Effect of starchy or fibrous carbohydrate supplementation of orchardgrass on ruminal fermentation and methane output in continuous culture

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ABSTRACT

A 4-unit dual-flow continuous culture fermentor system was used to assess the effects of supplementing orchardgrass (*Dactylis glomerata* L.) with 2 levels [5 and 10% of total dry matter (DM) fed] of starchy (barley grain, BAR) or fibrous (beet pulp, BP) carbohydrate sources on nutrient digestibility, volatile fatty acid (VFA) production, bacterial protein synthesis, and CH₄ output. Treatments were randomly assigned to fermentors in a 4 × 4 Latin square design with a 2 × 2 factorial arrangement using 7 d for microbial adaptation and 3 d for sample collection. Treatments included (1) 57 g of DM orchardgrass + 3 g of DM BAR, (2) 54 g of DM orchardgrass + 6 g of DM BAR, (3) 57 g of DM orchardgrass + 3 g of DM BP, or (4) 54 g of DM orchardgrass + 6 g of DM BP. Feedings occurred at 0900, 1030, 1400, and 1900 h throughout four 10-d periods. Gas samples for CH₄ analysis were collected 6 times daily at 0725, 0900, 1000, 1355, 1530, and 1630 h. Fermentor samples for pH, NH₃-N, and VFA analysis were taken on d 8, 9, and 10. Samples were also analyzed for DM, organic matter, crude protein, purines, neutral detergent fiber, and acid detergent fiber to determine nutrient digestibilities and estimation of bacterial protein synthesis. Apparent and true DM and organic matter digestibilities were not affected by supplement source. Apparent neutral and acid detergent fiber digestibilities were greater for BAR than BP. Conversely, apparent crude protein digestibility was greater for BP than BAR. Mean and maximum pH tended to be greatest for BAR than BP. Minimum pH was greater at the lower level (5% of diet DM) of supplementation. Barley produced greater concentrations of total VFA and acetate, whereas BP had greater daily outputs of CH₄. Significant supplement type × level interactions were found for bacterial N flow and efficiency. Overall, supplementing orchardgrass with BP improved crude protein digestibility, reduced fiber digestibility and

total VFA concentration, but increased CH₄ output compared with BAR.

Key words: barley, beet pulp, continuous culture fermentation, grazing

INTRODUCTION

High-quality pastures, such as those found on grazing dairy farms in the northeastern United States, often contain high concentrations of CP, much of which is NPN. Rapid and extensive degradation of CP in orchardgrass occurs in the rumen, and a portion of this soluble N is absorbed as NH₃-N through the ruminal wall with some portion subsequently excreted in the urine as urea, thereby reducing utilization of RDP. Energy supplementation of grass can increase the flow of NAN to the duodenum (García et al., 2000), reduce ruminal NH₃-N concentration (van Vuuren et al., 1986; Bargo et al., 2002), and improve milk production (Stockdale et al., 1987; Kellaway and Porta, 1993; Stockdale, 1999). Supplementation with cereal grains, which are high in starch, shifts ruminal fermentation toward more propionate, positively influencing the energetic status of the cow. However, previous studies have demonstrated adverse effects on ruminal fermentation of fiber when energy in the form of starch is supplemented to high-forage diets (El-Shazly et al., 1961; Mould and Orskov, 1984; Miller and Muntifering, 1985). van Vuuren et al. (1990) suggested that feeds containing a high fraction of insoluble, fermentable carbohydrates (such as beet pulp) may be a more suitable supplement for pasture-based diets than supplements such as barley, oats, and wheat, which have a greater proportion of starch. Beet pulp has been shown to have lower ruminal escape of OM than corn, meaning that beet pulp may provide increased energy for microbial growth to improve NH₃-N uptake in the rumen (van Vuuren et al., 1993). Compared with no supplementation or supplementation with a starchy energy source, several studies reported increased N utilization in the rumen and increased efficiency of microbial protein synthesis when beet pulp was supplemented to grass-based diets in vitro (Mount et al., 2009) and in vivo [e.g., grazing

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dairy cows (O'Mara et al., 1997) and grazing lambs (Mount et al., 2009)]. However, others have reported no effect of fibrous carbohydrate supplementation on N utilization in grazing dairy cows (e.g., Pulido et al., 2010).

To the best of our knowledge, no studies have directly compared supplementation of starchy versus fibrous sources of carbohydrate to a grass diet on ruminal fermentation and CH₄ production. Therefore, the objective of this study was to evaluate the effects of supplementing orchardgrass with 2 levels (5 or 10% of total DM fed) of starchy (barley grain, **BAR**) or fibrous (beet pulp, **BP**) carbohydrates on nutrient digestibility, VFA production, bacterial protein synthesis, and CH₄ output in continuous culture. We hypothesized that compared with BAR, BP supplementation of orchardgrass would improve ruminal fermentation through a more synchronous supply of fermentable energy relative to RDP in the orchardgrass.

