Interpretive summary: Effect of feeding warm-season annuals with orchardgrass on ruminal fermentation and methane output in continuous culture. *By Dillard et al.* The aim of this study was to assess the effects of feeding warm-season annuals ('brown midrib' sorghum × sudangrass, Japanese millet, or a mixture of both) with orchardgrass on ruminal fermentation and methane output in a dual-flow continuous culture fermentor system. Overall, there was little difference in nutrient digestibility among all treatments. However, sorghum × sudangrass and the mixture provided a lower acetate to propionate ratio and lower methane output than the millet. Use of improved warm-season annuals during summer months would provide alternative high-quality forage options for dairy cattle when forage productivity and quality of cool-season pastures decreases.

Effect of feeding warm-season annuals with 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 nutrient digestibility, volatile fatty acids (VFA) production, bacterial protein synthesis and methane (**CH_4**) output of warm-season annual grasses. Treatments were randomly assigned to fermentors in a 4 × 4 Latin square design using 7 d for treatment adaptation and 3 d for sample collection. Treatments were: 1) 100% orchardgrass [(*Dactylis glomerata* L.); **ORD**]; 2) 50% orchardgrass + 50% Japanese millet [*Echinochloa esculenta* (A. Braun H. Scholz); **MIL**], 3) 50% orchardgrass + 50% 'brown midrib' sorghum × sudangrass [(*Sorghum bicolor* L. *Moenc*h × *S. bicolor var. sudanense*); **SSG**]; or 4) 50% orchardgrass + 25% millet + 25% sorghum × sudangrass (**MIX**). Fermentors were fed 60 g dry matter (**DM**)/d in equal portions of herbage 4 times daily (0730, 1030, 1400, and 1900 h). In order to replicate a typical 12-h pasture rotation, fermentors were fed the orchardgrass at 0730 and 1030 h and individual treatment herbage (orchardgrass, Japanese millet, sorghum × sudangrass, or 50:50 Japanese millet and sorghum × sudangrass) at 1400 and 1900 h. Gas samples for CH_4 analysis were collected 6 times daily at 0725, 0900, 1000, 1355, 1530, and 1630 h. Fermentor pH was determined at the time of feeding and fermentor effluent samples for NH_3-N and VFA analyses were taken daily at 1030 h on d 8, 9, and 10. Samples were also analyzed for DM, organic matter (OM), crude protein, and fiber fractions to determine nutrient digestibilities. Bacterial efficiency was estimated by dividing bacterial N by truly digested OM. True DM and OM digestibilities, and pH were not different among treatments. Apparent OM digestibility was greater in ORD than MIL and SSG. The concentration of propionate was greater in ORD than SSG and MIX, and that of butyrate was greatest in ORD and MIL. Methane output was greatest in MIL, intermediate in ORD, and lowest in SSG and MIX. Nitrogen intake did not differ across treatments, whereas bacterial N efficiency per kg of truly digestible OM was greatest in MIL, intermediate in SSG and MIX, and lowest in ORD. True crude protein digestibility was greater in ORD versus MIL, and ORD had lower total N, non-NH_3-N, bacterial N, and dietary N in effluent flows than MIL. Overall, there was little difference in true nutrient digestibility; however, SSG and MIX provided the lowest acetate to propionate ratio and lower CH_4 output than MIL and ORD. Thus, improved warm-season annual pastures (i.e., ‘brown midrib’ sorghum × sudangrass) could provide a reasonable alternative to orchardgrass pastures during the summer months when such perennial cool-season grass species have greatly reduced productivity.

Keywords: continuous culture, ruminal fermentation, warm-season annuals

INTRODUCTION

Perennial cool-season grasses provide high-quality forage for pasture-based dairy systems in the temperate regions of the United States throughout the spring and fall grazing seasons. However, forage production and quality decline markedly during the hot summer months (July and August) with this occurrence frequently referred to as the “summer forage slump”. Winsten et al. (2010) reported that, of 987 dairy farms surveyed across the Northeast, 13% used management-intensive rotational grazing and 80% of farms used a combination of low-intensity grazing and traditional confinement systems. According to Stiglbauer et al. (2013), 85 and 95% of the conventional pasture-based and organic-certified dairies, respectively, used management-intensive rotational grazing across New York, Wisconsin, and Oregon. With the number of pasture-based dairies expected to increase as a result of increasing demand for grassfed and organic milk (Pereira et al., 2013), a requirement for pasture-based diets with some milk processors (e.g. certified organic labels through USDA-National Organic Program or labels through individual processors), and some climate models predicting warmer, drier summers for temperate regions during the next century (Wolfe et al., 2008), evaluation of heat- and drought-tolerant warm-season forages that can support profitable milk production in pasture-based dairy cattle is paramount.

