Running Head: Ruminal fermentation of forage brassicas

Enteric methane production and ruminal fermentation of forage brassica diets fed in continuous culture

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ABSTRACT

The aim of the current study was to determine nutrient digestibility, VFA production, N metabolism, and CH_4 production of canola (*Brassica napus* L.), rapeseed (*B. napus* L.), turnip (*B. rapa* L.), and annual ryegrass (*Lolium multiflorum* Lam.) fed with orchardgrass (*Dactylis glomerata* L.) in continuous culture. Diets were randomly assigned to fermentors in a 4 × 4 Latin square design using 7 d for adaptation and 3 d for collection. Diets were: 1) 50% orchardgrass + 50% annual ryegrass (ARG); 2) 50% orchardgrass + 50% canola (CAN); 3) 50% orchardgrass + 50% rapeseed (RAP); and 4) 50% orchardgrass + 50% turnip (TUR). Feedings (82 g DM/d) occurred 4 times daily throughout 4, 10-d periods at 730, 1030, 1400, and 1900 h. Methane samples were collected every 10 min using a photoacoustic gas analyzer (LumaSense Technologies, Inc.; Santa Clara, CA) during the last 3 d of the experiment. Effluent samples were collected on d 8, 9, and 10, composited by fermentor, and analyzed for VFA and pH as well as DM, OM, CP, and fiber fractions for determination of nutrient digestibility. Forage samples were analyzed for CP, NDF, ADF, minerals, and glucosinolate (GLS) concentrations. Data were analyzed using the GLIMMIX procedure of SAS. Apparent DM, OM, and NDF digestibilities and true DM and OM digestibilities were similar ($P > 0.28$) among diets (45.1, 63.2, 44.1, 67.1, and 87.2%, respectively). Total VFA (87.2 mol/100 mol), pH (6.47) and acetate (A: 44.6 mol/100 mol) were also not different ($P > 0.20$) among diets. The A:P (P = propionate) ratio was greater ($P < 0.01$) in ARG and CAN than RAP and TUR. Daily CH_4 production was greater ($P < 0.01$) in ARG than all other diets (68.9 vs. 11.2 mg/d). Methane, whether expressed as g per g of OM, NDF, digestible OM, or digestible NDF fed was greatest ($P < 0.01$) in ARG but similar ($P > 0.18$) among brassica diets. A significant negative correlation was observed between total GLS and CH_4 production. However, when multiple regression analysis on CH_4 production was

completed, neither total GLS nor individual GLS were a significant component of the model. Addition of brassicas provided similar nutrient digestibility to ARG while reducing daily CH_4 production, potentially making brassicas a more environmentally friendly alternative for ARG in pasture-based ruminant diets.

KEYWORDS: brassica, continuous culture, methane, ruminal fermentation

INTRODUCTION

Forage brassicas are cool-season annual forages that can be used for grazing during the summer and late fall when cool-season perennial pastures may not be as productive (Hall and Jung, 2008). Many brassica varieties exist, including vegetable, oilseed, and forage varieties; however, forage varieties have been bred to mature more quickly and produce greater above-ground biomass than their vegetable and oilseed counterparts. Brassica species include rapeseed (*Brassica napus* L.), turnip (*B. rapa* L.), kale (*B. oleracea* L.), radish (*Raphanus sativus* L.), and swede (*B. napobrassica* (L.) Mill). Forage variety trials throughout the eastern United States have shown the high biomass potential of forage brassicas (1500 – 5000 kg DM/ha; Darby et al., 2013, 2015; Simon et al., 2014). Further studies reported high CP concentrations (>20%), low NDF (20-35%) and high DM digestibility (>85%; Darby et al., 2013; Villalobos and Brummer, 2015). The combination of high biomass during periods of perennial forage shortages and high forage quality has increased producer interest in using brassicas for pasture-based ruminants with high nutrient requirements (i.e., beef stockers, finishing lambs, and lactating dairy cows).

Brassicas contain a class of sulfur-containing plant secondary metabolites called glucosinolates (GLS). The type and quantity of GLS in each brassica varies with variety, agronomic management, and climatic conditions (Gustine and Jung, 1985; Tripathi and Mishra, 2007). Ingestion of substantial amounts of GLS can have adverse effects on animal performance and health. This includes reduced DMI, ADG, I and Cu deficiencies, goiter, and kale anemia (Gustine and Jung, 1985). For this reason, nutritionists typically suggest limiting brassicas to ≤ 50% of daily DMI (Hall and Jung, 2008).