MATERIALS AND METHODS

Site, Experimental Design, and Treatments

This study was conducted at the USDA-Agricultural Research Service (ARS) Pasture Systems and Watershed Management Research Unit (University Park, PA) from February to April 2014. Orchardgrass (*Dactylis glomerata* L.) was harvested in the afternoon of September 2, 2011, at the Russell Larson Agricultural Research Farm (Rock Springs, PA) from a pure stand. A forage plot harvester (HEGE 212, Wintersteiger AG, Waldenburg, Germany; 1.5-m-wide swath) was used for harvesting, set at 10 cm above soil level, approximately 3 wk after the previous cutting such that resulting orchardgrass was in a vegetative stage of growth, typical of high-quality pastures used for grazing in the northeastern United States (25 to 30 cm tall). Within 30 min of harvest, the orchardgrass was placed in cloth bags and frozen (−4°C) until being freeze-dried (Ultra 35 Super ES; VirTis Co. Inc., Gardiner, NY). Barley (*Hordeum vulgare* L.) used for the barley supplement was a winter hullless variety purchased from Lakeview Organic Grain (Penn Yan, NY). Beet pulp pellets (Standlee Western) were purchased from Tractor Supply Company (State College, PA). Freeze-dried orchardgrass, barley and beet pulp were ground to pass through a 2-mm screen (Wiley mill, Thomas Scientific Inc., Swedesboro, NJ) to be used as feed for the fermentors. While it is recognized that freeze-dried forages are not nutritionally identical to fresh forages, the forage needed to be preserved and ground for use in this experiment, and Jones and Bailey (1972) reported that

oven-drying forages could denature protein in plant material, thereby depressing nutrient digestibility.

Total DM (orchardgrass plus supplement) fed to all fermentors was maintained at a constant 60 g of DM/d for the duration of each period. Supplements (BAR or BP) made up 5 or 10% of diet DM and were as follows: (1) 57 g of DM orchardgrass + 3 g of DM BAR, (2) 54 g of DM orchardgrass + 6 g of DM BAR, (3) 57 g of DM orchardgrass + 3 g of DM BP, or (4) 54 g of DM orchardgrass + 6 g of DM BP. Freeze-dried orchardgrass was fed in equal portions daily (0730, 1030, 1400, and 1900 h), and BAR and BP supplements were fed twice daily (0730 and 1400 h) in equal portions to simulate typical supplementation practices that are becoming more common in grazing dairy herds that are reducing the use of concentrates due to increased feed costs, farmer philosophy, or milk price incentives in the northeastern United States. Representative samples of freeze-dried orchardgrass, BAR, and BP were stored for later nutrient analysis. Nutrient analyses of dietary ingredients and treatments were obtained from chemical analyses conducted at a commercial laboratory (Dairy One Laboratories, Ithaca, NY; Table 1) before the start of the study.

Continuous Culture Operation

This study was a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Feeds were incubated in a 4-unit dual flow continuous culture fermentor system (OmniCulture Plus, VirTis Co. Inc.), similar to that described by Hoover et al. (1989), with the following modifications: pH was not controlled, feed ingredients were not pelleted, fermentor volumes ranged from 1,100 to 1,140 mL, and urea was added to the mineral buffer solution at a rate of 0.4 g/L to simulate recycled N (Weller and Pilgrim, 1974). Solid mean retention time, solid dilution rate, and liquid dilution rate of the fermentors were adjusted daily to approximately 24 h, 4%/h (±0.32%), and 11%/h (±0.41), respectively, and were achieved by regulation of buffer input and filtrate removal (Bargo et al., 2003).

Ruminal fluid and digesta samples were collected from a ruminally fistulated, nonlactating, multiparous Holstein cow (BW = 652 kg) cared for in accordance with the Pennsylvania State University Animal Care and Use (Institutional Animal Care and Use Committee #39513) guidelines. The donor cow was group housed and fed a diet of mixed grain and grass hay (65:35 forage-to-concentrate ratio) in a feed bunk for a total of 16.2 kg of DM of available feed per cow per day at the Pennsylvania State University Dairy Research Farm (University Park, PA). The mixed grain

Table 1. Chemical composition (% of DM) of diet ingredients and orchardgrass supplemented with barley grain (BAR) or beet pulp (BP) at 2 levels of supplementation (5% or 10% of total DM fed) during continuous culture fermentation

Item	Ingredient			Diet ¹			
	Barley	Beet pulp	Orchardgrass	5% BAR	10% BAR	5% BP	10% BP
OM	97.1	91.0	91.7	92.0	92.3	91.7	91.7
CP	12.9	9.0	15.5	15.4	15.2	15.2	14.9
RDP, % of CP	50.0	40.0	68.0	67.1	66.2	66.6	65.2
NDF	14.4	36.4	63.7	61.2	58.8	62.3	61.0
ADF	5.30	23.7	37.8	36.2	34.6	37.1	36.4
NFC ²	67.4	44.6	9.20	12.1	15.1	11.0	12.7
WSC ³	4.00	10.5	7.30	7.14	6.97	7.46	7.62
ESC ⁴	2.00	7.60	4.40	4.28	4.16	4.56	4.72
CP:NFC	0.18	0.27	1.08	1.03	0.99	1.04	1.00
Starch	64.2	1.50	0.70	3.68	6.65	0.74	0.78
NSC ⁵	68.2	12.0	8.00	10.8	13.6	8.20	8.40
NE _L , ⁶ Mcal/kg	1.96	1.58	1.08	1.12	1.17	1.11	1.13
Ether extract	2.40	1.00	3.30	3.26	3.21	3.19	3.07
Ca	0.05	0.97	0.39	0.27	0.36	0.42	0.45
P	0.43	0.10	0.31	0.32	0.32	0.30	0.29
Mg	0.13	0.19	0.33	0.32	0.31	0.32	0.32
K	0.49	1.38	2.61	2.50	2.40	2.50	2.50
Na	0.01	0.37	0.16	0.15	0.14	0.17	0.18

¹Calculated using actual nutrient composition and proportion of individual ingredients; barley (BAR) or beet pulp (BP) supplemented to orchardgrass at 5 or 10% of total DM fed.

²Calculated as $\text{NFC} = 100 - (\text{CP} \% + \text{NDF} \% + \text{ether extract} \% + \text{ash} \%)$.

³WSC = water-soluble carbohydrates.

⁴ESC = ethanol-soluble carbohydrate.

⁵NSC = WSC + starch.

