAB[2] AA flow, g/d | | | | Protozoal AA flow, g/d | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment[3] | | | | Treatment | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-value | G | G+RB | SEM | *P*-value | G | G+RB | SEM | *P*-value |
| EAA | | | | | | | | | | | | |
| Arg | 30.3 | 36.1 | 4.0 | 0.18 | 31.6 | 36.5 | 1.4 | <0.05 | 19.5 | 18.7 | 2.8 | 0.78 |
| His | 10.3 | 12.7 | 1.4 | 0.11 | 10.8 | 12.5 | 0.5 | <0.05 | 7.0 | 6.7 | 1.0 | 0.80 |
| Ile | 33.8 | 39.4 | 4.3 | 0.24 | 37.2 | 42.8 | 1.6 | <0.05 | 27.5 | 25.8 | 4.3 | 0.67 |
| Leu | 44.5 | 52.9 | 5.7 | 0.18 | 48.8 | 55.6 | 2.1 | <0.05 | 31.8 | 30.6 | 4.5 | 0.77 |
| Lys | 44.9 | 54.1 | 6.0 | 0.14 | 48.3 | 55.2 | 2.2 | <0.05 | 42.2 | 38.6 | 7.0 | 0.58 |
| Met | 19.5 | 23.8 | 2.7 | 0.13 | 19.1 | 21.9 | 0.8 | <0.05 | 9.9 | 9.3 | 1.4 | 0.67 |
| Phe | 25.5 | 30.6 | 3.3 | 0.17 | 28.5 | 32.5 | 1.2 | <0.05 | 19.6 | 18.7 | 2.9 | 0.74 |
| Thr | 33.7 | 39.5 | 4.4 | 0.21 | 35.6 | 40.5 | 1.5 | <0.05 | 20.0 | 18.9 | 2.9 | 0.71 |
| Trp | 12.1 | 14.4 | 1.6 | 0.21 | 13.6 | 14.7 | 0.5 | 0.11 | 5.7 | 5.7 | 0.8 | 0.98 |
| Val | 34.8 | 40.9 | 4.5 | 0.22 | 36.8 | 41.6 | 1.6 | <0.05 | 19.7 | 18.9 | 2.8 | 0.77 |
| Total EAA | 289.4 | 344.4 | 37.6 | 0.18 | 310.3 | 353.9 | 13.2 | <0.05 | 202.7 | 191.9 | 30.3 | 0.70 |
| NEAA | | | | | | | | | | | | |
| Ala | 44.1 | 51.4 | 5.6 | 0.23 | 44.3 | 50.3 | 1.9 | <0.05 | 18.5 | 18.1 | 2.4 | 0.84 |
| Asp | 70.1 | 82.5 | 9.1 | 0.20 | 75.0 | 86.0 | 3.4 | <0.05 | 54.7 | 51.3 | 8.3 | 0.66 |
| Cys | 6.9 | 8.1 | 0.9 | 0.23 | 8.0 | 9.2 | 0.3 | <0.05 | 7.0 | 6.5 | 0.9 | 0.61 |
| Glu | 84.1 | 99.0 | 10.7 | 0.19 | 88.1 | 102.0 | 3.8 | <0.05 | 66.5 | 64.2 | 9.6 | 0.80 |
| Gly | 31.9 | 37.7 | 4.1 | 0.19 | 34.3 | 38.9 | 1.5 | <0.05 | 18.1 | 17.5 | 2.5 | 0.82 |
| Pro | 20.7 | 25.5 | 2.6 | 0.11 | 24.1 | 27.1 | 1.1 | 0.05 | 14.7 | 15.5 | 2.1 | 0.70 |
| Ser | 26.8 | 31.8 | 3.4 | 0.17 | 28.9 | 33.2 | 1.2 | <0.05 | 17.9 | 17.3 | 2.6 | 0.79 |
| Tyr | 28.0 | 33.6 | 3.7 | 0.16 | 29.4 | 33.9 | 1.3 | <0.01 | 19.0 | 17.9 | 2.8 | 0.68 |
| Total NEAA | 312.5 | 369.5 | 40.0 | 0.19 | 332.0 | 380.6 | 14.4 | <0.05 | 216.7 | 208.0 | 31.3 | 0.76 |
| Total AA | 601.9 | 713.9 | 77.6 | 0.18 | 642.2 | 734.5 | 27.6 | <0.05 | 419.4 | 399.9 | 61.5 | 0.73 |

[1]PRG = perennial ryegrass.