Warm-season annuals such as sorghum, sudangrass, sorghum × sudangrass hybrids, and millets have been promoted as highly productive forages, but are considered to be highly lignified and have low leaf to stem ratios, resulting in lower-quality forage and reduced digestibility compared to perennial cool-season forages (Cowan and Lowe, 1998). Research in the northeastern United States reported that brown midrib (**BMR**) sorghum × sudangrass yielded 7,297 kg DM/ha and contained 12.9% CP, 61.7% NDF, with the NDF reported to be 77.5%

digestible (Ketterings et al., 2005). Using a prediction model (Milk2000, http://www. uwex edu/ces/forage/pubs/milk2000.xls), the estimated milk production was 1,552 kg milk/Mg forage DM and 11,301 kg milk/ha. However, this model does not consider air temperature which can have a negative effect on DMI and milk production when temperatures > 20°C (NRC, 2001). Fontaneli et al. (2001) observed that intensively managed warm-season annuals grown in a subtropical climate were of sufficient yield and quality (5.7 Mg DM/ha, 18.3% CP, and 61.6% in vitro OM digestibility to meet the nutritional demands of lactating dairy cows producing 20 kg of milk/d (NRC, 2001). Fontaneli et al. (2001) also stated that warm-season annuals would significantly contribute to the development of a year-round, grazing dairy system. However, there is currently no literature available on the performance of lactating dairy cows consuming warm-season annuals compared with perennial, cool-season forage.

Even though it has been considered standard practice to include warm-season annuals in the forage program of pasture-based dairy farms in temperate regions such as the northeastern United States since the 1960s (Clark et al., 1965), there is still limited information available on the digestibility and the animal production potential of warm-season annuals when used in the diet of lactating dairy cows. Furthermore, new varieties (e.g., BMR) have been developed that could prove to be of superior nutritional quality than older varieties of warm-season annuals (Ketterings et al., 2005). Therefore, the objective of the current study was to determine the effects of Japanese millet, BMR sorghum × sudangrass, and a mixture of both on nutrient digestibility, VFA production, bacterial protein synthesis and methane (CH_4) output in continuous culture compared with a typical, cool-season herbage (i.e., orchardgrass). We hypothesized that inclusion of the warm-season annual forages will provide greater digestible

OM compared with orchardgrass alone, resulting in improved ruminal fermentation and decreased CH_4 production per unit of OM digested.

MATERIALS AND METHODS

Site, Experimental Design, and Herbage Treatments

The study was conducted at the USDA-Agricultural Research Service Pasture Systems and Watershed Management Research Unit (University Park, PA) from February to April, 2015. On June 16, 2014, 'FSG 208 BMR' hybrid sorghum × sudangrass (*Sorghum bicolor* L. *Moench* × *S. bicolor* var. *sudanense*) and 'Japanese' millet [*Echinochloa esculenta* (A. Braun) H. Scholz] were planted into a prepared seedbed using a no-till drill (HEGE 1000; Wintersteiger AG, Waldenburg, Germany). Plots were fertilized with 46 kg N/ha in a split-application using ammonium sulfate; P and K were applied to plots according to soil test results. Plots were harvested twice during the growing season (July 17 and September 23, 2014), when herbage height was within the optimal range for grazing (45 to 75 cm; Hodgson et al., 1977). Orchardgrass (*Dactylis glomerata* L.) was harvested in the morning of July 2 and September 23, 2014, from a 1-yr old pure stand. Orchardgrass was harvested in a vegetative stage of growth, typical of high-quality pastures used for grazing in temperate regions of the United States (25 to 30 cm tall). A plot harvester (HEGE 212; Wintersteiger AG; 1.5-m wide swath), set to a 10-cm stubble height was used to harvest all plots. Within 30 min of harvest, herbage was placed in cloth bags and frozen (-4°C) until being freeze-dried (Ultra 35 Super ES; Virtis Co. Inc., Gardiner, NY). Freeze-dried herbage was ground to pass through a 2-mm sieve (Wiley Mill; Thomson Scientific Inc., Philadelphia, PA) to be used as feed for the fermentors. While it is recognized that freeze-dried forages are not nutritionally identical to fresh forages, the herbage 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 and depress digestibility.