Multiple studies (Brask et al., 2013a, 2013b; Storlien et al., 2017) have shown that feeding whole rapeseed or rapeseed meal reduces enteric methane emissions in cattle by 9 – 23%. Additionally, Storlien et al. (2017) determined that rapeseed concentrate was still effective at reducing enteric CH_4 39 d after the initial addition of brassicas into the diet, suggesting that brassicas have the potential for long-term CH_4 reduction. However, to date, only 2 studies (Sun et al., 2012, 2015) determined CH_4 emissions from ruminants grazing brassicas. Therefore, the objective of the current study was to determine the effects of three forage brassicas fed in a 50:50 with orchardgrass on ruminal fermentation, N metabolism, and enteric CH_4 production in continuous culture.

MATERIALS AND METHODS

Site, Experimental Design, and Diets

On August 26, 2015, 'KB Sumpreme' annual ryegrass (*Lolium multiflorum* Lam.; King's Agriseed, Inc., Ronks, PA), 'Inspiration' canola (*Brassica napus* L.; Rubisco Seed, LLC., Philpot, KY), 'Barisca' rapeseed (*B. napus* L.; King's Agriseed), and 'Appin' turnip (*B. rapa* L.; King's Agriseed, Inc.) were planted at the Pennsylvania State University Russell E. Larson Agricultural Research Center in Rock Springs, PA. Diet herbage was drilled into a prepared seedbed using a no-till drill (HEGE 1000; Wintersteiger AG, Waldenburg, Germany). Diet forage was harvested on November 3, 2015, when forage was optimal for grazing (approximately 70d after planting; Hall and Jung, 2008). Orchardgrass (*Dactylis glomerata* L.) was harvested from a 3-yr-old pure stand on April 27, 2016. 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; Dillard et al., 2017a). A plot harvester (HEGE 212; Wintersteifger AG; 1.5-m-wide swath), set to a 10-cm stubble height was used to harvest all forage. Within 30 min of harvest, forage was placed in cloth bags and frozen (-4°C) until being freeze-dried (Ultra 35 Super ES; Virtis Co. Inc., Gardiner, NY). Freeze-dried forage was ground to pass through a 1-mm sieve (Wiley mill; Thomson Scientific Inc., Philadelphia, PA). Freeze-dried forages are not nutritionally identical to fresh forages; however, in order to be preserved and ground for use in the experiment, drying was necessary. Jones and Bailey (1972) reported that oven-drying forages could denature protein in plant material and decrease digestibility.

The continuous culture fermentor study was conducted at the USDA-Agricultural Research Service, Pasture Systems and Watershed Management Research Unit (University Park, PA) from May to July 2016. Total DM fed to all fermentors was kept constant at 82 g/d for the duration of the experiment. Diets were as follows: (1) 50% orchardgrass + 50% annual ryegrass (ARG); (2) 50% orchardgrass + 50% canola (CAN); (3) 50% orchardgrass + 50% rapeseed (RAP); and (4) 50% orchardgrass + 50% turnip (TUR). Fermentors were fed forage 4 times daily (0730, 1030, 1400, and 1900 h) in equal proportions of the diet. Representative samples of freeze-dried forage were collected from each diet at the beginning of the study and analyzed for nutrient concentrations at a commercial laboratory (Dairy One Laboratories, Ithaca, NY; Table 1).

Continuous Culture System and Operation

The experiment was designed as a 4 × 4 Latin square. Diets were fermented in a 4-unit, continuous culture fermentor system (Applikon Biotechnology, B.V., Schideam, The Netherlands) as described by Dillard et al. (2017b). Solid mean retention time and liquid dilution