[2]LAB = liquid-associated bacteria; PAB = particle-associated bacteria.

[3]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

a higher rate of passage of the liquid phase compared with particulate phase allows similar contribution from LAB and PAB to total microbial N flow (Hristov and Broderick, 1996). The fresh PRG-based diets in the current study resulted in high liquid passage rates (Dineen et al., 2020) due to the high water content of the forage, which likely allowed the large contribution from LAB to microbial N omasal flow.

Supplementation of PRG with RB did not affect protozoal contribution to the microbial OM, NAN, and AA omasal flows (Table 4 and Table 6). On average, protozoa accounted for 21.7% of microbial NAN flow and 23.3% of microbial AA flow. The contribution of protozoa to microbial EAA flows ranged, on average, from 17.4 to 27.9% for Trp and Lys, respectively (Table 6). Protozoal omasal flow has not previously been quantified in pasture-fed cows. These measured values are higher than previously reported when compared with cows consuming diets based on corn silage (Sylvester et al., 2005; Fessenden et al., 2019a) and grass silage (Ahvenjärvi et al., 2002). The rapid liquid passage rate in the current study (0.20/h; Dineen et al., 2020), due to the fresh PRG-based diets, is a likely reason for the large contribution of protozoa to total microbial OM, NAN, and AA omasal flow. Sylvester et al. (2009) demonstrated, in vitro, that a higher ruminal passage rate can simultaneously increase the flow of protozoa and decrease their generation time, allowing more efficient protein synthesis to be achieved. In the companion paper (Dineen et al., 2020), low ruminal protozoal biomass relative to ruminal bacterial biomass and extremely high efficiency of protozoal growth was measured, which is in line with the predictions of Firkins et al. (2007). Sok et al. (2017) recently challenged the calculations of Firkins et al. (2007), arguing that as passage rates are typically first-order fractional rates, a decreased protozoal ruminal pool size would consequently decrease protozoal ruminal outflow rate, justifying their predictions that protozoa contribute less to the total microbial flow (16.5%). However, in the current study, direct measurement of protozoal ruminal pool size and protozoal omasal flow were conducted simultaneously, negating the assumptions required when using fractional outflow rates to describe protozoal passage (Karnati et al., 2007) and supporting the predictions of Firkins et al. (2007). More in vivo studies are required to further elucidate protozoal metabolism and to evaluate the ability of current mechanistic models to predict such behavior.

Broderick et al. (2010) demonstrated that if microbial samples did not include protozoal isolations, microbial N flow would be underestimated by approximately 5% due to differences in $^{15}$N enrichment. In the current study, $^{15}$N enrichment was similar among bacteria and protozoa (Table 2); therefore, accounting for protozoa did not considerably alter microbial N flow. However, as discussed above, the AA concentrations of the microbial N did differ among bacteria and protozoa (Table 3). Therefore, if protozoa were not accounted for in the current study, microbial AA flow would be under- (e.g., Lys) or overestimated (e.g., Met), depending on the specific AA.

### Omasal Flows of AA in OTD, Microbial, and Nonmicrobial Fractions

Rolled barley supplementation to a PRG diet increased the flow of all AA in OTD compared with the G diet, resulting in a 228 g/d increase in total AA flow in cows fed the G+RB diet (Table 7; 1,964 vs. 2,193 g/d for G vs. G+RB, respectively; $P < 0.01$). Van Vuuren et al. (1993) reported higher duodenal AA flows when a starch-based supplement was provided to pasture-fed cows, although supplementation level was much higher (7 kg of DM/d). In contrast, both O'Mara et al. (1997) and Younge et al. (2004) reported no difference in total AA flows when cows fed PRG were supplemented with a fiber-based concentrate; however, AA flows in both