Total DM fed to all fermentors was maintained at a constant 60 g/d for the duration of each period. Treatments were as follows: 1) 100% orchardgrass (**ORD**); 2) 50% orchardgrass + 50% Japanese millet (**MIL**); 3) 50% orchardgrass + 50% sorghum × sudangrass (**SSG**); or 4) 50% orchardgrass + 25% Japanese millet + 25% sorghum × sudangrass (**MIX**). Fermentors were fed equal portions of herbage 4 times daily (0730, 1030, 1400, and 1900 h). All fermentors were fed orchardgrass at 0730 and 1030 h and individual treatment herbage (orchardgrass, Japanese millet, sorghum × sudangrass, or 50:50 Japanese millet and sorghum × sudangrass) at 1400 and 1900 h. Feeding schedule was designed to mimic grazing patterns of dairy cattle, including a fresh allotment of pasture (in this case, warm-season annuals) after milking (Hoffman et al., 2000). Representative samples of freeze-dried herbage were collected from each treatment at the beginning of the study for nutrient analyses at a commercial laboratory (Dairy One Laboratories, Ithaca, NY; Table 1).

Continuous Culture System and Operation

The study was conducted as a 4 × 4 Latin square design. Treatments were incubated in a 4-unit, dual flow continuous culture fermentor system (OmniCulture Plus; VirTis), 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 1.10 to 1.14 L, urea was added to the mineral buffer solution at a rate of 0.4 g/L to simulate recycled N (Weller and Pilgrim, 1974), and fermentors were continually stirred at 255 rpm (Soder et al., 2016). Solid mean retention time, solid dilution rate, and liquid dilution rate of the fermentors were adjusted daily to approximately 24 h, 4.17%/h, and 11%/h, respectively, and was achieved by regulation of buffer input and filtrate removal (pore size = 104.14 µm; Hoover et al., 1976).

Ruminal fluid and digesta samples were collected from a ruminally fistulated, non-lactating, multiparous Holstein cow (BW = 652 kg) cared for in accordance with the Pennsylvania State University Animal Care and Use (IACUC #39513) guidelines. The donor cow was group housed and fed a diet of mixed grain and grass hay (35:65 concentrate-to-forage ratio) in a feed bunk for a total of 16.2 kg DM of available feed per cow per day at the Pennsylvania State University Dairy Research Farm (University Park, PA). A vitamin/mineral premix was included in order to meet NRC (2001) recommendations 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 and maintained at 39°C. 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 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 CO_2 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 pH was recorded manually immediately before each of the 4 daily feedings.

Fermentors were operated for 4 consecutive 10-d periods according to the methods of Soder et al. (2016). In brief, each period consisted of a 7-d adaptation period followed by a 3-d sampling period. During the adaptation period, liquid and solid overflow were collected daily, weighed, and then discarded. After being emptied on d 7 and continuing through d 10, liquid and solid overflow containers were chilled in a 4°C water bath and each received 20 mL of 50% H_2SO_4 (vol/vol) daily prior to overflow collection. During the last 3 d of each period, liquid and solid effluent overflows from each fermentor were combined and mixed. 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 and a subsample taken for VFA (Erwin et al., 1961) and NH_3-N analyses using the methods of Chaney and Marbach (1962). An additional 1.10 L sample of 3-d composited effluent was freeze-dried, ground to pass through a 1-mm sieve, and analyzed for DM, OM, NDF, and CP (AOAC, 2006), and total purines (Zinn and Owens, 1986). During the last day of each period, the contents of each fermentor were mixed, strained through 2 layers of a 53-µm Nitex fabric (Wildco, Buffalo, NY), combined with 5 mL of 50% H_2SO_4 (vol/vol), and centrifuged 3 times at 20,000 × *g* for 20 min at -4°C with the pellet re-suspended in 0.9% saline (wt/vol) and 50% methanol (vol/vol), respectively, for the last 2 centrifugations (Griswold et al., 1996). The final pellet was freeze-dried and analyses for DM, OM, CP (AOAC, 2006) and total purines (Zinn and Owens, 1986) were completed and used to calculate nutrient digestibility, bacterial protein synthesis and N metabolism (Soder et al., 2016).

Gas 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). Ten min prior to gas collection, a rubber stopper (size 1) was placed in the effluent overflow port to prevent gas escape. 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 gas chromatography (Varian CP 3800; Agilent Technologies, Santa Clara, CA) as described in Soder et al. (2012). A separate needle and syringe were designated for each fermentor. Estimates of daily CH_4 output (mmol/d) were calculated using the following equation: CH_4 concentration in fermentor headspace (mmol/L) × CO_2 gas flow through the fermentor headspace (20 mL/min) × 60 min × 24 h (Johnson et al., 2009).