⁶Estimated by the NRC (2001) model.

contained a vitamin/mineral premix with the following composition (% as-is basis): trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix, 0.48; limestone, 37.2; selenium premix, 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg and was fed at 1.8% of total DMI. Approximately 3 h after feeding, 6 L of ruminal fluid was collected with a hand pump into a prewarmed insulated container. Solid digesta was collected by hand from the ventral, central, and dorsal areas of the rumen. Liquid and whole digesta samples were transported to the USDA-ARS laboratory in separate containers. Within 15 min of collection, fluid was strained through 4 layers of cheesecloth and poured into each of the prewarmed fermentation jars until it cleared the overflow spout. Solid digesta was mixed by hand and 25 g was added to each fermentor. Each fermentor was continuously purged with CO₂ gas at a rate of 20 mL/min (model MC50, Alicat Scientific, Tucson, AZ) to maintain anaerobiosis and the temperature was maintained at 39°C. Fermentor contents were continually stirred at a rate of 255 rpm. Fermentor pH was recorded immediately before each of the 4 daily feedings.

Fermentors were operated for 4 consecutive 10-d periods and re-inoculated with fresh rumen fluid at the beginning of each period. Each period consisted of a 7-d adaptation period followed by a 3-d sampling period. Liquid and solid overflow were collected in 4-L plastic containers. During the 7-d adaptation period, the overflow containers were weighed daily and the contents discarded. After being emptied on d 7 and continuing through d 10, the containers were immersed in a chilled (4°C) water bath [Pennsylvania State University Agricultural Bio-Engineering Department custom design, stainless steel (dimensions: 2.75 cm deep × 3.5 cm wide × 21.3 cm long) insulated on the bottom and sides with 1.2 cm of spray foam insulation] with cold water circulator (model 9005, Polyscience, Niles, IL) and each received 20 mL of 50% sulfuric acid daily (after being emptied) to inhibit microbial fermentation. During the last 3 d of each period, liquid and solid effluent overflows from each fermentor were combined and mixed in a blender (model 38LL52 Waring, Torrington, CT) for 30 s on the lowest setting and speed controlled further with a PowerStat variable transformer (model 3PN126, Superior Electric, Bristol, CT) set at 35 to achieve a slight vortex in the top 1 cm of the blender contents. This provided for adequate mixing of sample without too much abrasive agitation. After mixing, a

100-mL effluent sample was taken and composited over the 3 d to determine overflow DM. A 50-mL sample of effluent was strained through 8 layers of cheesecloth before a 15-mL sub-sample was placed in a 30-mL plastic bottle for determination of VFA [Varian 3300 Gas Chromatograph (FID detector), Varian 4290 Integrator; Rumen Fermentation Profiling Laboratory, West Virginia University, Morgantown, WV; Supelco, 1975, modified to use a 80/120 Carbowax B-DA/4% Carbowax 20M column; Erwin et al., 1961] and $\text{NH}_3\text{-N}$ (Yang and Varga, 1989). The GC parameters used were 2 m $\times$ 2 mm i.d. glass column packed with 80/120 Carbowax B-DA/4% Carbowax 20M (catalog no. 1-1889, Supelco Inc.), column temperature of 175°C, injector temperature of 200°C, detector temperature of 200°C, N gas used as a carrier at a flow rate of 24 mL/min and injection volume of 1 μL . An additional 1,100-mL sample of effluent was composited over the 3-d sample period, freeze-dried, ground to pass through a 1-mm sieve, and stored in a sealed plastic bag at ambient temperature for later analyses of DM, OM, NDF, CP, and total purines. On d 10, in addition to the previously reported sampling, the contents of each fermentor jar were processed according to the methods of Griswold et al. (1996) with the following modification: in lieu of the initial centrifugation, contents were mixed in the blender as described above for 30 s and strained through 2 layers of 53- μm Nitex fabric (Wildco, Buffalo, NY) into a 2-L plastic container with 5 mL of 50% sulfuric acid added to retard microbial growth. Contents were then centrifuged 3 times at 20,000 $\times g$ for 20 min at -4°C with the pellet re-suspended in 0.9% saline and 50% methanol, respectively, for the last 2 centrifugations (Griswold et al., 1996). The final pellet was placed in a plastic container and stored in the freezer until subsequent freeze-drying and analyses for DM, OM, and CP [per previously described AOAC International (2006) procedures described in the Nutrient Analyses section below] and purines (Zinn and Owens, 1986). Purine data were used to calculate bacterial efficiencies as described in the Statistical Analysis and Calculations section below.

Methane Collection and Measurements

Gas samples for CH_4 analysis were collected 6 times daily in duplicate (0725, 0900, 1000, 1355, 1530, and 1630 h) using a 25-gauge needle attached to a 30-mL syringe (Vibart et al., 2007). A separate needle and syringe were designated for each fermentor. After the syringe was plunged 3 times to purge any residual gas, the needle was inserted through a rubber septum (part #608010, Sigma-Aldrich, St. Louis, MO) located in a port on top of the fermentor, and 30 mL of gas was

withdrawn from the headspace. A stopcock attached between the needle and syringe ensured that gas did not escape upon transference to an evacuated 15-mL glass vial through a septum in the cap. Gas samples were analyzed for CH_4 using a GC (Varian CP 3800, Agilent Technologies, Santa Clara, CA) as described in Soder et al. (2012). The daily average of the 6 temporal CH_4 samplings (averaged within treatment and across all 3 sampling days) was used in the calculation of mean daily CH_4 output (Johnson et al., 2009; see calculation in Statistical Analysis and Calculations section below). For graphing temporal CH_4 output (Figure 1), the noncumulative average of CH_4 output at each of the 6 individual sampling times was used (averaged within treatment and across all 3 sampling days).