**Table 6.** Protozoal proportion of omasal microbial AA flow in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| Protozoal AA flow, % of microbial AA | Treatment[2] | | SEM | *P*-value |
|---|---|---|---|---|
| | G | G+RB | | |
| EAA | | | | |
| Arg | 23.4 | 20.7 | 2.9 | 0.36 |
| His | 24.3 | 21.4 | 3.0 | 0.32 |
| Ile | 27.0 | 23.8 | 3.3 | 0.31 |
| Leu | 24.8 | 22.1 | 2.9 | 0.35 |
| Lys | 29.9 | 25.9 | 3.8 | 0.26 |
| Met | 20.2 | 17.5 | 2.8 | 0.30 |
| Phe | 25.8 | 22.9 | 3.1 | 0.33 |
| Thr | 22.0 | 19.6 | 2.9 | 0.38 |
| Trp | 18.1 | 16.7 | 2.2 | 0.54 |
| Val | 21.2 | 19.0 | 2.7 | 0.41 |
| Total EAA | 24.6 | 21.7 | 3.1 | 0.34 |
| NEAA | | | | |
| Ala | 17.3 | 15.6 | 2.3 | 0.45 |
| Asp | 26.7 | 23.5 | 3.3 | 0.31 |
| Cys | 31.3 | 27.6 | 3.1 | 0.22 |
| Glu | 27.2 | 24.3 | 3.2 | 0.35 |
| Gly | 21.2 | 19.0 | 2.6 | 0.41 |
| Pro | 24.2 | 22.7 | 2.7 | 0.60 |
| Ser | 23.8 | 21.2 | 2.9 | 0.35 |
| Tyr | 24.2 | 21.2 | 3.1 | 0.30 |
| Total NEAA | 24.6 | 22.0 | 3.0 | 0.36 |
| Total AA | 24.6 | 21.9 | 3.0 | 0.35 |

[1]PRG = perennial ryegrass.

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

studies were numerically increased in supplemented cows. In the current study, the increase in AA flow was most likely due to the greater amount of fermentable CHO digested in the rumen and the greater efficiency of microbial protein synthesis in cows supplemented with RB (Dineen et al. 2020). This increased total AA omasal flow when cows consumed the G+RB diet can help explain, at least in part, an increase in milk protein concentration (Dineen et al., 2020).

The effects of RB supplementation on total microbial AA flow and nonmicrobial AA flow are presented in Table 8. Microbial AA flow increased for all AA when cows were supplemented with RB, and this was associated with the increase in PAB AA flow. Rolled barley supplementation did not affect the nonmicrobial AA flow (Table 8). The nonmicrobial AA portion of the total AA flow accounted for 16.5% and 14.7% in cows fed the G and G+RB diets, respectively. For cows consuming the G diet, this indicated that 83.5% of the PRG AA were degraded in the rumen. This ruminal degradability is likely underestimated because endogenous AA will contribute to a proportion of nonmicrobial AA; however, the contribution of endogenous AA at the omasal canal is small (Ørskow et al., 1986; Lapierre et al., 2008). To our knowledge, no other data set exists pertaining to the nonmicrobial AA contribution to total AA omasal flow in pasture-fed cows. However, one other study previously reported the contribution of NANMN to total NAN flow at the omasal canal in pasture-fed cows (Sairanen et al., 2005), with several others reporting the NANMN contribution to duodenal NAN flow (Berzaghi et al., 1996; O'Mara et al., 1997; Peyraud et al., 1997; Kolver et al., 1999; Younge et al., 2004). The ruminal degradation of pasture N {i.e., [1 − (NANMN flow/N intake)]} reported in the omasal flow studies varied from 87.5 to 88.5%, whereas in the duodenal flow studies, it varied from 53.5 to 79.0%. In the duodenal flow studies, the lower estimates of pasture N ruminal degradation is likely, at least in part, due to larger endogenous N contributions compared with omasal flow studies. Ruminal degradation of pasture N, measured with in situ procedures, support the omasal flow data (Beever et al., 1986; Van Vuuren et al., 1991; Chaves et al., 2006) and reported rapid ruminal degradation rates of PRG N, which exceeded 0.20/h. Using the data reported in Dineen et al. (2020), the ruminal degradation rate of PRG N in vivo could be calculated as both NANMN ruminal pool size and NANMN omasal flow were reported. This calculation indicates that the PRG N degraded in vivo at 0.16/h. Altogether, these observations demonstrate the low contribution from nonmicrobial AA to total AA flow in cows fed fresh PRG-based diets.