Nutrient Analyses

Samples of orchardgrass, Japanese millet, and sorghum × sudangrass were analyzed by wet chemistry (Dairy One Laboratories, Ithaca, NY) according to the following procedures: DM (method 930.15; AOAC, 2006), CP (method 990.03; AOAC, 2006), RDP (Cornell *Streptomyces griseus* enzymatic digestion; Coblentz et al., 1999), NDF [Ankom model A200; Mertens (2002), with heat-stable alpha-amylase and sodium sulfite used in the NDF procedure (inclusive of ash)]. Water-soluble carbohydrates (**WSC**) were determined by incubating samples in a 40°C water bath for 1 h, extracting WSC (simple sugars and fructans). The WSC was determined using a Thermo Scientific Genesys 10S Vis Spectrophotometer after acid hydrolysis with H_2SO_4 (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 intrepid inductively coupled plasma radial spectrometer after microwave digestion; CEM Application Note for Acid Digestion, CEM, Matthews, NC), and ether extract (method 2003.05; AOAC, 2006) were also determined. The NFC concentration was calculated using the equation NFC% = 100% − (CP% + NDF% + ether extract% + ash%). Pectins were included in the NFC calculation but not included in WSC or NSC analyses.

Effluent samples were analyzed for DM and OM (methods 930.15 and 942.05, respectively; AOAC, 2006) and CP concentrations (micro-Kjeldahl digestion using 75-mL calibrated tubes with $CuSO_4/K_2SO_4$ catalyst, method 976.06; AOAC, 2006). The NDF concentration of the effluent was determined as done for herbage NDF reported above. Concentrations of total purines (Zinn and Owens, 1986) in effluent and bacterial isolates were used to partition effluent N flow into bacterial and non-bacterial fractions and to calculate true DM and OM digestibilities and flows.

Statistical Analysis

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

$$Y_{ijk} = \mu + P_i + F_j + T_k + e_{ijk}.$$

Where, Y_{ijk} = observations for dependent variables, μ = population mean, P_i = mean effect of *i*th period, F_j = mean effect of *j*th fermentor, T_k = mean effect of *k*th treatment, and e_{ijk} = residual error. Treatment was considered a fixed effect, and fermentor, period, and error were random effects.

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

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

Where, Y_{ijkl} = observations for dependent variables, μ = population mean, P_i = mean effect of *i*th period, F_j = mean effect of *j*th fermentor, T_k = mean effect of *k*th treatment, $E1_{ijk}$ = whole plot error, H_l = mean effect of *l*th hour sampled analyzed as repeated measures, HT_{lk} = interaction between *l*th hour and *k*th treatment, and $E2_{ijkl}$ = subplot residual error. Treatment, hour sampled, and their interaction were considered fixed effects, and period, fermentor, whole plot error, and subplot error, which were considered random. A first-order autoregressive covariance structure, which showed the lowest Akaike information criterion values, was retained in the final model. Least squares means were compared by least squared minimum difference. Pearson correlation coefficients between N metabolism variables and forage characteristics were conducted according to the PROC CORR procedure of SAS and stepwise linear regression analysis was conducted according to the PROC REG procedure of SAS to detect predictive statistical associations between forage characteristics and N metabolism metrics. For all statistical analyses, significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Apparent (DM, OM, NDF, and ADF) and true (DM, OM, and CP) digestibilities of nutrients were calculated according to equations from Soder et al. (2016).

RESULTS AND DISCUSSION

Treatment Composition

The chemical composition of dietary ingredients and treatments are presented in Table 1. Statistical comparison of treatments was not conducted because treatment nutrient compositions were based on pooled samples. The nutritive value of orchardgrass in the current study was similar to that reported by Hafla et al. (2016). The CP, ADF, and mineral concentrations of Japanese millet were similar to previous literature reports (Muldoon, 1985; Darby et al., 2016). Similarly, the CP, fiber fractions, and mineral concentrations of the sorghum × sudangrass were similar to previous studies (Fontaneli et al., 2001; Ketterings et al., 2005). Overall, the nutrient concentration of the treatments used in the current study was considered to be moderate quality with CP of 18% and a moderately high NDF concentration of 55 to 60% (Bargo et al., 2003). The OM, CP, and NE_L of all treatments were numerically similar. The NDF and ADF concentrations were numerically greater in ORD than the warm-season annual treatments (MIL, SSG and MIX). Concentrations of WSC and ethanol-soluble carbohydrate were numerically greater (20 and 30%, respectively) in SSG than ORD and MIL; conversely, starch was 69% numerically greater in MIL than SSG and ORD. The CP to NFC ratio was numerically greater in ORD than any other treatment and MIL had the numerically lowest ratio.

Nutrient Digestibility

There was no effect ($P \geq 0.11$) of treatment on apparent digestibilities of DM, NDF, and ADF, as well as true digestibilities of DM and OM (57.0, 80.1, 78.4, 70.4, and 76.4%, respectively; Table 2). The apparent OM digestibility was 14% and 9.7% greater in ORD than MIL ($P = 0.01$) and SSG ($P = 0.04$), respective. Apparent OM digestibility also tended ($P = 0.06$) to be greater in ORD than MIX.

