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Rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh perennial ryegrass (*Lolium perenne* L.) not supplemented or supplemented with rolled barley grain

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ABSTRACT

The objective of this study was to evaluate the effect of rolled barley grain (RB) supplementation on rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh perennial ryegrass (*Lolium perenne* L.; PRG)-based diets. Ten ruminally cannulated 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 (G) or PRG plus 3.5 kg of dry matter RB (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 labeled ^{15}N-ammonium sulfate to measure bacterial, protozoal, and nonmicrobial N flow. Rumen evacuation techniques were used to determine nutrient and microbial pool size, allowing the calculation of fractional rates of digestion and microbial growth. There was no difference in daily milk yield or energy-corrected milk yield between treatments. Milk fat concentration and milk urea N decreased, whereas milk protein concentration increased in cows fed the G+RB diet. During the omasal sampling phase, dry matter intake was higher in cows fed the G+RB diet. Ruminal and total-tract neutral detergent fiber digestibility was lower in G+RB cows; however, no difference was observed in reticulorumen pH. The rumen pool size of fermentable carbohydrate was increased in cows fed the G+RB diet; however, the fractional rate of digestion was decreased. Flow of nonammonia N and bacterial N at the omasal canal increased in cows fed the G+RB diet compared with the G diet. Protozoa N flow was not different between diets; however, protozoa appeared to supply a much larger amount of microbial N and exhibited shorter generation time than previously considered. Feed N ruminal digestibility, corrected for microbial contribution, was similar for both treatments (88.4 and 89.0% for G and G+RB, respectively). In conclusion, RB supplementation did not benefit overall animal performance; however, it reduced ruminal neutral detergent fiber digestibility and increased bacterial N flow. The results demonstrate the large dependence of cows consuming PRG-based diets on microbial N as the main source of nonammonia N supply. Additional quantitative research is required to further describe the supply of nutrients and microbial dynamics in cows consuming PRG-based diets in an effort to determine most limiting nutrients.

Key words: pasture, fermentable carbohydrate, omasal flow, protozoa

INTRODUCTION

In pasture-based systems, there is potential to increase the efficiency and productivity on a per-cow basis by incorporating more nutrients (i.e., N and carbon) into milk and meat products. In a review of supplementation of pasture-based diets, individual milk production increased linearly as the amount of supplemental concentrate increased (Bargo et al., 2003). However, wide variation in milk yield response exists among studies with little mechanistic explanation of how or why this variation occurs (Penno, 2002). In temperate regions, where a large proportion of the pastures are primarily perennial ryegrass (*Lolium perenne* L.; **PRG**), modern grazing management practices aim to maintain the plant at an immature stage (O'Donovan et al., 2002), resulting in PRG forage with OM digestibility in excess of 85% (Smit et al., 2005; Wims et al., 2013). Despite well-managed PRG being highly digestible, ME supply is considered first limiting for milk production of graz-

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ing cows (Kolver and Muller, 1998; Nicol and Brookes, 2007). A breadth of reports have investigated the effects of supplementing cereal grains to cows consuming PRG-based diets (Bargo et al., 2003; Baudracco et al., 2010). However, the majority of these reports examine the effects at a whole-animal level, which prevents the determination of the mechanistic nutritional physiology involved at the rumen or postruminal level. As a result, understanding of the in vivo ruminal digestion and passage of nutrients in cows consuming PRG-based diets is limited.

The supply of AA to the small intestine is also considered to colimit milk production in cows consuming PRG-based diets (Delaby et al., 1995; O'Mara et al., 2000). In some cases, this can be attributed to extensive rumen proteolysis and substantial preduodenal losses of the PRG N (Beever et al., 1986, 1987). Consequently, microbial protein contributes a large proportion of the NAN flow in cows consuming pasture-based diets (Younge et al., 2004; Sairanen et al., 2005). This contribution, however, is likely underestimated in pasture-fed cows as protozoal N has not been accounted for. Ahvenjärvi et al. (2002) and Fessenden et al. (2019a) reported that protozoal N contributed 15 to 20% of the microbial protein flow in silage-based diets.

Although it is evident that protozoa have a significant effect on ruminant physiology (Newbold et al., 2015), an understanding of their metabolism is currently incomplete (Firkins et al., 2020). Direct measurement of protozoal rumen pool size and omasal flow simultaneously are rarely conducted even though this is the most correct way to represent protozoal generation time in vivo (Karnati et al., 2007). Cows consuming PRG-based diets exhibit rapid rumen turnover, which is suggested to be a principal factor associated with protozoal generation time (Potter and Dehority, 1973; Sylvester et al., 2009). Furthermore, the rumen of a grazing cow seems optimal for efficient protozoal growth due to an ample supply of sugars, soluble true protein, and moderate pH levels across the day (Clarke, 1965; Williams and Coleman, 1988). Thus, cows fed PRG-based diets could be an effective model to enhance our current understanding of protozoal metabolism.

Therefore, the objective of this study was to evaluate the effect of rolled barley grain (**RB**) supplementation as a source of additional rumen fermentable carbohydrate on rumen metabolism, omasal flow of nutrients, and microbial dynamics in lactating dairy cows fed fresh PRG-based diets. To determine the effect of RB supplementation on nutrient and microbial growth and passage, the omasal sampling technique (Huhtanen et al., 1997; Reynal and Broderick, 2005) was used in combination with rumen evacuation and microbial isolation procedures. The hypothesis of this experiment was that the inclusion of RB would increase the fermentable carbohydrate (**CHO**) supply for rumen microbes and thereby increase the microbial N flow at the omasal canal compared with a PRG-only diet.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted from April to July 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).

Sward Management and Measurements

An area of ground dedicated to the study (5.5 ha) was divided into 6 subplots and managed as described by O'Donovan et al. (2002) to ensure high pasture quality. Nitrogen fertilizer was applied to the subplots in 2 applications of 17 kg of N/ha approximately 21 and 14 d before each harvesting in the form of calcium ammonium nitrate with added S (Goulding Chemicals Ltd., Centre Park Road, Marina, Cork, Ireland).

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. The prestudy diet consisted of fresh PRG, offered ad libitum and indoors to all cows to allow a 10% refusal rate, and 3.5 kg DM of RB. At the end of the 3-wk period, cows were 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 periods, where each period consisted of 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. Cows were milked twice daily (0730 and 1530 h) in a parlor except during the 8-d sampling phase, when they were milked in the tiestalls. Milk yield was recorded and milk samples were taken at each milking on d 21, 22, and 23 of each pe-

riod and analyzed for fat, CP, lactose, SCC, and MUN using mid-infrared spectroscopy analysis (Milkoscan 203, DK-3400; Foss Electric, Hillerød, Denmark). Body weights were measured weekly after the 0730 h milking using an electronic portable weighing scale and Winweigh software package (Tru-Test Ltd., Auckland, New Zealand). Body condition score was recorded weekly as the average of 2 trained scorers using a 1-to-5 scale with 0.25-unit increments (Edmonson et al., 1989). The changes in BW and BCS were calculated as the difference between measurements taken on d 29 of each period.