Generally, AA supply is assumed to be in excess of requirements of pasture-fed cows, as fertilized pastures typically exhibit high CP content (Pacheco and Waghorn, 2008; Griffiths et al., 2020). However, several studies have demonstrated increased milk production when grazing dairy cows have been supplemented with rumen-protected protein ingredients such as protected casein (Stobbs et al., 1977; Rogers et al., 1980; Minson, 1981), formaldehyde-treated soybean meal (Delaby et al., 1995; Delagarde et al., 1997; Astigarraga et al., 2002), or fishmeal (O'Mara et al., 2000). Furthermore, extensive ruminal protein degradation, as measured in the current study, has previously been indicated to limit the milk production of cows fed high-quality temperate legume forages (Broderick, 1995). Therefore, the currently held view that N-fertilized PRG provides an excess of metabolizable AA requires further investigation. Although cows fed the G+RB diet in the current study did not increase milk yield, there was an increase in milk protein concentration, which might have been supported via increased AA supply achieved through increased microbial protein supply. The effect of increasing postruminal AA supply or reducing ruminal protein degradation of pasture-based diets should be evaluated with feeding trials to determine the effects on the productivity and efficiency of lactating dairy cows.

**Table 7.** Omasal true digesta flow of AA in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | Treatment[2] | | | |
|---|---|---|---|---|
| AA flow, g/d | G | G+RB | SEM | *P*-value |
| EAA | | | | |
| Arg | 86.2 | 97.6 | 3.9 | <0.01 |
| His | 32.9 | 37.6 | 1.5 | <0.01 |
| Ile | 107.4 | 118.9 | 4.8 | <0.01 |
| Leu | 155.2 | 173.5 | 7.0 | <0.01 |
| Lys | 154.4 | 168.5 | 6.9 | <0.01 |
| Met | 51.8 | 58.2 | 2.6 | <0.01 |
| Phe | 92.8 | 103.5 | 4.2 | <0.01 |
| Thr | 105.8 | 116.5 | 4.7 | <0.01 |
| Trp | 36.7 | 40.2 | 1.6 | <0.01 |
| Val | 109.4 | 121.3 | 5.0 | <0.01 |
| Total EAA | 932.4 | 1,035.9 | 41.8 | <0.01 |
| NEAA | | | | |
| Ala | 144.9 | 157.9 | 6.8 | <0.01 |
| Asp | 220.2 | 243.8 | 9.9 | <0.01 |
| Cys | 25.2 | 29.1 | 1.4 | <0.01 |
| Glu | 276.7 | 317.7 | 12.2 | <0.01 |
| Gly | 109.7 | 122.1 | 5.1 | <0.01 |
| Pro | 76.2 | 86.0 | 3.8 | <0.01 |
| Ser | 86.9 | 97.7 | 3.9 | <0.01 |
| Tyr | 92.0 | 102.1 | 4.1 | <0.01 |
| Total NEAA | 1,031.7 | 1,156.6 | 46.6 | <0.01 |
| Total AA | 1,964.1 | 2,192.5 | 88.3 | <0.01 |

[1]PRG = perennial ryegrass.