Apparent and true OM digestibilities of ORD were similar to that observed by Soder et al. (2012); however, apparent and true DM digestibilities in the current study were 25% and 19% lower, respectively, and apparent NDF digestibility was 9% greater, than Soder et al. (2012). Conversely, true DM and OM digestibilities of ORD in the current study were 39 and 53% greater, respectively, than a 50% orchardgrass, 25% red clover (*Trifolium pretense* L.), and 25% alfalfa (*Medicago sativa* L.) pasture mixture (Bach et al., 1999). Other studies reported digestible DM of 48.6 to 51.6% for pure Japanese millet and sorghum × sudangrass (Muldoon, 1985; Darby et al., 2016) and were similar to the results of the current study. Apparent NDF digestibility was similar to previous studies using pure sorghum × sudangrass and Japanese millet (Ketterings et al., 2005; Darby et al., 2016). However, data on true digestibility of DM or OM for warm-season annuals are lacking in the literature.

Fermentor pH, VFA, and CH_4 Output

There was no effect ($P \geq 0.23$) of treatment on mean (of the data points collected), maximum, or minimum fermentor pH (6.76, 6.89, and 6.61, respectively; Table 3). Mean, maximum, and minimum pH for ORD was similar to previous continuous culture studies (Kolver et al., 1998; Bargo et al., 2003; Soder et al., 2012). Kolver and de Veth (2002) predicted that the mean pH for optimal pasture digestion was 6.35, lower than all treatments in the current study; however, they also reported that nutrient digestion and synthesis of microbial protein were largely insensitive to pH across a broad range of pH values (5.8 to 6.8). It must be noted that the pH (mean, minimum, maximum) result of this study are reflective of the 4 data points measured immediately prior to each feeding, which may not be representative of diurnal changes in pH.

However, these data still provide valid information about mean and fluctuations in pH as affected by treatments.

Concentrations of total VFA were greater (*P* = 0.04) in SSG than ORD (Table 3), with MIL and MIX having intermediary values. Total VFA concentrations observed in the current study were similar to values reported for cool-season perennial grasses in continuous culture (Bargo et al., 2003; Soder et al., 2012). Furthermore, no effect ($P \geq 0.40$) of treatment was observed in molar proportions of valerate or isovalerate (1.90 and 0.05 mol/100 mol, respectively). Molar proportions of acetate were greater ($P \leq 0.01$) in ORD and MIL than SSG and MIX. Conversely, butyrate was lower ($P \leq 0.01$) in ORD and MIL than SSG and MIX; likely due to the increase of NFC in SSG and MIX compared with ORD, which agrees with data reported by Stokes et al. (1991) and Bach et al. (1999). Molar proportions of propionate were lower ($P \leq 0.01$) in MIL than SSG and MIX. The acetate to propionate ratio was greater ($P \leq 0.012$) in ORD and MIL than SSG. Furthermore, the acetate plus butyrate to propionate plus valerate ratio was greater ($P \leq 0.02$) in MIL than MIX and SSG, with ORD having an intermediate value. Acetate to propionate ratios in the current study were lower than those reported for cool-season grasses in continuous culture (Bach et al., 1999; Soder et al., 2012), but in agreement with other studies (Bargo et al., 2003; Wales et al., 2004). However, due to the elevated concentrations of butyrate, acetate plus butyrate to propionate ratios were similar to previously reported values for ORD (Soder et al., 2012). Differences in VFA ratios were likely due to lower WSC and ESC in ORD and MIL than SSG and MIX (Liu et al., 2009).

Methane output, expressed as mmol of CH_4 per day, was greatest ($P \leq 0.01$) in MIL compared with all other treatments (Table 3). The CH_4 output of SSG was lower ($P < 0.03$) than MIL. The same pattern was observed when CH_4 output was expressed per gram of OM fed and per gram of NDF fed. However, when reported as CH_4 per gram of NDF fed, or digestible OM or NDF fed, there was no difference ($P \geq 0.11$) between SSG and MIX treatments. Greater CH_4 output in MIL was likely due to shifts in molar proportions of individual VFA. While formation of propionate uses reducing equivalents, acetate and butyrate formation in the rumen produce H_2 for methanogenesis (Hungate, 1966; Owens and Goetsch, 1988). The acetate to propionate ratio was greatest in MIL than the other warm-season annual treatments (SSG and MIX) as discussed previously and due to similar forage digestibility among treatments, this pattern was repeated when CH_4 was calculated per gram digestible OM and NDF fed.