Treatment diets were PRG only (**G**) or PRG plus 3.5 kg DM of RB (**G+RB**; Table 1). In addition, cows assigned to 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. 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). Swards were cut at 4 cm above ground level with no additional processing to minimize disruption of the cellular structure of the plant, which resulted in particle sizes ranging from 26 to 37 cm. The cut forage was collected and weighed, and a subsample was taken to determine DM concentration in an oven at 60°C for 48 h. 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 quantity offered to each animal was recorded, and refusals were collected the following morning at 0730 h and weights were recorded. The feeding rates were adjusted daily to yield refusals of 5 to 10% of intake. The RB was offered to the respective cows at the time of milking (0730 and 1530 h) as 2 equal meals. Daily, samples of PRG and RB were dried at 105°C for 15 h and analyzed for DM. Additional samples were either freeze dried (LS40+chamber, MechaTech Systems Ltd., Bristol, UK) at −55°C for 120 h or oven dried at 60°C for 48 h. Dried samples were ground through a 1-mm screen using a Cyclotech 1093 Sample Mill (Foss, DK-3400) and stored for subsequent nutrient composition analysis.

Sampling Procedures

Each experimental period contained an infusion, omasal sampling, and rumen evacuation phase, which occurred in the final 8 d of the period. The double

Table 1. Nutrient composition[1] (mean ± SD) of feeds, selected supplement, and experimental diets used in the experiment

	Period[2]				Treatment[4]	
Nutrient	GP1	GP2	GP3	RB[3]	G	G+RB
DM, %	19.5 ± 2.3	18.4 ± 1.5	22.6 ± 1.0	86.9 ± 0.8	20.2	34.2
CP, % of DM	12.5 ± 1.1	18.1 ± 1.5	18.3 ± 1.6	11.6 ± 0.4	16.3	15.3
NPN, % of N	25.7 ± 2.5	24.2 ± 0.1	20.0 ± 1.9	—	23.3	—
Soluble N, % of N	37.6 ± 2.5	36.1 ± 2.5	32.2 ± 1.8	17.1 ± 1.9	35.3	31.7
NDIN, % of N	13.8 ± 0.2	14.1 ± 0.4	16.8 ± 0.9	8.0 ± 1.0	14.9	13.5
ADIN, % of N	2.8 ± 0.1	2.3 ± 0.2	2.3 ± 0.2	3.0 ± 0.7	2.5	2.6
Starch, % of DM	3.1 ± 1.9	2.3 ± 0.7	2.2 ± 0.7	60.7 ± 0.7	2.5	14.7
Water-soluble carbohydrates, % of DM	27.1 ± 2.4	20.9 ± 1.8	19.2 ± 1.4	7.1 ± 0.6	22.4	19.2
NFC, % of DM	43.6 ± 2.6	35.9 ± 1.5	33.0 ± 3.8	65.0 ± 0.5	37.5	43.3
aNDFom,[5] % of DM	35.2 ± 2.1	35.6 ± 1.2	37.6 ± 1.4	19.2 ± 1.0	36.1	32.6
12-h uNDFom,[6] % of aNDFom	56.5 ± 8.2	54.6 ± 10.4	49.7 ± 7.4	71.0 ± 0.3	53.6	—
30-h uNDFom, % of aNDFom	24.1 ± 6.6	25.0 ± 8.4	19.0 ± 1.8	—	22.7	—
72-h uNDFom, % of aNDFom	—	—	—	38.4 ± 1.4	—	—
120-h uNDFom, % of aNDFom	11.6 ± 0.4	11.9 ± 1.1	11.4 ± 0.8	32.9 ± 0.6	11.6	—
240-h uNDFom, % of aNDFom	9.7 ± 0.5	9.9 ± 1.0	9.9 ± 0.3	—	9.8	—
ADF, % of DM	19.0 ± 0.6	22.2 ± 1.2	20.5 ± 0.7	5.0 ± 0.7	20.6	17.3
ADL, % of NDF	3.8 ± 1.1	4.3 ± 0.4	4.7 ± 0.4	11.8 ± 2.7	4.2	5.8
Ether extract, % of DM	2.4 ± 0.2	3.3 ± 0.5	3.7 ± 0.6	1.7 ± 0.2	3.1	2.8
Ash, % of DM	6.6 ± 0.4	7.1 ± 1.0	7.4 ± 0.5	2.6 ± 0.6	7.0	6.1
Pre-cutting yield, kg of DM/ha	2,018 ± 389	1,383 ± 191	1,421 ± 166	—	1,608	

[1]Analyzed values from 12 samples (4 d × 3 periods).

[2]GP1 = perennial ryegrass period 1; GP2 = perennial ryegrass period 2; GP3 = perennial ryegrass period 3.

[3]RB = rolled barley grain.

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

[5]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue.

[6]uNDFom = undigested amylase- and sodium sulfite-treated NDF corrected for ash residue.

marker method using CoEDTA (Udén et al., 1980) and undigested amylase- and sodium sulfite-treated NDF corrected for ash residue after 240 h of in vitro fermentation (**uNDFom**; Raffrenato et al., 2018) were used to quantify liquid and particle flow entering the omasal canal, respectively. From d 21 at 1400 h until the end of the period, CoEDTA was dissolved in distilled water and continuously infused into the rumen at a rate of 2.7 g of Co/d in 2.5 L of solution/d via peristaltic pump (Masterflex, Cole-Parmer Instrument Co. LLC, Vernon Hills, IL). All cows received a 3-L priming dose of CoEDTA (3.3 g of Co) into the rumen via the rumen cannula immediately before starting infusion. To enrich microbial N with ^{15}N, 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 CoEDTA infusate. Prior to starting the infusion, samples of ruminal contents were taken randomly from several regions of the rumen for determination of ^{15}N background.

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). Omasal sampling began approximately 74 h after the beginning of marker infusion to allow uniform marker and isotope distribution. Samples of omasal contents were collected from the omasal canal during three 8-h intervals: 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. This sampling schedule encompassed every 2 h of the 24-h cycle. The sampling device was placed in position at the beginning of each interval and before each time point within the interval; the location of the sampling device was confirmed and repositioned if necessary. At the end of each interval, the sampling device was removed. 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 point 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. One of the 50-mL samples (omasal fluid) was filtered through a single layer of large-pore polyethylene cheesecloth (Graytec, GD Textile, Manchester, UK), acidified with 50% H_2SO_4, and stored at −20°C for subsequent NH_3-N and VFA analysis, and the other was processed and stored for a separate investigation. The 125-mL subsample was placed on ice and combined within interval, yielding a 500-mL sample for bacterial isolation. Bacteria were isolated using a modification of the procedure of Whitehouse et al. (1994). The omasal contents were squeezed through a single layer of cheesecloth, and the retained solids were washed once with saline solution and squeezed again through a single layer of 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 solid-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 a single layer of 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 freeze drying and later analysis. The bacterial pellets recovered from filtrates A and B represented the omasal liquid-associated bacteria (**OLAB**) and omasal particle-associated bacteria (**OPAB**). The bacterial isolations from each 8-h interval were subsequently combined within period to generate an OLAB and an OPAB sample per cow per period. The 200-mL subsamples were combined within period and stored at −20°C, generating a 2.4-L composite. This omasal composite was subsequently thawed and separated into omasal large particle, small particle, and liquid phases as described in Reynal and Broderick (2005), and these were stored at −20°C until freeze dried. The additional 250-mL subsample obtained on the final time point of each interval was processed immediately to isolate omasal protozoa (**OP**) as described by Denton et al. (2015).