122 rates were adjusted daily to 24 h and 10%/h, respectively, by regulation of buffer input and
123 effluent volume removal. Buffer input was adjusted to 20.6 ± 0.2 mL per 7.5 min and the
124 average vat volume was 1.56 ± 0.014 L.
125 A ruminally fistulated, non-pregnant, non-lactating Holstein cow (BW = 794 kg) was
126 used for ruminal fluid and digesta collections in accordance with the Pennsylvania State
127 University Animal Care and Use guidelines (IACUC #46212). The donor cow was group housed
128 and fed a diet of mixed grass hay and grain (75:25 forage-to-concentrate ratio) in a feed bunk for
129 a total of 13.8 kg DM of available feed per cow per day at the Pennsylvania State University
130 Dairy Research Farm (University Park, PA). A vitamin-mineral premix was fed at 1.8% of total
131 DMI in order to meet NRC (2001) recommendations. Approximately 3 h after feeding, 7 L of
132 ruminal fluid was collected with a hand pump into a prewarmed container and maintained at
133 39°C. Hand-grab samples of solid digesta were taken from the ventral, central, and dorsal areas
134 of the rumen. Liquid and solid digesta samples were transported to the laboratory in separate
135 containers. Within 15 min of collection, fluid was strained through 4 layers of cheesecloth and
136 1.5 L poured into each prewarmed fermentation vat. Each fermentor vat was then sealed and
137 flushed with 20 mL/min CO_2 for 1 h. After 1 h, CO_2 was reduced to 1 mL/min and vats were
138 continuously purged with 1 mL/min CO_2 for the remainder of the experimental period.
139 Temperature was maintained at 39°C. Fermentor pH and temperature were recorded every 2 min
140 using an automated sensor (Applikon Biotechnology, B.V.).
141 The experiment was operated for 4 consecutive, 10-d periods and re-inoculated with fresh
142 ruminal fluid at the beginning of each period. Each 10-d period consisted of a 7-d adaptation
143 period followed by a 3-d collection period. Effluent was collected in 4-L plastic containers
144 located inside a freezer to maintain an effluent temperature of 4°C and inhibit microbial

145 fermentation. During the adaptation period, effluent was weighed daily, volume recorded, and
146 then contents were discarded. During the collection period, daily effluent collections were mixed
147 in a blender (model 38LL52 Waring, Torrington, CT) for 30 s on the lowest setting and speed
148 controlled further with a PowerStat variable transformer (model 3PN126, Superior Electric,
149 Bristol, CT) set at 35 to create an 1-cm vortex at the top of the blender contents in order to
150 provide adequate mixing without too much abrasive agitation. After mixing, a 100-mL sample
151 was taken and composited over the collection period to determine effluent DM. A 50-mL sample
152 was strained through 8 layers of cheesecloth and a 15-mL subsample was added to 3-mL of 25%
153 (vol/vol) *m*-phosphoric acid for determination of VFA [Varian 3300 Gas Chromatograph (FID
154 detector), Varian 4290 Integrator; Rumen Fermentation Profiling Laboratory, West Virginia
155 University, Morgantown, WV; Supelco, 1975, modified to use a 80/120 Carbopack B-DA/4%
156 Carbowax 20M column; Erwin et al., 1961] and NH_3-N (Yang and Varga, 1989). The GC
157 parameters used were 2 m × 2 mm i.d. glass column packed with 80/120 Carbopack B-DA/4%
158 Carbowax 20M (catalog no. 1-1889, Supelco, Inc.), column temperature of 175°C, injector
159 temperature of 200°C, detector temperature of 200°C, N gas used as a carrier at a flow rate of 24
160 mL/min, and an injection volume of 1 µL. An additional 1.00-L sample was composited from
161 each of the 3 collection days, freeze-dried, ground to 1 mm, and stored in a sealed plastic bag at
162 ambient temperature for analyses of DM, OM, NDF, CP, and total purines. In addition to the
163 previously described sampling, on d 10 the contents of each fermentor were processed according
164 to the methods of Griswold et al. (1996), except for the initial centrifugation, when contents were
165 mixed in the above-described blender for 30 s and strained through 2 layers of 53-µm Nitex
166 fabric (Wildco, Bufflao, NY) into a 2-L plastic container with 5 mL of 50% sulfuric acid added
167 to inhibit microbial growth. Contents were centrifuged 3 times at 20,000 × *g* for 20 min at -4°C

and then the pellet was re-suspended in 0.9% saline during the first 2 centrifugations with 50% methanol during the 3rd centrifugation (Griswold et al., 1996). The final pellet was stored at -4°C until freeze-drying and analyses for DM, OM, and CP [per AOAC (2006) procedures described in Nutrient Analyses section below] and purine (Zinn and Owens, 1986). Purine data were used to calculate bacterial efficiencies using the equations described by Soder et al. (2016).