Nutrient Analyses

Samples of orchardgrass, barley, and beet pulp were analyzed by wet chemistry (Dairy One Laboratories, Ithaca, NY) according to the following sample analyses: DM (method 930.15; AOAC International, 2006), CP (method 990.03; AOAC International, 2006), RDP (Cornell *Streptomyces griseus* enzymatic digestion; Colblentz et al., 1999), NDF [Ankom model A200; Ankom Technology method 6 (NDF in Feeds—Filter Bag Technique for A200; Ankom Technology, Fairport, NY); solutions as in Van Soest et al. (1991) with heat-stable α -amylase and sodium sulfite used in the NDF procedure (inclusive of ash)]. Water-soluble carbohydrates were determined by incubating samples in a 40°C water bath for 1 h, extracting WSC (simple sugars and fructan). The WSC was determined using a Thermo Scientific Genesys 10S Vis Spectrophotometer after acid hydrolysis with sulfuric acid (Smith, 1969) using potassium ferricyanide for the colorimetric reaction rather than potassium iodide-potassium oxalate as cited in Smith (1969), as potassium ferricyanide provides a more stable reaction for detecting reducing sugars (Miller-Webster et al., 2002). Ethanol-soluble carbohydrates (Hall et al., 1999), starch (Application Note Number 319; YSI Inc. Life Sciences, Yellow Springs, OH), minerals (Ca, P, Mg, K, Na; Thermo IRIS Advantage HX or inductively coupled plasma radial spectrometer after microwave digestion; CEM Application Note for Acid Digestion, CEM, Matthews, NC), and ether extract (method 2003.05; AOAC International, 2006) were also determined. The NFC concentration was calculated using the equation $\text{NFC}\% = 100\% - (\text{CP}\% + \text{NDF}\% + \text{ether extract}\% + \text{ash}\%)$. Pectins were included in the NFC calculation but were not included in WSC or NSC analyses.

Effluent samples and microbial pellets were analyzed for DM and OM (methods 930.15 and 942.05, respec-

tively; AOAC International, 2006) and CP content (micro-Kjeldahl digestion using 75-mL calibrated tubes with $\text{CuSO}_4/\text{K}_2\text{SO}_4$ catalyst, method 976.06; AOAC International, 2006). The NDF content of the effluent was determined using the Ankom model A200 with heat-stable α -amylase and sodium sulfite used in the NDF procedure (inclusive of ash; Ankom Technology method 6 (NDF in Feeds—Filter Bag Technique for A200; Ankom Technology, Fairport, NY); solutions as in Van Soest et al. (1991) with heat-stable α -amylase and sodium sulfite used in the NDF procedure (inclusive of ash)). Concentrations of total purines (Zinn and Owens, 1986) in effluent and microbial isolates were used to partition effluent N flow into microbial and nonmicrobial fractions and to calculate true CP, DM, and OM digestibilities and flows.

Statistical Analysis and Calculations

Data were analyzed as a 4×4 Latin square design with a 2×2 factorial arrangement of treatments using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC), fitted to the following model:

$$Y_{ijk} = \mu + P_i + F_j + T_k + PT_{ik} + e_{ijk},$$

where Y_{ijk} = the observations for dependent variables, μ = the population mean, P_i = the mean effect of the i th period, F_j = the mean effect of the j th fermentor, T_k = the mean effect of the k th treatment, PT_{ik} = the interaction between the i th period and k th treatment, and e_{ijk} = the residual error. Preplanned orthogonal contrasts were used to test the main effect of supplement type (BAR vs. BP), supplement level (5 vs. 10%), and the supplement source $\times$ supplement level interaction. Period and treatment were fixed effects and fermentor and error were random.

Measures of CH_4 concentrations were analyzed for temporal patterns using the following model:

$$Y_{ijkl} = \mu + P_i + F_j + T_k + E1_{ijk} + H_l + HT_{kl} + E2_{ijkl},$$

where Y_{ijkl} = the observations for dependent variables, μ = the population mean, P_i = the mean effect of i th period, F_j = the mean effect of the j th fermentor, T_k = the mean effect of the k th treatment, $E1_{ijk}$ = the whole plot error, H_l = the mean effect of the l th hour

Figure 1. Noncumulative temporal CH_4 output of orchardgrass supplemented with barley grain (BAR) or beet pulp (BP) at 2 levels of supplementation (5 or 10% of total DM fed) during continuous culture fermentation. Fermentors were fed (see arrows) at 0730 h (orchardgrass plus supplement), 0930 h (orchardgrass only), 1400 h (orchardgrass plus supplement), and 1900 h (orchardgrass only; not depicted on this figure because no CH_4 samples were taken past 1630 h). Methane samples were collected at 0725, 0900, 1000, 1355, 1530, and 1630 h. Error bars represent standard errors.

sampling analyzed as repeated measures, HT_{lk} = the interaction between the l th hour and k th treatment, and $E2_{ijkl}$ = the subplot residual error. All terms were considered fixed effects except fermentor, whole plot error, and subplot error, which were considered random. An autoregressive covariance structure, which showed the lowest Akaike information criterion values, was retained in the final model.

All reported values are least squares means. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$ for all analyses. No period or period $\times$ treatment interaction effects were found; therefore, only the main effects and supplement $\times$ level interactions were reported.