In parallel with the omasal sampling, fecal and rumen fluid samples were also obtained. Fecal samples were composited by period and stored at −20°C, and rumen fluid was acidified with 50% H_2SO_4 and stored at −20°C. Blood samples were obtained at the second time point of each interval via coccygeal vein puncture. Blood samples were collected into tubes containing sodium heparin and centrifuged (3,000 × g for 20 min at 4°C), and plasma was harvested and stored at −20°C. On d 28 and 29 of each period, rumen contents were evacuated 2 h before (0630 h) and 2 h after (1030 h) the main meal. The rumen contents were weighed and mixed, and a representative sample was obtained and stored at −20°C. Rumen contents were returned to the cow via the rumen cannula. Prior to beginning rumen evacuations, random composite samples of rumen contents from multiple sites in the rumen were removed for isolation of rumen liquid-associated bacteria, rumen particle-associated bacteria, and rumen protozoa (**RP**) as described above for the omasal isolations.

Laboratory Analysis

Feed samples were analyzed for chemical composition using wet chemistry methods (CPM Plus Package) by Cumberland Valley Analytical Services (Waynesboro, PA). In addition to this analysis, feed samples were analyzed at our laboratory for amylase- and sodium sulfite-treated NDF corrected for ash residue (**aNDFom**; Mertens, 2002) and uNDFom after in vitro incubation with rumen fluid according to Raffrenato et al. (2018). The time points selected for PRG were 12, 30, 120, and 240 h with the 12-h time point included to capture the rate of digestion in the linear phase of digestion as the rate of degradation of the immature PRG was quite high (Dineen et al., 2020). For the RB, time points of 12, 72, and 120 h were selected as described by Zontini (2016). Water-soluble CHO (**WSC**) were determined according to the procedures of Hall (2014). Finally, NPN, NDIN, and ADIN of the feed samples were determined according to Licitra et al. (1996). The chemical composition of the PRG by period, RB, and treatment diets is presented in Table 1.

All omasal phase samples were freeze dried and either ground through a 1-mm screen on a Cyclotech mill (large particle phase) or homogenized with a mortar and pestle (small particle and liquid phases) before analysis. The concentration of Co was determined by inductively coupled plasma-MS in all phase samples (Cornell University Nutrient Analysis Laboratory, Ithaca, NY), and the large and small particle phases were analyzed for uNDFom as described above. All omasal samples were analyzed for DM, aNDFom, and WSC as described previously for feed samples as well as ash (AOAC International, 2005), total N (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI), and starch (Hall et al., 2015) to determine the ruminal digestion and omasal flow of each nutrient. As digesta was fractionated into 3 phases, the small particle phase was considered to be part of the particulate matter in the double-marker system. Concentrations of Co and uNDFom were then used to calculate the concentration of each nutrient in a sample theoretically representing omasal true digesta (**OTD**; France and Siddons, 1986). Subsamples of rumen contents obtained from the rumen evacuations were freeze dried. Composite fecal samples were thawed and thoroughly mixed, and a subsample was placed in a forced-air oven at 60°C until completely dried. Both the rumen contents and feces were then ground to pass a 1-mm screen on a Cyclotech mill and analyzed for DM, OM, total N, WSC, starch, aNDFom, and uNDFom as described above and then used for both ruminal pool size and fecal excretion calculations. Volatile fatty acid (acetic, propionic, butyric, valeric, iso-butyric, and isovaleric) concentrations in rumen fluid and omasal fluid were determined using a Varian CP-3000 GC analyzer (Varian Inc., Palo Alto, CA) as described by Ranfft (1973). Samples were first thawed and centrifuged, and a 250-μL subsample was mixed with 3.75 mL of distilled water and 1 mL of 0.5-g 3-methyl-*N*-valeric acid in 1 L of 0.15 *M* oxalic acid solution. Ammonia N concentration was also determined in rumen fluid and omasal fluid using an ABX Horiba Pentra 400 chemistry analyzer (Horiba-ABX Diagnostics, Kyoto, Japan).

Omasal digesta phases, rumen contents, rumen liquid-associated bacteria, rumen particle-associated bacteria, RP, OLAB, OPAB, and OP were analyzed for NAN and ^{15}N. The concentration of NAN and abundance of ^{15}N were determined 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. (2019a). Samples of rumen contents taken in each period immediately before initiation of marker infusion were prepared and analyzed separately in the same manner as the enriched samples to evaluate natural abundance of ^{15}N. Last, the plasma samples were analyzed for urea using an enzymatic assay (kit no. UR3825, Randox Laboratories Ltd., Antrim, Northern Ireland; University College Dublin, Co. Dublin, Ireland).

Reticulorumen pH

At the beginning of the experiment, a wireless telemetry bolus that included a pH sensor (Mottram et al., 2008; eBolus, eCow Ltd., Exeter, UK) was orally administered to all animals using a balling gun. The boluses were removed via the rumen cannulate immediately after the completion of the first experimental period. The boluses were evaluated with several pH standards to ensure that no measurement drift had occurred. After completion of the evaluation, the boluses were returned to the animals for the remainder of the experiment.

Calculations

Ruminal apparent digestibility of OM, aNDFom, WSC, starch, and N was determined by first subtracting the omasal flow of each nutrient from its respective intake and then dividing by the respective intake. Ruminal true digestibility of OM and N were determined by correcting apparent digestibility for microbial nutrient flow with an additional correction applied to OM for VFA flow (Ahvenjärvi et al., 2002). For aNDFom, the apparent digestibility was assumed to be the true digestibility, as no metabolic loss is associated with

these nutrients (Van Soest, 1994). To calculate fecal output and apparent total-tract digestibility of OM, aNDFom, WSC, starch, and N, the fecal concentration of uNDFom was used as an internal marker.

Rumen turnover of OM, potentially digestible aNDFom (**pdNDFom**; aNDFom − uNDFom), and uNDFom were calculated according to Van Soest et al. (1992) using the following equation:

$$\text{Turnover (h)} = \text{rumen pool size (kg)}/[1/24 \times \text{intake (kg/d)}].$$

Turnover of OM is the apparent turnover due the presence of metabolic matter. For pdNDFom and uNDFom, the rate of intake and rate of passage over a 24-h period were calculated as follows:

$$\text{Rate of intake} = 1/24 \times \text{intake (kg/d)}/\text{rumen pool size (kg)};$$

$$\text{Rate of passage} = 1/24 \times \text{omasal flow (kg/d)}/\text{rumen pool size (kg)}.$$

From this, the rate of pdNDFom digestion was calculated as the difference:

$$\text{Rate of digestion} = \text{rate of intake} - \text{rate of passage}.$$

The concentration of ammonia N in the omasal fluid sample in combination with the flow of liquid determined by the double marker system was used to calculate 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 particle-associated bacteria N, fluid-associated bacteria N, protozoa N, and nonmicrobial N. This nonammonia nonmicrobial N (**NANMN**) was assumed to contain primarily undigested feed N and a smaller contribution of endogenous N. To determine microbial NAN flow, ^{15}N atom percent excess (**APE**) for the OTD, OLAB, OPAB, and OP samples was calculated as follows:

$$^{15}\text{N APE} = \text{enriched } ^{15}\text{N atom \%} - \text{mean natural } ^{15}\text{N atom \%}.$$