There was no sampling time × treatment interaction ($P = 0.39$) for CH_4 output variables (Figure 1); however, a significant effect ($P < 0.01$) of sampling time was observed. Methane output at 1000 h was greater ($P \leq 0.02$) than 0900 and 1530 h and tended ($P = 0.08$) to be greater than 0725 h (8.5, 3.5, 5.9, 6.5 mmol of CH_4/d, respectively). Methane samples taken at 0900 h had the lowest ($P \leq 0.04$) mmol of CH_4 per day of all sampling times. There was no difference ($P \geq 0.36$) in CH_4 output at 1000, 1355, or 1630 h (8.5, 7.7, and 7.5 mmol of CH_4/d, respectively). Methane expressed based on OM, NDF, digestible OM, and digestible NDF fed showed the same diurnal fluctuation in CH_4 production as expressed on mmol of CH_4/d. Methane output (mmol/d) displayed a diurnal variation throughout the day for all treatments as shown previously (Hafla et al., 2014; Brask et al., 2015; Soder et al., 2016).

Nitrogen Metabolism

There was no effect ($P = 0.44$) of treatment on N intake (averaging 2.30 g N/d across all treatments; Table 4). Concentration of NH_3-N was 19% greater ($P < 0.01$) in ORD than MIL and MIX; SSG tended ($P = 0.08$) to be 10% lower than ORD. True CP digestibility was 10% greater

($P = 0.02$) in ORD than MIL and tended ($P = 0.07$) to be 7% lower in MIX; conversely, SSG was not different ($P \geq 0.14$) from any other treatment. Effluent total N and dietary N flows were lower ($P \leq 0.02$) in ORD than MIL (12 and 16%, respectively), but MIL, SSG, and MIX were not different ($P \geq 0.16$). There was no difference ($P \geq 0.13$) in bacterial N among treatments (0.31 g/d). Concentrations of NH_3-N in effluent were 19% greater ($P \leq 0.01$) in ORD than MIL or MIX. Effluent flow of NAN was 41% lower ($P \leq 0.02$) in ORD than all other treatments. There was no effect of treatment ($P = 0.29$) on bacterial efficiency when expressed as grams of bacterial N per kilograms of DM truly digested. Previous studies (Kolver et al., 1998; Soder et al., 2012) have reported similar values for concentrations of NH_3-N, total N, and NH_3-N effluent flows to that reported for ORD. True CP digestibility of ORD and SSG were 6 and 19% greater in the current study than previous reports, respectively (Liu et al., 2009; Soder et al., 2012). Total effluent N, effluent NAN, and bacterial effluent N were at least half of what was reported in Soder et al. (2012), and is likely due to the greater true CP digestibility of treatments in the current study.

Bacterial efficiency expressed as grams of bacterial N per kilograms of truly digested OM was 44% greater ($P \leq 0.02$) in MIL than ORD (Table 4). Bacterial N efficiency per truly digestible OM was 83% greater in the current study than that reported by Soder et al. (2012), but was 74% less than those reported by Bargo et al. (2003), both of which reported numerically greater treatment CP concentrations (19.9 and 25.3% , respectively) than the current study. Previous studies (Brito et al., 2006; Soder et al., 2012) demonstrated that ruminal NH_3-N concentrations below 5 to 8.5 mg of N/dL can depress bacterial N synthesis; however, since NH_3-N was not a limiting factor in any treatment in the current study, this likely explains the lack of biologically significant differences among treatments.

Pearson's correlation coefficient showed a positive relationship ($P \leq 0.02$; Table 5) between CP and NH_3-N, true CP digestibility, and effluent NH_3-N, but a negative relationship ($P \leq 0.03$) between CP and total effluent N, effluent NAN, effluent dietary N, and bacterial N efficiency per true OM digestibility across all treatments. Furthermore, there was no correlation ($P \geq 0.37$) between degradable or soluble protein of treatments and any N metabolism variables. Devant et al. (2000) showed that degradability of CP in the rumen affects N digestibility and retention; however, in the current study, the lack of biological differences in RDP among treatments was likely the reason why no significant correlation was observed. Additionally, for all N metabolism traits, with the exceptions of total N intake and effluent NH_3-N flow, only NDF entered the stepwise regression model ($P \leq 0.04$). Neutral detergent fiber explained between 21.4 and 58.1% of the variability among treatments for each N metabolism variable. Microbial protein degradation is largely impacted by the presence and type of carbohydrate in the feedstuff and typically results in greater N utilization with increasing amounts of readily digestible fiber (NDF) in the diet (Bach et al., 2005). In the current study, ADF of all treatments was greater than that reported by Bach et al. (2005), despite treatment NDF concentrations being similar.