Apparent (DM, OM, NDF, and ADF) and true (DM, OM, CP) digestibilities of nutrients were calculated as follows (DM as an example):

$$\begin{aligned} \text{DM apparently digested (\% of total DM)} = \\ [(g \text{ of DM fed} - g \text{ of effluent flow DM}) \div g \text{ of DM fed}] \\ \times 100, \text{ with effluent corrected for g of buffer DM.} \end{aligned}$$

$$\begin{aligned} \text{DM truly digested (\% of total DM)} = \\ \{[(g \text{ of DM fed} - (g \text{ of effluent flow DM} \\ - g \text{ of microbial DM})) \div g \text{ of DM fed}] \times 100, \\ \text{with effluent corrected for g of buffer DM.} \end{aligned}$$

Nitrogenous fraction flows were calculated as follows:

$$\begin{aligned} \text{Total N intake (g/d)} = \text{infused urea (g/d)} \\ + \text{dietary N (g/d).} \end{aligned}$$

$$\begin{aligned} \text{NH}_3\text{-N flow (g/d)} = [(\text{effluent NH}_3\text{-N in mg/dL} \\ \times (\text{total effluent flow in mL} \times 1 \text{ dL}/100 \text{ mL}))]. \end{aligned}$$

$$\begin{aligned} \text{Non-NH}_3\text{-N flow (g/d)} = \\ (g \text{ of total effluent N flow}) - (g \text{ of effluent NH}_3\text{-N}). \end{aligned}$$

$$\begin{aligned} \text{Microbial N flow (g/d)} = (g \text{ of microbial DM}) \\ \times (\% \text{ N in microbes} \div 100). \end{aligned}$$

$$\begin{aligned} \text{Dietary N flow (g/d)} = (g \text{ of effluent non-NH}_3\text{-N}) \\ - (g \text{ of effluent microbial N}). \end{aligned}$$

$$\begin{aligned} \text{Total N flow (g/d)} = (g \text{ of effluent NH}_3\text{-N flow}) \\ + (g \text{ of non-NH}_3\text{-N flow}). \end{aligned}$$

$$\begin{aligned} \text{Rumen-degradable N (mg/d)} = (g \text{ of total N intake}) \\ - (g \text{ of dietary N flow}). \end{aligned}$$

Bacterial efficiency was calculated as follows:

$$\begin{aligned} \text{bacterial efficiency (mg of bacterial N/kg} \\ \text{OM truly digested)} = (g \text{ of effluent bacterial N} \div g \\ \text{of OM truly digested}) \times 1,000, \text{ where} \end{aligned}$$

$$\begin{aligned} \text{effluent bacterial N (g)} = [\text{effluent total N (g/d)} \\ \times (\% \text{ bacterial N in effluent total N}/100)], \text{ and} \end{aligned}$$

$$\begin{aligned} \% \text{ bacterial N in effluent total n} = [\text{effluent purine} \\ (\text{mg/g of N}) \div \text{bacterial purine (mg/g of N)}] \times 100. \end{aligned}$$

Daily CH_4 output (mmol/d) was estimated using the following equation:

$$\begin{aligned} \text{CH}_4 \text{ concentration in fermentor headspace (mmol/mL)} \\ \times \text{CO}_2 \text{ gas flow through the fermentor headspace} \\ (20 \text{ mL/min}) \times 60 \text{ min} \times 24 \text{ h.} \end{aligned}$$

Methane output (mmol/g of OM fed, NDF fed, or digestible NDF or OM fed) were calculated as follows (OM fed used as an example):

$$\begin{aligned} \text{mmol of CH}_4 \text{ output/g of OM fed} = \\ [\text{daily CH}_4 \text{ output (mmol/d)}/g \text{ of OM fed/d}]. \end{aligned}$$

RESULTS

Treatment Composition and Nutrient Digestibility

Chemical composition of dietary ingredients and treatments are presented in Table 1. The use of pooled samples for compositional analysis of dietary ingredient samples precluded statistical comparison of treatments. The CP of the treatments ranged from 14.9% for 10% BP to 15.4% for 5% BAR. The NDF numerically ranged from 58.8% for 10% BAR to 62.3% for 5% BP with ADF showing similar numerical differences (34.6 and 37.1% for 10% BP and 5% BAR, respectively). Concentration of NSC ranged from 8.20% for 5% BP to 13.6% for 10% BAR with starch following a similar numerical difference (0.74 and 6.65% for 5% BP and 10% BAR, respectively). The NE_L was numerically greater for the 10% BAR (1.17 vs. 1.11 to 1.12 Mcal/kg for the other 3 treatments). Additionally, orchardgrass supple-

Table 2. Nutrient digestibility of orchardgrass supplemented with barley grain (BAR) or beet pulp (BP) at 2 levels of supplementation (5 or 10% of total DM fed) during continuous culture fermentation

Item	Diet ¹				SEM	Contrast (<i>P</i> -value) ²		
	5% BAR	10% BAR	5% BP	10% BP		5% vs. 10% ³	BAR vs. BP	Supplement × level
Apparent digestibility								
DM, %	64.2	65.2	62.8	65.0	0.99	0.10	0.39	0.48
OM, %	72.5	76.0	71.4	72.5	0.64	0.25	0.25	0.72
NDF, %	85.1	85.1	80.0	78.9	0.89	0.55	<0.001	0.54
ADF, %	81.7	81.5	75.2	75.4	1.06	0.98	<0.001	0.84
True digestibility								
DM, %	74.3	77.0	74.4	75.4	0.82	0.07	0.42	0.33
OM, %	81.4	83.9	81.3	81.2	0.78	0.21	0.15	0.19

¹Barley (BAR) or beet pulp (BP) supplemented to orchardgrass at 5 or 10% of total DM fed.²Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.³Level of supplement as a % of total DM fed.

mented with BAR had numerically greater starch content (3.68 and 6.65 for 5% and 10% BAR, respectively) compared with orchardgrass supplemented with beet pulp (0.74 and 0.78 for 5% and 10% BP, respectively), whereas orchardgrass supplemented with BP had 10% (numerically) greater ethanol-soluble carbohydrates than orchardgrass supplemented with BAR (4.64 vs. 4.22%, respectively). Both BAR treatments had 31% (numerically) greater NSC concentration than the BP treatments (15.4 vs. 11.8%, respectively).