The mean natural abundance of ^{15}N in rumen contents was 0.3686 (SD: ±0.0002) and the natural abundance of ^{15}N in rumen contents was assumed to be representative of OLAB, OPAB, OP, and OTD (Ahvenjärvi et al., 2002). Omasal protozoa OM (g/L) was calculated using gravimetric determinations in a known quantity of omasal liquid as described by Fessenden et al. (2019b), assuming that protozoa only leave the rumen in the liquid phase (Ahvenjärvi et al., 2002; Karnati et al., 2007). To calculate OP OM flow (g/d), the quantity of OP OM (g/L) was multiplied by the daily volume of liquid flow (L/d) at the omasal canal. To calculate OP NAN flow (g/d), the OP OM flow (g/d) was multiplied by OP NAN concentration (g/g of OM). Accounting for ^{15}N APE in OP, the OLAB NAN (g/d) flow was calculated as follows:

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

Omasal particle-associated bacteria (PAB) NAN flow was calculated as follows:

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

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

$$\text{Omasal bacterial NAN flow (g/d)} = \text{OLAB NAN flow (g/d)} + \text{OPAB NAN flow (g/d)};$$

$$\text{Omasal microbial NAN flow (g/d)} = \text{OP NAN flow (g/d)} + \text{omasal bacterial NAN flow (g/d)}.$$

The isolated OLAB and OPAB 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 OLAB, OPAB, and OP 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 rumen pool size of digestible OM and total fermentable CHO was calculated as described by Fessenden et al. (2019b). The RP OM (g/L) was calculated using gravimetric determinations of protozoa OM in rumen liquid (g/L). To calculate RP OM pool size (g), the quantity of RP OM (g/L) was multiplied by the rumen liquid pool size (L). To calculate RP NAN pool size (g), RP OM (g) was multiplied by RP NAN concentration (g/g of OM). Accounting for ^{15}N APE in RP,

the rumen bacteria (**RuB**) and microbial NAN pool size were calculated as follows:

$$\text{RuB NAN (g)} = \{[\text{rumen contents NAN (g)} \times \text{rumen contents } ^{15}\text{N APE (g/g of NAN)}] - [\text{RP NAN (g)} \times \text{RP } ^{15}\text{N APE (g/g of NAN)}]\}/\text{RuB } ^{15}\text{N APE (g/g of NAN)};$$

$$\text{Rumen microbial NAN (g)} = \text{RP NAN (g)} + \text{RuB NAN (g)}.$$

The fractional growth rate of total microbial, bacterial, and protozoal fractions was then calculated as follows:

$$\text{Fractional growth rate (h}^{-1}) = \text{flow of microbial, bacterial, or protozoal N (g/h)/rumen pool size of microbial, bacterial, or protozoal N (g)}.$$

The ruminal true digestion rate (g/h) and fractional rates of digestion (h^{-1}) of OM and CHO were calculated as described by Fessenden et al. (2019b). These results in combination with the microbial fractional growth rates were used to calculate yield of microbial DM per gram of CHO degraded (**Yg**; g of cell DM/g of CHO degraded):

$$\text{Yg} = \text{fractional rate of microbial growth/fractional rate of CHO degradation.}$$

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) by the following model:

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

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of sequence i, $C_{j:i}$ = random effect of cow within sequence, P_k = fixed effect of period k, T_l = fixed effect of treatment l, PT_{kl} = fixed interaction effect of period k and treatment l, and ε_{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, and means were determined using the least squares means statement. Data describing pH measurements were analyzed in a repeated measures model using SAS MIXED procedures. The model included tests for the fixed effects of sequence, period, treatment, time, the interaction of period and treatment, and the interaction of time and treatment. Repeated measures (time) and random effects (cow within sequence) were also included in the model. The cow was considered the experimental unit. Effects were removed from the model when $P > 0.1$. Using Akaike's information criterion, an autoregressive of order 1 covariance structure provided the best fit to the data. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Diet Nutrient Composition

The CP concentration of the harvested PRG was slightly lower than anticipated, averaging 16.3% across the 3 experimental periods due to the lower than anticipated CP concentration in period 1 (Table 1). During April 2017, directly before the period 1 sampling phase, the monthly rainfall was 19.3 mm, an 85% reduction of the April average. This reduction in rainfall affected plant N concentration (He and Dijkstra, 2014), which we hypothesize is the likely reason for the reduced CP observed in period 1. As a result, several significant period by treatment interactions were detected, which are discussed throughout the paper. The predictions from the rate and pool size calculations, as described by Raffrenato et al. (2019), partitioned 76.3, 13.8, and 9.9% of the aNDFom into the fast, slow, and indigestible pools with rates of 12.9, 2.1, and 0.0%/h, respectively. The precutting yield across the experiment was 1,608 kg of DM/ha (above 4 cm horizon), which was close to the optimal target (O'Donovan et al., 2002). The CP, WSC, and aNDFom concentrations were all lower in the G+RB diet compared with the G diet (Table 1). The starch concentration, as was intended in diet formulation, was greater for the G+RB diet, which resulted in an increase of NFC.

Animal Performance

During the milk sampling phase (d 21–23; Table 2), total DMI was numerically higher in cows fed the G+RB diet compared with the G diet; however, a large substitution rate of 0.88 kg of pasture DMI per kg of RB DMI was observed. This high substitution rate is in accordance with other studies when a starch-based supplement was offered to cows consuming fresh pasture (Delagarde and Peyraud, 1995; Sheahan et al., 2013).

The inclusion of RB had no effect on daily milk yield, ECM, or milk fat and protein yield (Table 2). This is inconsistent with previous studies supplementing RB to pasture-based diets (Stakelum, 1986; Khalili and

Table 2. Effect of rolled barley inclusion on DMI, milk production, and animal performance of pasture-fed lactating dairy cows

Item[1]	Treatment[2] G	Treatment[2] G+RB	SEM	*P*-value
DMI, kg/d	17.2	17.6	0.3	0.11
Milk yield, kg/d	21.2	21.4	1.0	0.81
ECM,[3] kg/d	24.6	24.1	0.8	0.41
Milk fat, %	4.52	4.29	0.16	<0.05
Milk fat, kg/d	0.96	0.90	0.03	0.09
Milk CP, %	3.44	3.54	0.07	<0.05
Milk CP, kg/d	0.73	0.75	0.02	0.19
MUN, mg/dL	16.5	12.7	0.9	<0.01
Plasma urea N, mg/dL	9.2	7.6	0.3	<0.01
Feed efficiency[4]	1.45	1.36	0.05	<0.05
BW change, kg/d	0.27	0.23	0.15	0.85

[1]Values calculated from data collected on d 21 to 23 of each experimental period.

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

[3]Estimated according to Tyrrell and Reid (1965).

[4]Calculated as ECM/DMI.