Methane Collection and Measurements

Methane measurements were taken every 10 min using a photoacoustic gas monitor (LumaSense Technologies, Inc., Santa Clara, CA) connected to a multiport sampler (CAI, Inc., Orange, CA) that directed the flow of gas from each fermentor vat. Each cycle (sampling and line flush) required 140 cm^3 of the approximately 1.50 L of headspace gas available. Total daily CH_4 production was calculated by determining the difference in CH_4 volume (measured CH_4 concentration multiplied by the headspace volume) between each of the 10-min samples and then summing these values for every 24-h period during the collection days (Dillard et al., 2017b).

Nutrient Analyses

Samples of orchardgrass, annual ryegrass, canola, rapeseed, and turnip 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), fat (method 2003.05; AOAC, 2006) and NDF [Ankom model A200; Mertens (2002) with heat stable α-amylase and sodium sulfite used in the NDF procedures (inclusive of ash)]. Water soluble carbohydrates (WSC) were determined using a Thermo Scientific Genesys 10S Vis Spectrophotometer after incubation in a 40°C water

bath for 1 h, and acid hydrolysis with H_2SO_4 (Smith, 1969). Potassium ferricyanide was used for the colorimetric reaction rather than iodide-potassium oxalate, as potassium ferricyanide proves a more stable reaction for detecting reducing sugars (Miller-Webster et al., 2002). Ethanol-soluble carbohydrates (Hall et al., 1999), starch (Application Note #319; YSI, Inc. Life Sciences, Yellow Springs, OH), minerals (Ca, P, Mg, K, Na, S; Thermo IRIS Advantage HX; CEM Application Note for Acid Digestion, CEM Corp., Matthews, NC), and ether extract (method 2003.045; AOAC, 2006) were also determined. Individual and total GLS concentrations were determined by extracting ground, freeze-dried forage in 70% methanol at 60°C for 15 min. The extracted liquid was filtered through a 0.45 μm syringe filter and analyzed using an ICS-5000+ chromatography system (Thermo-Fisher Scientific, Sunnyvale, CA) interfaced to a Q-Exactive orbitrap mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) equipped with an ultra aqueous polar end-capped analytic column (Restek Corp., Bellefonte, PA; Dillard et al., 2017c).

Samples from effluent and microbial pellets were analyzed for DM (method 930.15; AOAC, 2006), OM (method 942.05, AOAC, 2006) and CP (micro-Kjeldahl digestion using 75-mL calibrated tubes with CuSO4/K2SO4 catalyst, method 976.06; AOAC, 2006). Neutral detergent fiber of effluent was determined in the same manner as forage. Concentrations of total purines (Zinn and Owens, 1986) in bacterial isolates and effluent were used to partition effluent N flow into bacterial and nonbacterial fractions and to calculate true digestibilities and flows. Apparent (DM, OM, NDF, and ADF) and true (DM, OM, and CP) nutrient digestibilities were calculated using the equations of Soder et al. (2016).

Statistical Analysis

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

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

where Y_{ijk} = the observations for dependent variables, μ = population mean, P_i = mean effect of ith period, F_j = mean effect of jth fermentor, T_k = mean effect of kth diet, and e_{ihk} = residual error. Fermentor, period and error were considered random effects and diet was considered a fixed effect.

Temporal analyses of CH_4 concentrations were analyzed using the following model:

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

where Y_{ijk} = the observations for dependent variables, μ = population mean, P_i = mean effect of ith period, F_j = mean effect of jth fermentor, T_k = mean effect of kth diet, $E1_{ijk}$ = whole-plot error, H_l = mean effect of lth hour of day analyzed as repeated measures, HT_{lk} = interaction between lth hour of day and kth diet, and $E2_{ijkl}$ = subplot residual error. Diet was considered a fixed effect and all other parameters were considered to be random. All reported values are least squares means and were compared by least squares minimum difference. Pearson correlation coefficients between dependent variables and forage characteristics were conducted using PROC CORR of SAS. Stepwise linear regression was conducted using PROC REG of SAS to detect predictive statistical associations between forage characteristics and dependent variables. Statistical significant was declared at $P \leq 0.05$, and tendencies at $0.05 < P \leq 0.10$ for all analyses. There were no period or period × diet interactions; therefore, only main effects are reported.