CONCLUSIONS

Warm-season annuals provide a productive grazing alternative to cool-season grass pastures during the summer months. While orchardgrass had slightly greater nutrient digestibility than the warm season annuals (millet and BMR sorghum-sudangrass), the warm-season annuals showed benefits in bacterial efficiency. Results were mixed for CH_4 output with millet having the highest CH_4 output and sorghum-sudangrass (and the mix of the 2 warm-season species) having the lowest CH_4 output. It is important to note that forage quality of perennial cool-season grass pastures would likely be lower than that used in this study due to mid-summer heat and drought stress. This would make warm-season annuals even more valuable in grazing-based dairy systems as an alternative mid-summer forage. Additional research is needed to fully assess the potential of using warm-season annuals in the diets of lactating dairy cattle during the summer months to evaluate animal productivity and economics while grazing these forages.

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Table 1. Chemical composition (% of DM) of orchardgrass and orchardgrass/warm-season annual mixtures fed during continuous culture fermentation

	Ingredient			Treatment[1]			
Item	Orchard-grass	Japanese millet	Sorghum × sudangrass	ORD	MIL	SSG	MIX
OM	93.2	90.6	92.5	93.2	92.0	92.9	92.5
CP	17.9	17.8	17.8	17.9	17.9	17.9	17.9
RDP, % of CP	73.0	71.0	80.0	73.0	72.0	76.5	74.3
NDF	59.2	49.2	54.0	59.2	54.2	56.6	55.4
ADF	37.4	30.7	34.5	37.4	34.1	34.5	35.0
NFC[2]	12.0	19.9	17.5	12.0	16.0	14.8	15.4
WSC[3]	9.3	9.0	13.8	9.3	9.2	11.6	10.4
ESC[4]	6.3	6.4	11.9	6.3	6.4	9.1	7.7
CP:NFC	1.49	0.89	1.02	1.49	1.19	1.25	1.22
Starch	1.6	9.2	1.3	1.6	5.4	1.5	3.4
NSC[5]	10.9	10.1	2.3	10.9	14.6	13.1	13.8
NE_L[6], Mcal/kg	1.23	1.39	1.30	1.23	1.32	1.28	1.30
Ca	0.37	0.46	0.30	0.37	0.42	0.34	0.38
P	0.24	0.26	0.24	0.24	0.25	0.24	0.25
Mg	0.28	0.55	0.27	0.28	0.42	0.28	0.38
K	1.85	2.09	2.49	1.85	1.97	2.17	2.07
Na	0.20	0.01	0.00	0.20	0.11	0.10	0.11

[1] Calculated using actual nutrient composition and proportion of individual ingredients (DM basis); ORD = 100% orchardgrass; MIL = 50% orchardgrass + 50% Japanese millet; SSG = 50% orchardgrass + 50% sorghum × sudangrass; MIX = 50% orchardgrass + 25% Japanese millet + 25% sorghum × sudangrass.

[2] Calculated as NFC = 100 – (CP% + NDF% + ether extract% + ash%).

[3] WSC = water-soluble carbohydrate.

[4] ESC = ethanol-soluble carbohydrate.

[5] NSC = WSC + starch.

[6] Estimated by the NRC (2001) model.

Table 2. Nutrient digestibility of orchardgrass and orchardgrass/warm-season annual mixtures fed during continuous culture fermentation

	Treatment[1]				
Item	ORD	MIL	SSG	MIX	SEM
Apparent digestibility					
DM, %	56.0	55.5	57.5	58.8	3.03
OM, %	71.1^{a}	62.3^{b}	64.8^{b}	65.0^{ab}	2.36
NDF, %	84.8	75.6	76.0	83.9	3.08
ADF, %	80.2	78.4	76.5	78.5	2.39
True digestibility					
DM, %	67.8	70.6	70.2	73.1	4.10
OM, %	80.2	74.1	75.0	76.4	2.49

[1] ORD = 100% orchardgrass; MIL = 50% orchardgrass + 50% Japanese millet; SSG = 50% orchardgrass + 50% sorghum × sudangrass; MIX = 50% orchardgrass + 25% Japanese millet + 25% sorghum × sudangrass.