No supplement type × supplement level interactions were observed for apparent and true nutrient digestibilities (Table 2). Apparent DM and OM digestibilities, as well as true OM digestibility, were not affected by supplement type or level. True DM digestibility tended ($P = 0.07$) to be greater with 10% versus 5% of supplementation with either BAR or BP. Whereas apparent NDF and ADF digestibilities were not affected by supplement level, both were greater ($P \leq 0.05$) for BAR than for BP.

Table 3. Fermentor pH, VFA production, and CH₄ output of orchardgrass supplemented with barley grain (BAR) or beet pulp (BP) at 2 levels of supplementation (5% or 10% of total DM fed) during continuous culture fermentation

Item	Diet ¹				SEM	Contrast (<i>P</i> -value) ²		
	5% BAR	10% BAR	5% BP	10% BP		5% vs. 10% ³	BAR vs. BP	Supplement × level
pH								
Mean	6.76	6.74	6.73	6.70	0.04	0.30	0.08	0.88
Minimum	6.57	6.50	6.54	6.50	0.04	0.03	0.43	0.57
Maximum	7.01	7.03	6.94	6.93	0.06	0.95	0.06	0.76
VFA, mM								
Total	67.1	67.2	70.6	73.7	1.71	0.36	0.03	0.39
Individual VFA								
Acetate (A)	43.6	43.3	48.3	50.4	1.08	0.33	<0.01	0.23
Propionate (P)	16.1	16.4	15.6	16.7	0.66	0.28	0.87	0.51
Butyrate (B)	5.50	5.40	5.20	5.10	0.19	0.68	0.18	0.96
Isobutyrate	0.34	0.34	0.30	0.28	0.02	0.64	0.18	0.43
Valerate	1.44	1.65	1.11	1.31	0.16	0.20	0.07	0.96
Isovalerate	0.19	0.13	0.11	0.09	0.03	0.25	0.10	0.62
A:P	2.72	2.67	3.10	3.04	0.07	0.42	<0.01	0.95
A+B:P	3.06	3.00	3.44	3.35	0.08	0.31	<0.01	0.87
CH ₄								
mmol of CH ₄ /d	18.3	16.7	25.5	24.3	1.78	0.46	<0.01	0.88
mmol of CH ₄ /g of OM fed	5.30	4.80	7.40	7.10	0.52	0.44	<0.01	0.88
mmol of CH ₄ /g of NDF fed	8.00	7.30	10.9	10.6	0.77	0.53	<0.01	0.82
mmol of CH ₄ /g of digestible OM fed	5.20	5.47	7.29	6.48	0.68	0.51	<0.001	0.83
mmol of CH ₄ /g of digestible NDF fed	10.6	9.7	15.6	16.0	1.05	0.50	<0.001	0.88

¹Barley (BAR) or beet pulp (BP) supplemented to orchardgrass at 5 or 10% of total DM fed.²Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.³Level of supplement as a % of total DM fed.

Table 4. Nitrogen metabolism of orchardgrass supplemented with barley grain (BAR) or beet pulp (BP) at 2 levels of supplementation (5 or 10% of total DM fed) during continuous culture fermentation

Item	Diet ¹				SEM	Contrast (<i>P</i> -value) ²		
	5% BAR	10% BAR	5% BP	10% BP		5% vs. 10% ³	BAR vs. BP	Supplement × level
N intake, ⁴ g/d	2.00	1.97	2.02	2.00	0.01	0.01	<0.01	0.46
NH ₃ -N, mg of N/dL	13.7	12.9	14.1	13.6	0.33	0.09	0.14	0.63
True CP digestibility, %	49.0	56.0	75.4	75.2	2.53	0.26	<0.001	0.24
N flows, g/d								
Total N	1.53	1.48	1.24	1.18	0.04	0.23	<0.001	0.93
NH ₃ -N	0.40	0.38	0.41	0.40	0.01	0.10	0.10	1.00
NAN	1.12	1.10	0.83	0.78	0.04	0.38	<0.001	0.82
Bacterial N	0.41	0.47	0.47	0.42	0.02	0.71	0.81	0.03
Dietary N	0.74	0.63	0.37	0.36	0.04	0.19	<0.001	0.21
Bacterial efficiency								
g of N/kg of OM truly digested	9.69	10.8	11.0	9.87	0.41	0.92	0.62	0.04

¹Barley (BAR) or beet pulp (BP) supplemented to orchardgrass at 5 or 10% of total DM fed.

²Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

³Level of supplement as a % of total DM fed.

⁴N intake (g/d) = dietary N (g/d) + urea-N from buffer (g/d).

Fermentor pH, VFA, and Methane Output

No supplement type × supplement level interactions were observed for mean, minimum, and maximum fermentor pH (Table 3). Minimum fermentor pH was greater ($P = 0.025$) with 5% versus 10% supplementation. Mean and maximum fermentor pH tended ($P = 0.08$ and $P = 0.06$, respectively) to be greater for BAR than for BP.

No supplement type × supplement level interactions were observed for concentrations of total or individual VFA or ratios of VFA (Table 3). No effect was found of supplement level on total VFA concentration, individual VFA concentrations, or ratios of VFA. Total VFA and acetate concentrations were greater ($P < 0.05$) for BP than for BAR. Valerate concentrations tended ($P = 0.07$) to be greater for BAR than for BP treatments. The ratio of acetate to propionate, and the ratio of acetate plus butyrate to propionate were greater ($P < 0.05$) for BP than for BAR, primarily due to the shift in acetate.