Sairanen, 2000). However, the PRG swards used in the current study were considerably higher in WSC and lower in NDF concentrations than in previous studies. This indicates a greater ME supply from the forage in the current experiment and that the difference in ME supply might explain the lack of milk response to additional energy-dense supplements. The milk fat concentration decreased and milk protein concentration increased in cows fed the G+RB diet, which is similar to the results discussed in reviews of studies providing energy-dense supplements to pasture-based diets (Peyraud and Delaby, 2001; Bargo et al., 2003). In cows fed the G+RB diet, the MUN and plasma urea N (**PUN**) were lower compared with the cows fed the G diet (Table 2; 12.7 vs. 16.5 mg/dL, $P < 0.01$ for MUN; 7.6 vs. 9.2 mg/dL, $P < 0.01$ for PUN). This was likely due to the lower rumen ammonia pool size and concentration in cows fed G+RB (Table 3). A significant period by treatment interaction effect was detected for MUN and PUN. In period 1, no effect was observed for MUN and PUN, whereas there was a significant treatment effect in periods 2 and 3. This was likely due to the lower N concentration of the PRG forage during period 1. Feed efficiency (ECM/DMI) was reduced in cows fed the G+RB diet compared with the G diet ($P <$ 0.05); this was unexpected given the added fermentable CHO. Sairanen et al. (2005) reported increased milk yield from cows fed pasture-based diets supplemented with concentrates, suggesting that energy supply was the limiting factor for the nonsupplemented diet. The pasture-only treatment described by Sairanen et al. (2005) included the pasture species timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* L.). The NDF concentration was higher compared with the G treatment in this experiment (509 vs. 360 g/kg,

Table 3. Effect of rolled barley inclusion on rumen pool size[1] and concentration of ammonia N, VFA, and reticulorumen pH of pasture-fed lactating dairy cows

Item	Treatment[2] G	Treatment[2] G+RB	SEM	*P*-value
Ammonia N pool size, g	6.4	3.9	0.5	<0.01
Ammonia N concentration, mg/dL	9.0	5.9	0.5	<0.01
VFA pool size, mol				
Total VFA	8.26	8.27	0.51	0.96
Acetate	5.14	4.87	0.29	0.10
Propionate	1.74	1.99	0.13	<0.01
Butyrate	1.11	1.05	0.07	0.25
Isobutyrate	0.06	0.05	0.01	0.11
Valerate	0.12	0.16	0.02	<0.01
Isovalerate	0.12	0.12	0.01	0.89
Branched-chain VFA	0.18	0.17	0.02	0.75
Acetate:propionate ratio, mol/mol	2.95	2.50	0.07	<0.01
VFA concentration, m*M*				
Total VFA	121.8	126.0	2.0	<0.05
Acetate	75.8	74.6	1.1	0.32
Propionate	25.7	30.2	0.8	<0.01
Butyrate	16.0	16.2	0.3	0.67
Isobutyrate	0.9	0.8	0.1	0.43
Valerate	1.7	2.4	0.2	<0.01
Isovalerate	1.6	1.8	0.1	<0.05
Branched-chain VFA	2.5	2.7	0.1	0.09
Reticulorumen pH	6.34	6.35	0.02	0.51

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

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

respectively), likely diluting the energy availability of the diet.

Rumen Characteristics

The inclusion of RB reduced ruminal NH_3 pool sizes and concentration (Table 3). This was likely due to the increased incorporation of feed N into microbial N in G+RB cows as indicated by a higher microbial N flow. A significant period by treatment interaction effect was detected for ruminal NH_3, similar to that described above for MUN and PUN. Throughout the experiment, the reticulorumen pH was not different among treatments, averaging 6.35 (Table 3).

The mean pH was slightly higher than the ruminal mean reported by Kolver and deVeth (2002) of 6.15 for several pasture-based treatments. However, Falk et al. (2016) concluded that reticulorumen pH recordings are on average 0.24 pH units higher than in the rumen. Applying this correction to the current study would bring the calculated ruminal pH into agreement with that reported for cows consuming high-quality PRG forage (Delagarde et al., 1997; Rius et al., 2012). The reduction in ruminal pH of pasture-based cows when supplemented with starch-based concentrates is inconsistent (Bargo et al., 2003). The results of this study are consistent with several reports in which offering a supplement high in starch at a moderate level in pasture-based diets did not affect mean ruminal pH (Van Vuuren et al., 1993; Khalili and Sairanen, 2000). Overall, the mean rumen pH for the cows fed the G diet in this study was higher than that previously reported (Stakelum and Dillon, 2003; McEvoy et al., 2010). Other variables such as timing of rumen sample collection and method of detection might also influence these results.

The concentration of total VFA in the rumen was increased in cows fed the G+RB diet; however, the pool size determined from rumen evacuations was not affected by treatment (Table 3). In cows fed the G+RB diet, the concentration and pool size of propionate were increased compared with the G diet; this is consistent with previous reports of barley supplementation to cows being fed fresh pasture (Garcia et al., 2000). The rumen pool size of acetate tended to be reduced with supplementation of RB. This combined with the effect on propionate resulted in a lower ruminal acetate:propionate ratio in cows fed the G+RB diet compared with the G diet. The increased proportion of propionate, when cows consumed the G+RB diet, can help explain the increased milk protein concentration observed through the mechanism of increased glucose supply and potentially the influence on the insulin mammalian target of rapamycin pathway (Rius et al., 2010). Overall, changes in the concentration of ruminal VFA of pasture-based cows in response to supplementation are inconsistent. This might be due to the considerable diversity of pasture chemical composition and digestibility among studies (Bargo et al., 2003). Further, the level of supplementation will influence the absolute change in supply of rumen-fermentable CHO, ultimately dictating VFA concentrations and pool sizes (Sairanen et al., 2005).

Digestion of OM, aNDFom, WSC, and Starch

During the omasal sampling portion of the study, cows were exposed to increased human contact. This might have modestly reduced their DMI; therefore, separate intakes are reported for the milk yield data versus the omasal sampling data (Tables 2 and 4, respectively). During the omasal sampling phase (d 24–28), the inclusion of RB increased DM and OM intake and the flow of OM at the omasal canal ($P < 0.01$; Table 4). The amount of OM truly degraded in the rumen was greater in cows fed the G+RB diet ($P < 0.01$). However, on a percent of OM intake basis, the ruminal and total-tract digestibility of OM was reduced in cows fed the G+RB diet ($P < 0.01$). The total-tract digestibility of OM was calculated using uNDFom as an internal marker, and values are similar to those previously reported for PRG diets using total fecal collection (Rius et al., 2012; Beecher et al., 2014).