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

**Table 8.** Omasal flow of microbial and nonmicrobial AA in lactating dairy cows fed fresh PRG[1] not supplemented or supplemented with rolled barley

| | Microbial AA flow, g/d | | | | Nonmicrobial AA flow, g/d | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment[2] | | | | Treatment | | | |
| Item | G | G+RB | SEM | *P*-value | G | G+RB | SEM | *P*-value |
| EAA | | | | | | | | |
| Arg | 80.0 | 92.6 | 4.1 | <0.01 | 5.7 | 5.4 | 1.0 | 0.82 |
| His | 27.6 | 32.3 | 1.4 | <0.01 | 5.3 | 5.2 | 0.5 | 0.87 |
| Ile | 97.0 | 109.6 | 4.8 | <0.01 | 10.1 | 9.6 | 1.3 | 0.81 |
| Leu | 123.2 | 141.1 | 6.0 | <0.01 | 31.9 | 32.5 | 1.8 | 0.82 |
| Lys | 136.3 | 146.9 | 6.5 | <0.05 | 20.7 | 18.8 | 2.3 | 0.56 |
| Met | 47.9 | 55.6 | 2.3 | <0.01 | 3.9 | 2.6 | 0.9 | 0.27 |
| Phe | 72.4 | 82.9 | 3.5 | <0.01 | 20.3 | 20.6 | 1.3 | 0.83 |
| Thr | 88.1 | 100.1 | 4.1 | <0.01 | 17.7 | 16.5 | 1.3 | 0.52 |
| Trp | 30.9 | 35.3 | 1.5 | <0.01 | 5.8 | 4.9 | 0.4 | 0.13 |
| Val | 90.0 | 102.6 | 4.4 | <0.01 | 19.3 | 18.8 | 1.3 | 0.80 |
| Total EAA | 790.1 | 902.5 | 38.1 | <0.01 | 140.8 | 134.9 | 9.9 | 0.67 |
| NEAA | | | | | | | | |
| Ala | 105.9 | 120.8 | 5.3 | <0.01 | 39.2 | 36.9 | 2.4 | 0.28 |
| Asp | 196.8 | 222.8 | 9.4 | <0.01 | 23.0 | 21.4 | 2.9 | 0.70 |
| Cys | 21.4 | 24.2 | 1.0 | <0.01 | 3.9 | 4.8 | 0.8 | 0.32 |
| Glu | 235.0 | 268.9 | 11.2 | <0.01 | 41.3 | 49.3 | 3.4 | 0.11 |
| Gly | 83.1 | 95.3 | 4.1 | <0.01 | 26.7 | 26.8 | 1.5 | 0.92 |
| Pro | 59.1 | 68.6 | 2.9 | <0.01 | 17.4 | 17.1 | 1.9 | 0.90 |
| Ser | 72.5 | 83.3 | 3.4 | <0.01 | 14.3 | 14.4 | 1.1 | 0.94 |
| Tyr | 75.3 | 86.5 | 3.6 | <0.01 | 16.7 | 15.7 | 1.0 | 0.43 |
| Total NEAA | 849.0 | 970.4 | 40.1 | <0.01 | 182.6 | 186.4 | 12.4 | 0.82 |
| Total AA | 1,639.0 | 1,872.9 | 78.1 | <0.01 | 323.6 | 321.1 | 21.9 | 0.93 |

[1]PRG = perennial ryegrass.

[2]G = 100% (DM basis) perennial ryegrass; G+RB = 79% perennial ryegrass and 21% rolled barley.

## CONCLUSIONS

In this study, supplementation of PRG with RB appeared to have a minimal effect on the microbial composition of cows that consumed fresh PRG-based diets. However, differences in the AA composition among bacteria and protozoa were pronounced, emphasizing the importance of including these observations in mechanistic models designed to predict AA flows in lactating dairy cows. Rolled barley supplementation increased microbial AA flow, which is likely due to a greater amount of fermentable CHO digested in the rumen and greater efficiency of microbial protein synthesis, as reported in Dineen et al. (2020). Overall, protozoal flow was not affected by diet; however, protozoa contributed 23% of the microbial EAA flow. Rolled barley supplementation had no effect on the nonmicrobial AA flow, and the nonmicrobial AA flow modestly contributed to total AA flow, accounting for 16.5% and 14.7% in cows fed G and G+RB, respectively. These observations indicate that extensive ruminal degradation of PRG AA occurs (83.5%) and that cows consuming PRG-based diets exhibit a large dependence on microbial AA to support metabolizable AA supply. We found that RB supplementation can increase the omasal flow of microbial AA in cows consuming PRG-based diets. However, more research is required to investigate if this increased AA supply can stimulate higher milk production performance under such dietary conditions.

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