[a-b] Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 3. Fermentor pH, VFA concentration and molar proportion, and CH_4 output of orchardgrass and orchardgrass/warm-season annual mixtures during continuous culture fermentation

	Treatment[1]				
Item	ORD	MIL	SSG	MIX	SEM
pH					
Mean	6.80	6.76	6.74	6.75	0.037
Minimum	6.68	6.60	6.58	6.58	0.035
Maximum	6.92	6.88	6.84	6.90	0.047
Total VFA, m*M*	55.8^{a}	57.1^{ab}	59.2^{b}	57.9^{ab}	1.06
Individual VFA, mol/100 mol					
Acetate (A)	68.4^{a}	68.7^{a}	64.8^{b}	66.1^{b}	0.544
Propionate (P)	21.9^{ab}	21.1^{a}	23.2^{c}	22.8^{bc}	0.367
Butyrate (B)	7.16^{a}	7.55^{a}	9.69^{b}	8.72^{b}	0.353
Isobutyrate	0.58^{a}	0.45^{b}	0.49^{ab}	0.42^{b}	0.038
Valerate (V)	1.78	1.99	1.81	2.02	0.115
Isovalerate	0.12	0.08	0.00	0.00	0.071
A:P	3.12^{ab}	3.26^{a}	2.79^{c}	2.91^{bc}	0.074
A+B:P+V	3.19^{ab}	3.31^{a}	2.98^{b}	3.02^{b}	0.073
CH_4					
mmol of CH_4/d	7.4^{a}	10.2^{b}	3.5^{c}	5.1^{c}	1.34
mg of CH_4/g of OM fed	2.13^{a}	2.96^{b}	1.01^{c}	1.49^{c}	0.388
mg of CH_4/g of NDF fed	3.36^{a}	5.02^{b}	1.66^{c}	2.48^{ac}	0.642
mg of CH_4/g of digestible OM fed	2.48^{a}	3.67^{b}	1.25^{c}	1.80^{ac}	0.453
mg of CH_4/g of digestible NDF fed	2.35^{a}	3.60^{b}	1.24^{c}	1.64^{ac}	0.441

[1] ORD = 100% orchardgrass; MIL = 50% orchardgrass + 50% Japanese millet; SSG = 50% orchardgrass + 50% sorghum × sudangrass; MIX = 50% orchardgrass + 25% Japanese millet + 25% sorghum × sudangrass.

[a-c] Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 4. Nitrogen metabolism of orchardgrass and orchardgrass/warm-season annual mixtures during continuous culture fermentation

	Treatment[1]				
Item	ORD	MIL	SSG	MIX	SEM
N intake, g/d[2]	2.31	2.31	2.29	2.30	0.009
NH_3-N, mg/dL	19.6^{a}	15.9^{b}	17.7^{ab}	15.9^{b}	0.71
True CP digestibility, %	98.3^{a}	88.8^{b}	92.7^{ab}	91.4^{ab}	2.61
N flows, g/d					
Total N	0.91^{a}	1.03^{b}	0.97^{ab}	0.99^{ab}	0.030
NH_3-N	0.63^{a}	0.51^{b}	0.55^{ab}	0.51^{b}	0.027
NAN	0.28^{a}	0.52^{b}	0.42^{b}	0.48^{b}	0.046
Bacterial N	0.25	0.33	0.30	0.34	0.035
Dietary N	0.03^{a}	0.19^{b}	0.12^{ab}	0.15^{ab}	0.045
Bacterial efficiency					
g N/kg DM truly digested	6.23	7.85	7.00	7.58	0.596
g N/kg OM truly digested	5.95^{a}	8.57^{b}	7.44^{ab}	8.25^{ab}	0.814

[1] ORD = 100% orchardgrass; MIL = 50% orchardgrass + 50% Japanese millet; SSG = 50% orchardgrass + 50% sorghum × sudangrass; MIX = 50% orchardgrass + 25% Japanese millet + 25% sorghum × sudangrass.

[2] N intake (g/d) = dietary N (g/d) + urea-N from buffer (g/d).

[a-b] Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 5. Correlation coefficients (r) between CP and N metabolism variables of orchardgrass and orchardgrass/warm-season annual mixtures during continuous culture fermentation

Item	r	*P*-value
N intake	0.1992	0.46
NH_3-N	0.6906	< 0.01
True CP digestibility	0.5563	0.02
N flows		
Total N	-0.5608	0.02
NH_3-N	0.6680	< 0.01
NAN	-0.6900	< 0.01
Bacterial N	-0.4187	0.01
Dietary N	-0.5553	0.02
Bacterial efficiency		
N/DM truly digested	-0.4490	0.08
N/ OM truly digested	-0.5331	0.03

FIGURE CAPTIONS

Figure 1. Temporal CH_4 output of orchardgrass and orchardgrass/warm-season annual mixtures during continuous culture fermentation. All fermentors were fed orchardgrass at 0730 and 1000 h and orchardgrass (ORD), Japanese millet (MIL), sorghum × sudangrass (SSG), or a 50:50 mixture of Japanese millet and sorghum × sudangrass (MIX) were fed at 1400 and 1900 h. CH_4 output (mmol/d) = mmol of CH_4/ml × 20 ml CO_2/min × 60 min/h × 24 h/d.

Dillard, Figure 1