The intake of aNDFom was reduced in cows fed the G+RB diet; however, aNDFom flow at the omasal canal was increased relative to cows fed the G diet (Table 4). Accordingly, aNDFom digestibility decreased, both ruminally and in the total tract, in cows fed the G+RB diet. These results agree with those of Sairanen et al. (2005) and several reviews investigating the response to concentrate supplementation in dairy cows (Huhtanen, 1998; Bargo et al., 2003). This reduction in aNDFom digestibility explains the majority of the reduction in digestibility observed in the OM fraction. A reduction in rumen pH due to the addition of rapidly fermentable supplements to pasture-based diets is a mechanism commonly cited to explain this reduction in feed digestibility (Dixon and Stockdale, 1999). In the current study, reticulorumen pH was not affected by treatment, suggesting that this mechanism was not responsible for the negative effect on feed digestion observed. Reduced aNDFom digestibility can be a multifaceted issue. The concentration of uNDFom was higher in the RB grain compared with PRG due to the barley grain containing hull material (Table 1; Firkins et al., 2001). This might have contributed to some of the reduction in aNDFom digestibility, as reported in other studies (Van Vuuren et al., 1993; Sairanen et al., 2005). In a review,

Hoover (1986) suggested that the rumen ammonia N concentration required to optimize nutrient digestion was 6.2 mg/dL, whereas microbial growth was optimized at a lower ammonia N concentration of 3.3 mg/dL. Others have suggested that the rumen ammonia N concentration required by the particulate-associated microbes digesting fiber might be greater than that of the fluid-associated microbes (Allison, 1980; McAllan and Smith, 1983). Further, Satter and Slyter (1974) demonstrated that a rumen ammonia level of 5 mg/dL was the minimum required to maintain adequate microbial growth. In the current experiment, rumen ammonia N concentration was close to the threshold of 5 mg/dL in cows fed the G+RB diet, potentially explaining a portion of the reduced aNDFom digestibility. This suggests that on a dynamic basis, with variable rumen ammonia levels throughout the day, there might have been periods when the NFC bacteria could likely outcompete the fiber bacteria for ammonia, decreasing aNDFom digestion. The results of the current experiment suggest that pH was not the main driver of reduced feed digestion in cows fed the G+RB diet. Further work is required to better describe the variables and mechanisms involved in the reduction of pasture digestibility when supplements are provided.

Feed intake is closely related to rumen pool size and rumen turnover (Van Soest, 1994; Forbes, 1995). In pasture-based systems, physical capacity of the reticulorumen has been suggested to limit DMI due to the relatively high aNDFom concentration of the diet (Allen, 1996; Baudracco et al., 2010). In the current experiment, the average aNDFom concentration of PRG was 36% of DM, resulting in a rumen aNDFom pool size of 1% of BW. The inclusion of RB increased rumen OM, aNDFom, and uNDFom pool size ($P < 0.05$; Table 4), which suggests that DMI intake of the G diet was not limited by physical fill capacity of the rumen. The rumen aNDFom pool size in cows fed the G+RB in the current study (1.1% of BW) was similar to that previously reported for cows consuming pasture-based diets (Sairanen et al., 2005; Taweel et al., 2005). Sairanen et al. (2005) remarked that the rumen NDF pools observed when cows consumed highly digestible pasture were considerably lower compared with those of cows fed grass silage or red clover–grass silage (Khalili and Huhtanen, 2002; Rinne et al., 2002). This suggests that

Table 4. Effect of rolled barley inclusion on rumen pool size, nutrient flow to the omasum, and digestibility of DM, OM, aNDFom, and uNDFom of pasture-fed lactating dairy cows[1]

Item	Treatment[2]			
	G	G+RB	SEM	P-value
DM				
Intake, kg/d	16.1	17.1	0.4	<0.01
OM				
Intake, kg/d	15.1	16.1	0.4	<0.01
Rumen pool, kg	8.3	9.1	0.5	<0.05
Flow at omasal canal, kg/d	6.9	7.7	0.3	<0.01
Apparently digested in the rumen, kg/d	8.2	8.4	0.2	0.28
Truly digested in the rumen,[3] kg/d	13.2	13.9	0.3	<0.01
% of OM intake	87.9	86.1	0.6	<0.01
Total-tract apparent digestibility, %	85.2	82.0	0.3	<0.01
aNDFom[4]				
Intake, kg/d	5.8	5.6	0.2	<0.05
Rumen pool, kg/d	4.8	5.5	0.3	<0.01
Flow at omasal canal, kg/d	1.6	2.0	0.1	<0.01
Apparently digested in the rumen, kg/d	4.2	3.6	0.1	<0.01
% of aNDFom intake	72.3	63.1	0.9	<0.01
% of pdNDFom[5] intake	80.4	72.3	1.0	<0.01
Total-tract apparent digestibility, %				
% of aNDFom intake	83.2	74.5	0.6	<0.01
% of pdNDFom intake	92.5	85.4	0.7	<0.01
uNDFom[6]				
Intake, kg/d	0.58	0.71	0.02	<0.01
Rumen pool, kg/d	1.55	1.75	0.10	<0.01

[1]Values calculated from data collected on d 24 to 28 of each experimental period.

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

[3]Corrected for microbial and VFA contribution to flows.

[4]aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue.

[5]pdNDFom = potentially digestible aNDFom.

[6]uNDFom = undigested amylase- and sodium sulfite-treated NDF corrected for ash residue.

Table 5. Effect of rolled barley inclusion on nutrient flow to the omasum and digestibility of water-soluble carbohydrates (WSC) and starch of pasture-fed lactating dairy cows

Item[1]	Treatment[2] G	Treatment[2] G+RB	SEM	*P*-value
WSC				
Intake, kg/d	3.55	3.25	0.10	<0.01
Flow at omasal canal, kg/d	0.24	0.37	0.03	<0.01
Apparently digested in the rumen, kg/d	3.32	2.87	0.08	<0.01
% of WSC intake	93.2	88.7	0.5	<0.01
Total-tract apparent digestibility, %	99.4	98.6	0.1	<0.01
Starch				
Intake, kg/d	0.38	2.42	0.01	<0.01
Flow at omasal canal, kg/d	—	0.26	—	—
Apparently digested in the rumen, kg/d	—	2.16	—	—
% of starch intake	—	89.2	—	—
Total-tract apparent digestibility, %	—	97.9	—	—

[1]Values calculated from data collected on d 24 to 28 of each experimental period.

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

in the current study, the G+RB cows had the potential for greater rumen fill capacity if other environmental conditions allowed.

The average WSC concentration of the PRG in this experiment was comparable with that of grass cultivars selected for high sugar concentration (Taweel et al., 2005; Moorby et al., 2006). Water-soluble CHO intake was reduced in cows fed the G+RB diet (Table 5) due to the high substitution rate and a lower WSC concentration in the RB. Further, both the amount and proportion of WSC digested in the rumen and in the total tract were decreased with the supplementation of RB. This might indicate lower digestibility of WSC in G+RB cows compared with G cows; however, for cows fed the G+RB diet, intermediates of starch degradation might have been detected as, and contributed to, WSC omasal flow. In cows fed the G diet, more than 90% of the WSC was degraded in the rumen, which has important implications for both the cow's energy supply via VFA production and the synthesis of bacterial and protozoal N. To the authors' knowledge, no other data quantifying ruminal digestion of WSC in pasture-based diets are available. The ruminal digestibility of starch in cows fed the G+RB diet was 89%, similar to the results suggested in a review by Nocek and Tamminga (1991). In the current experiment, the apparent total-tract digestibility of starch was 98%, which was comparable with and slightly higher than that reported by Firkins et al. (2001) and Overton et al. (1995).