No supplement type × supplement level interactions were observed for CH₄ output expressed as millimoles per day (Table 3). Methane output was lowest for all treatments when sampled immediately before the 0730 h feeding of orchardgrass and supplement, and increased ($P < 0.05$) throughout the day when expressed as daily CH₄ output (Figure 1), millimoles per gram of OM fed, NDF fed, or digestible NDF and OM fed (data not shown for these last 4 variables). Temporal patterns showed a treatment by sampling time interaction ($P = 0.054$) for daily CH₄ output for all treatments (Figure 1). The BP treatments had a greater increase in CH₄ output between samplings than did BAR. This resulted

in BP having significantly greater CH₄ output at the 1355, 1530, and 1630 samplings than BAR.

No effect was found of supplement level on any of the CH₄ output variables. Orchardgrass supplemented with BP had greater ($P < 0.01$) output of daily CH₄, as well as greater ($P < 0.05$) CH₄ output when expressed as millimoles per gram of OM fed, NDF fed, or digestible NDF or OM fed compared with orchardgrass supplemented with BAR.

Nitrogen Metabolism

No supplement type × supplement level interactions were found for N intake, NH₃-N concentration, true CP digestibility, and nitrogenous fractions flows (i.e., total N, NH₃-N, NAN, and dietary N flow; Table 4). However, significant ($P < 0.05$) supplement type × supplement level interactions were found for bacterial N flow and bacterial efficiency (mg of bacterial N/kg of OM truly digested). Nitrogen intake was greater ($P < 0.01$) at the lower level of supplementation, whereas NH₃-N concentration tended ($P = 0.10$) to be greater at the lower level of supplementation. Nitrogen intake and true CP digestibility were greater ($P < 0.05$) for BP than for BAR. Total N, NAN, and dietary N flows were greater ($P < 0.05$) for BAR than for BP.

DISCUSSION

Treatment Composition

Crude protein and NDF values were typical of feed-grade barley and beet pulp (NRC, 2001). Nutrient concentration of the forage used in this study

was considered moderate quality with CP of 15% and moderately high NDF concentrations (64%; Bargo et al., 2003). The nutrient content of the treatments reflects that of the ingredients. For example, the CP of the barley-supplemented treatments was an average of 0.2 percentage units greater than the beet pulp-supplemented treatments due to the increased CP of the barley supplement compared with the beet pulp supplement (12.9 and 9.0%, respectively). The greater starch content of the barley supplement (60.2 vs. 1.5% for beet pulp) resulted in a 5- to 9-fold numerical increase in starch values for the barley-supplemented treatments compared with beet pulp-supplemented treatments.

Nutrient Digestibility

Apparent DM and OM digestibilities ranged from 62.8 to 65.2% and 71.4 to 76.0%, respectively, across treatments. These values are in general agreement with previously reported values for perennial ryegrass (*Lolium perenne* L.)- and orchardgrass-only treatments fermented in continuous culture, with apparent DM and OM digestibilities ranging from 49 to 70.2% and 58 to 72.4%, respectively (de Veth and Kolver, 2001; Bargo et al., 2003; Soder et al., 2012). Although supplement type did not affect apparent or true DM and OM digestibilities, apparent NDF and ADF digestibilities were greater for BAR than for BP. The increased digestion of fiber fractions and slightly lesser amounts of NDF to digest in BAR may be explained by a greater concentration of starch compared with BP (average of 5.17 vs. 0.76%, respectively, for BAR and BP) and, as a result, more fermentable energy available to stimulate cellulolytic bacteria growth. For instance, replacing dietary starch with neutral detergent-soluble fiber (12.7, 16.4, 20.1, or 23.8% NDSF, DM basis) by inclusion of sugar beet pulp increased carboxymethylcellulase and xylanase activities in the solid fraction and apparent disappearance of ADF, but decreased the 16S rDNA copy numbers of *Ruminococcus albus* in both liquid and solid fractions and *Ruminococcus flavefaciens* in the solid fraction using the rumen simulation technique apparatus (Zhao et al., 2013). Nutritional interdependence or “cross-feeding” among key ruminal microorganisms involved in starch and fiber utilization such as *Ruminobacter amylophilus* (former *Bacteroides amylophilus*), *Megasphaera elsdenii*, and *Ruminococcus albus* as reported by Miura et al. (1980) should be also considered to explain current results.

Fermentor pH, VFA, and Methane Output

Barley tended to produce greater mean and maximum fermentor pH than BP. The 10% level of supplementa-

tion resulted in lower minimum pH than the 5% level of supplementation, which may be explained by the additional amount of fermentable energy provided with the greater supplementation rate (Dixon and Stockdale, 1999). However, minimum fermentor pH was greater than 6.4 for all treatments, which has been shown to be the optimal pH for cellulose digestion (Hoover, 1986; Wales et al., 2004).

The VFA results reported in this study are within range of those reported in other continuous culture fermentation studies evaluating grass diets supplemented with beet pulp (Bach et al., 1999) or barley (Fellner et al., 2008; Wales et al., 2009). Total VFA and acetate concentrations were greater for BP than for BAR, whereas valerate tended to be greater for BAR than for BP. Total VFA results in the present study are corroborated by previous studies in which readily digestible fiber sources such as beet pulp were fed (Grigsby et al., 1992; Carey et al., 1993). The greater proportion of acetate observed in the BP treatments was not surprising as ruminal acetate is derived primarily from the fermentation of structural carbohydrates (Feng et al., 1995). Fermentation of pectin by known pectinolytic bacteria strains (e.g., *Prevotella ruminicola* and *Butyrivibrio fibrisolvens*) yielded acetate as the major end product, whereas butyrate, formate, or lactate were produced when glucose was used as a substrate (Marounek and Dušková, 1999; Dušková and Marounek, 2001). Liu et al. (2015) reported that when comparing corn stover plus citrus peel pectin with corn stover plus corn starch, as well as pectin with starch, pectin supplementation increased acetate production, whereas starch increased butyrate production during an in vitro study using mixed rumen microbial populations. In the current study, fiber digestibility decreased despite increased acetate production with feeding beet pulp. These results suggest that pectin from beet pulp led to more acetate production than barley or fiber from orchardgrass or both. Ariza et al. (2001) demonstrated in continuous culture that citrus pulp (rich in pectin) resulted in greater acetate concentrations than hominy feed (rich in starch) without an effect on butyrate formation, which agrees with our results. Strobel and Russell (1986) found increased acetate production with pectin than with other carbohydrate sources, with little effect on propionate production, which agrees with the results of the current study.