Rumen Digestion Kinetics

There was no difference in the apparent turnover of OM between treatments (Table 6). Compared with the G diet, the inclusion of RB increased the turnover time required for pdNDFom in the rumen. This is consistent with the decreased rumen ammonia concentration of those cows and previous studies that increased the starch concentration of the diet (Van Vuuren et al., 1993). Conversely, when cows consumed the G+RB diet, the turnover time for uNDFom was reduced due to the inclusion of RB in the diet, and the rate of passage increased for pdNDFom and uNDFom. The rate of digestion of pdNDFom was decreased with the inclusion of RB, which is similar to that previously discussed for ruminal pdNDFom digestibility with fermentable CHO supplementation (Sairanen et al., 2005). The liquid flow tended to decrease when cows consumed the G+RB diet; however, the liquid rate of passage was not different between treatments (Table 6). The liquid

Table 6. Effect of rolled barley inclusion on rumen turnover, rate of passage, and rate of digestion of OM, pdNDFom, uNDFom, and liquid flow of pasture-fed lactating dairy cows

Item	Treatment[1] G	Treatment[1] G+RB	SEM	*P*-value
OM				
Apparent turnover, h	13.3	13.6	0.6	0.44
pdNDFom[2]				
Turnover, h	15.4	18.9	1.0	<0.01
Rate of passage, %/h	1.3	1.5	0.1	<0.01
Rate of digestion, %/h	5.6	4.2	0.3	<0.01
uNDFom[3]				
Turnover, h	64.0	59.0	2.6	<0.05
Rate of passage, %/h	1.6	1.8	0.1	<0.01
Liquid				
Liquid flow, L/d	336	309	18	0.08
Rate of passage, %/h	20.9	19.9	0.9	0.39

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

[2]pdNDFom = potentially digestible amylase- and sodium sulfite-treated NDF corrected for ash residue.

[3]uNDFom = undigested amylase- and sodium sulfite-treated NDF corrected for ash residue.

rate of passage measured in the current experiment (approximately 0.20 h^{-1}) was similar to that previously estimated for cows consuming pasture-based diets (Van Vuuren et al., 1992; Rius et al., 2012).

Digestion and Omasal Flow of N

During the sampling phase, N intake was similar between treatments due to the lower N concentration but higher DMI of cows consuming the G+RB diet (Table 7). Inclusion of RB increased the flow of NAN at the omasal canal ($P < 0.01$) compared with the G diet. This is consistent with the results observed by Van Vuuren et al. (1993), who offered a starch supplement, and Sairanen et al. (2005), who offered a low-CP pelleted supplement. In the current experiment, the increase in NAN can be attributed to an increased flow of microbial N in cows fed the G+RB diet compared with the G diet ($P < 0.01$). This increase in microbial N flow when cows consumed the G+RB diet can help explain the increased milk protein concentration observed (Table 2) through the mechanism of increased AA supply and greater propionate concentration. There was no difference in NANMN flow between the treatments; however, the contribution of NANMN to the total NAN flow was relatively low compared with previous studies (O'Mara et al., 1997; Younge et al., 2004). The NANMN flow is typically estimated by difference (i.e., NAN flow − microbial N flow); therefore, any error in either of these estimations will be partitioned into the NANMN flow. Key differences between the current study and previous studies were that in both Younge et al. (2004) and O'Mara et al. (1997), protozoal N flow was not determined and purine derivatives were used to determine microbial N. Estimates using purine derivatives as microbial markers have been shown to have lower precision and accuracy compared with techniques using ^{15}N (Firkins and Reynolds, 2005; Del Valle et al., 2019). These inaccuracies have further implications in regards to the determination of ruminal digestible feed N, as an underestimated microbial N flow will underestimate digestibility. In the present study, the average feed N ruminal digestibility, corrected for microbial contributions, was 89%. This was not different between treatments and was comparable with that reported by Sairanen et al. (2005; 85%) and Kolver and Muller (1998; 84%). The results of the current experiment, using the omasal sampling technique and ^{15}N isotope labeling, confirm that extensive rumen proteolysis of ingested N occurs when PRG is consumed (Beever et al., 1986; Delagarde et al., 1997; Table 7). This highlights the significant dependence of cows grazing such swards on microbial N as their main NAN supply and source of metabolizable AA.

Of the total microbial N flow, protozoal N contributed on average 22% and was not different between treatments. Few quantitative data describe protozoal N flow in pasture-fed cows. The levels reported in this study are within the range proposed by Dijkstra et al. (1998; 10.7–26.1%) in computer simulations of cows consuming similar amounts of DMI. Supplementation with RB did not increase protozoal N flow in the current experiment, which is in contrast to previous reports for cows consuming diets based on grass (Khalili and Sairanen, 2000) and grass silage (Ahvenjärvi et al., 2002). It is difficult to ascertain the reason for this; however, the high WSC concentration of the fresh PRG might have provided ample sugar to sustain high protozoal growth (Clarke, 1965; Williams and Coleman, 1988). Denton et al. (2015) recently demonstrated that mixed protozoa could sequester sugar away from bacteria, giving protozoa a competitive advantage under such ruminal conditions.

Microbial Dynamics

Compared with the G diet, the inclusion of RB increased the rumen pool size and true ruminal digestion rate of both digestible OM and fermentable CHO (Table

Table 7. Effect of rolled barley inclusion on the N flow of pasture-fed lactating dairy cows[1]

	Treatment[2]			
Item	G	G+RB	SEM	P-value
N intake, g/d	429	424	11	0.53
Flow at omasal canal				
Total N, g/d	394	436	18	<0.01
Ammonia N, g/d	21	14	1	<0.01
NAN				
g/d	373	422	18	<0.01
% of N intake	90.9	99.3	2.8	<0.05
NANMN[3]				
g/d	49.1	47.7	4.1	0.78
% of N intake	11.6	11.0	0.9	0.65
Microbial NAN				
g/d	324	374	15	<0.01
% of total NAN	87.1	88.8	0.8	0.17
Bacterial NAN				
g/d	248	298	18	<0.01
% of microbial NAN flow	76.5	80.1	3.2	0.24
Protozoa NAN				
g/d	79	73	11	0.55
% of microbial NAN flow	23.5	20.0	3.2	0.24
Microbial N, g/kg of OTDR[4]	24.4	26.6	0.7	<0.05
True ruminal N digestibility, %	88.4	89.0	0.9	0.65

[1]Values calculated from data collected on d 24 to 28 of each experimental period.

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

[3]NANMN = nonammonia nonmicrobial N.

[4]OTDR = OM truly digested in the rumen.