A significant supplementation type $\times$ level of supplementation interaction was found for CH₄ output. The lowest CH₄ output was observed for all treatments when sampled immediately before the first daily feeding of forage and supplement (Figure 1). This observation was likely due to depletion of substrate in the continuous culture system, as the previous feeding (forage only)

occurred 12 h prior. All treatments increased in CH₄ output throughout the day, which has been shown by others (e.g., Hafla et al., 2014; Brask et al., 2015).

Beet pulp produced 42% more CH₄ (mmol/d) than BAR. Moreover, BP produced more CH₄ when expressed as millimoles of CH₄ per gram of OM fed, millimoles of CH₄ per gram of NDF fed, millimoles of CH₄ per gram of digestible NDF fed, and millimoles of CH₄ per gram of digestible OM fed than BAR. Greater CH₄ output for BP than for BAR may have been due to shifts in ruminal proportions of individual VFA, which have been shown to affect CH₄ production. Whereas propionate formation consumes reducing equivalents, acetate and butyrate formation generate H₂ for methanogenesis (Hungate, 1966; Owens and Goetsch, 1988). Acetate concentrations were greater for BP than for BAR as discussed earlier.

When ruminal NDF digestibility is decreased, greater amounts of undigested NDF pass out of the rumen and are fermented in the hind gut, resulting in increased CH₄ production (Moe and Tyrrell, 1979). Because the continuous culture fermentor system simulates the rumen but not the hind gut, this may not be the most plausible explanation for the noted changes in CH₄ in the current study. Rather, changes in individual VFA, specifically acetate, likely resulted in the observed difference in CH₄. When the actual CH₄ values were compared with the CH₄ prediction equation of Owens and Goetsch (1988), which is based on VFA production [$\text{CH}_4 \text{ (mmol/d)} = (0.5 \times \text{acetate}) + (0.5 \times \text{butyrate}) - (0.25 \times \text{propionate})$], the equation underpredicted CH₄ for BP (8 and 2% for the 5 and 10% supplementation levels, respectively) and overpredicted CH₄ for BAR (14 and 18% for the 5 and 10% supplementation levels of BAR, respectively). These differences were primarily due to the differences noted in acetate production among the treatments, as the acetate fermentation process provides a methyl group for methanogenesis (Ferry, 1992).

Nitrogen Metabolism

Significant supplement type $\times$ level of supplementation interactions were observed for bacterial N flow and efficiency with the 5% BAR and 10% BP showing the lowest values. Organic matter truly digested was not different across treatments (mean = 82%), thus suggesting that fermentable energy did not limit bacterial growth in the current study. It is conceivable that individual or a combination of factors such as types of substrates (pectin versus starch), associative effects between the basal diet and substrates, fermentation patterns, or dilution of ryegrass with substrates may explain to some extent these observed interactions. For instance, Hall

and Herejk (2001) fed isolated bermudagrass (*Cynodon dactylon*) NDF alone or in combination (60:40 ratios) with sucrose, pectin, or corn starch to mixed rumen microbial populations in vitro, and observed maximal microbial yields (measured as TCA-precipitable protein) for each substrate as starch > sucrose = pectin > bermudagrass NDF (Hall and Herejk, 2001).

Despite fairly similar intakes of CP and RDP across treatments, the small variation in N input into the system when orchardgrass was a greater proportion of the treatment (at the lower supplementation levels) resulted in tendencies for increased N intake and NH₃-N concentrations in these treatments. However, these differences are not likely to be biologically important. Despite lower N intake, BAR-fed fermentors had a greater total N flow than BP-fed fermentors. The 89% greater dietary N flow for BAR than for BP explains the greater total N and NAN flows when NH₃-N flow was similar across treatments.

CONCLUSIONS

Supplementing orchardgrass with beet pulp improved CP digestibility, reduced fiber digestibility, and increased total VFA concentration and CH₄ output compared with barley-supplemented orchardgrass. Increased CH₄ output with beet pulp may have negative environmental effects and must be weighed against potential benefits. Significant supplement type $\times$ level of supplementation interactions were observed for bacterial N flow and efficiency in fermentors fed barley at the lowest level of supplementation or beet pulp at the greatest level of supplementation. Bacterial growth was not limited by fermentable energy (similar OM truly digested in the rumen across treatments), suggesting that types of substrates (e.g., pectin vs. starch), associative effects between the basal diet and substrates, fermentation patterns, or dilution of ryegrass with substrates may explain to some extent the bacterial N flow and efficiency interactions. The greatest apparent CP digestibility with beet pulp without an accompanying effect on the concentration of NH₃-N appears to indicate that RDP-N was efficiently captured by the microbes, as beet pulp may have provided a more synchronous supply of fermentable energy relative to orchardgrass soluble N. Further research is needed to better understand N use efficiency in grass-based diets supplemented with ruminally fermentable fiber sources.

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