Table 8. Effect of rolled barley inclusion on rumen pool sizes, fractional rates of microbial growth and nutrient digestion, and microbial generation time of pasture-fed lactating dairy cows

Item	Treatment[1]		SEM	P-value
	G	G+RB		
Rumen pool size				
Digestible OM,[2] kg	4.35	4.98	0.33	<0.05
Total fermentable CHO,[3] kg	3.43	4.09	0.29	<0.01
Total NAN, g	333	325	15	0.45
Microbial NAN, g	239	233	11	0.54
Microbial OM proportion of rumen OM pool, %	29.3	26.5	0.8	<0.01
Bacteria NAN,[4] g	226	221	12	0.60
Protozoa NAN, g	13	12	3	0.73
Protozoa NAN pool, % of total microbial NAN pool	5.3	5.2	1.1	0.92
Rumen kinetics				
Fractional growth rate of bacteria,[5] h^{-1}	0.046	0.057	0.003	<0.05
Fractional growth rate of protozoa,[5] h^{-1}	0.301	0.299	0.035	0.98
Fractional growth rate of all microbes, h^{-1}	0.058	0.067	0.003	<0.01
Ruminal true OM digestion rate, g/h	551	580	13	<0.01
Ruminal true CHO digestion rate, g/h	453	479	11	<0.01
Fractional rate of OM digestion,[6] h^{-1}	0.133	0.122	0.007	<0.05
Fractional rate of CHO digestion,[6] h^{-1}	0.141	0.124	0.008	<0.01
Observed Yg,[7] g of cells/g of CHO degraded	0.43	0.56	0.03	<0.01
Generation time of bacteria,[8] h	22.6	18.5	1.2	<0.05
Generation time of protozoa,[8] h	4.0	4.1	0.5	0.93
Generation time of microbes,[8] h	18.0	15.4	0.8	<0.05
Fluid retention time,[9] h	5.0	5.1	0.2	0.71

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

[2]Measured OM from rumen evacuation, corrected for microbial OM and undigested NDF after 240 h of in vitro digestion and analyzed with amylase, sodium sulfite and ash corrected (Raffrenato et al., 2018).

[3]CHO = carbohydrate. Calculated as rumen digestible OM pool − (rumen CP pool − microbial CP pool) − (rumen DM pool × diet fat concentration).

[4]Calculated as microbial NAN pool − protozoal NAN pool.

[5]Calculated as bacterial or protozoal daily flow (g/h)/bacterial or protozoal pool size (g).

[6]Calculated as OM or carbohydrate degraded (g/h)/OM or carbohydrate rumen pool size (g).

[7]Calculated as fractional microbial growth rate/fractional rate of CHO digestion.

[8]Reciprocal of fractional growth rate of bacteria, protozoa, or all microbes.

[9]Reciprocal of liquid passage rate.

8). The rumen bacterial pool size was not affected by treatment; however, cows fed the G diet had a greater microbial OM contribution to the total OM rumen pool than cows fed the G+RB diet (29.3 vs. 26.5% for G vs. G+RB, respectively; $P < 0.01$). Consistent with the observed increase in bacterial N flow (Table 7), fractional growth rate of bacteria increased in cows fed the G+RB diet, with several studies reporting similar effects (Nocek and Russell, 1988). The Yg increased in cows fed the G+RB diet compared with G (0.56 vs. 0.43, respectively). Variable Yg values have previously been reported due to differing CHO sources (Nocek, 1988); however, values greater than 0.5, the theoretical maximum (Isaacson et al., 1975), are rare. Using a biochemical approach, Stouthamer (1973) reported a maximal Yg of approximately 0.8 g/g of glucose, indicating the potential for higher yields to be achieved in vivo.

Protozoa are expected to sequester in the rumen and contribute about 50% of the microbial biomass (Jouany, 1996). However, these inferences are primarily based on in vitro data or data that were often generated from procedures with low accuracy (Sylvester et al., 2005; Karnati et al., 2007). Until recently, no measurements of in vivo protozoal generation time existed for high-producing dairy cows (Karnati et al., 2007). In the current study, protozoal rumen pool size, fractional growth rate, and generation time were not affected by treatment (Table 8). For both treatments, however, protozoa N contributed considerably less to the total microbial N pool in the rumen (5%) compared with at the omasal canal (22%). This resulted in an average protozoal generation time of 4.1 h, which is extremely short compared with current expectations (Jouany, 1996). Using protozoa cell count procedures, Karnati et al. (2007) determined a protozoa generation time of 16.4 h. The authors noted, however, that low ruminal pH appeared to reduce protozoal diversity, and this low rumen pH might have also influenced protozoal metabolism (Franzolin and Dehority, 2010). Firkins et

al. (2007) suggested that a higher rumen passage rate could allow less lysis of protozoa in vivo than in vitro, lower ruminal pool size of protozoa relative to bacteria, and improve efficiency of protozoal cell growth (i.e., faster cell division). In agreement, Sylvester et al. (2009) demonstrated that rumen ciliated protozoa could decrease generation time in response to increasing dilution rate; other researchers reported similar effects (Harrison et al., 1976; Dehority, 2004). In the current study, the fluid passage rate averaged 0.20 h^{-1} (Table 6), a 27% increase compared with Karnati et al. (2007). This might provide a mechanism to help explain the high protozoal growth efficiency and low rumen pool size relative to bacteria observed in the current study. However, based on any published information, an in vivo generation time as short as the current study has only once been previously reported (Warner, 1962).

To maintain viable populations, protozoa must have a generation time that is shorter than the retention time of the phase in which they leave the rumen (Dehority, 2003). Hence, passage of protozoa from the rumen has previously been estimated by comparing the calculated protozoal generation time with the retention time of the fluid or particle rumen phases (Sylvester et al., 2005; Karnati et al., 2007). In the current experiment, fluid retention time and protozoa generation time averaged 5.0 and 4.1 h, respectively, which demonstrates the protozoa's ability to leave the rumen in the fluid phase. Others have demonstrated that the mean generation time of protozoa seems to approximate the mean retention time of particulate matter in the rumen (Sylvester et al., 2005; Karnati et al., 2007), which has been interpreted to reflect chemotaxis of protozoa for feed particles (Diaz et al., 2014). In the current study, the fractional rate of CHO digestion was quite high, averaging 0.13 h^{-1}, as fresh, immature PRG can have a high digestion rate. This implies that the CHO in the rumen turned over every 8 h. Thus, even if a portion of the protozoa exhibits a chemotaxis toward digestible particulates, they still need to have a generation time that is shorter than previously characterized other than that reported by Warner (1962).

Calculation of protozoa generation time involves both rumen pool size and omasal flow of protozoa; therefore, error in either measurement can influence the result obtained. Using a real-time PCR assay, Sylvester et al. (2005) reported low protozoal proportions in the rumen (9%), similar to the current study. However, RP, especially isotrichids, have been reported to follow a diurnal cycle (Potter and Dehority, 1973; Dehority, 2003). As RP were sampled at a lower frequency in the current study compared with Karnati et al. (2007), this might have contributed to an underestimation of the rumen pool size and hence an underestimation of protozoal generation time. Further studies investigating both the rumen pool size and omasal flow of protozoa are required to confirm the dynamics observed in the current study.

CONCLUSIONS

In this study, RB supplementation did not benefit overall performance and reduced ruminal aNDFom digestibility; however, this was not mediated through a reduction in reticulorumen pH. Rumen pool size and fractional digestion rate of digestible OM and fermentable CHO were increased in cows fed the G+RB diet. It seems likely that this increased fermentable CHO supply mediated an increased fractional growth rate and omasal flow of bacteria in cows fed G+RB diet. For both diets, the contribution of microbial N to the total flow of NAN, together with high ruminal digestibility of feed N, underlines the large dependence of cows consuming fresh PRG-based diets on microbial N. Further quantification of the specific AA contributing to this NAN flow is required. Protozoa N flow was not different between diets, although protozoa appear to supply a much larger amount of microbial N and exhibit shorter generation time than previously considered. This was most likely due to the rapid rumen turnover and high sugar concentration of PRG-based diets. The data generated in this study can be used to evaluate predictions from nutrition models and modify predictions of bacterial and protozoal growth and passage under conditions of high-quality pasture intake.

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