RESULTS AND DISCUSSION

Diet Composition and Nutrient Digestibility

The chemical composition of dietary ingredients and diets are reported in Table 1 and GLS concentration in Table 2. The use of composite sampling for nutrient and GLS analyses precluded the ability for statistical comparison among diets. The orchardgrass used in the current study was of higher quality (greater CP and lower NDF and ADF) than that reported in previous studies (Hafla et al., 2016; Dillard et al., 2017a) and is of very high quality based on the description of Cherney and Allen (1995; 18- 24% DM, 18 – 25% CP, 40 – 50% NDF, and 1.53 – 1.67 Mcal/kg NE_L). Among the brassicas, all quality parameters were numerically similar, while ARG had numerically greater CP and fiber fractions than the brassica forages. All forage used in the current study had numerically higher CP (> 23%) and lower NDF and ADF (16.1 – 29.7% and 10.8 – 21.2%, respectively) compared to typical cool-season perennial forages (National Academies of Sciences, Engineering, and Medicine, 2016), and contained 1.61 to 1.98 Mcal/kg NE_L. This resulted in all 4 diets being of extremely high quality (> 28% CP, < 36% NDF, and < 22% ADF) and of higher quality than the 3-yr average of 14 pasture-based dairy farms throughout the northeastern United States (19.5, 51.0, and 31.4% CP, NDF, and ADF, respectively; Hafla et al. 2016). All diets used in the current study were of sufficient quality to meet the CP and energy requirements of a mid-lactation dairy cow (680 kg BW, 45 kg milk/d; NRC, 2001) or a yearling stocker (300 kg BW, 1.35 kg ADG; National Academies of Sciences, Engineering, and Medicine, 2016). However, due to their low NDF concentrations, these diets may not have sufficient fiber to optimize nutrient utilization due to rapid passage rate (van Soest, 1994).

As expected, no GLS were observed in either orchardgrass or annual ryegrass (Table 2). The turnip diet had, numerically, the greatest concentration of GLS of all diets; both CAN and

RAP also contained GLS, but in lesser quantities. Among all diets containing GLS, glucobrassicanapin, gluconapin, gluconasturtiin, and progoitrin represented the largest fraction of individual GLS. Sinigrin, glucoeruian, glucoraphanin, and glucoraphenin were also observed in diets, but in lesser amounts. However, sinalbin, glucohlearin, glucoiberin, and glucoiberverin were not observed in any of the forage samples. While GLS concentrations vary considerably with species, variety, and growing conditions (Gustine and Jung, 1985), both total and individual GLS present in all brassicas tested were within the range of those reported in the literature (Font et al., 2005; Cartea and Velasco, 2008; Velasco et al., 2008).

There were no differences ($P > 0.28$) in apparent DM, OM, and NDF digestibilities or true DM and OM digestibilities among diets (Table 3). Apparent ADF digestibility was greatest ($P \leq 0.04$) in CAN, while no differences ($P > 0.27$) were observed among the other diets. Both Cassida et al. (1994) and Lambert et al. (1987) reported greater apparent DM, OM, and NDF digestibilities for diets containing 40 to 52% brassica and 60 to 48% mixed grass hay. The apparent ADF digestibilities of ARG, RAP, and TUR were similar to that reported by Cassida et al. (1994) and Lambert et al. (1987), but the CAN diet used in the current study was greater than that reported in either study.

Fermentor pH, VFA, and CH_4 Production

There were no differences ($P > 0.11$) in mean, minimum, and maximum pH among diets (Table 4). Lower fiber diets tend to have higher rates of digestion and acid production, thereby decreasing ruminal pH (van Soest, 1994); however, lack of differences in pH suggest that fiber differences between the diets were not great enough to exceed the buffering capacity of the system. Keogh et al. (2009) reported that pregnant, non-lactating dairy cows fed 60% forage kale

had a mean ruminal pH of 6.32, similar to the mean pH of the brassica diets in the current study. However, ruminal pH of lambs fed 100% forage rapeseed was lower than that of lambs fed 100% perennial ryegrass (6.02 and 6.71, respectively; Sun et al., 2015), likely due to less effective fiber in the 100% rapeseed diet.

Neither total VFA nor acetate (A) concentrations were different ($P > 0.34$) among diets (Table 4). Propionate (P) was greater ($P \leq 0.05$) in ARG than CAN or RAP, and butyrate (B) was greater ($P = 0.03$) in RAP than ARG. Valerate (V) was greater ($P = 0.011$) in RAP than ARG. Isobutyrate and isovalerate concentrations were greater ($P < 0.01$) in ARG than the brassica diets. All VFA ratios (A:P and A+B:P+V) were greater ($P < 0.01$) in RAP than ARG, with CAN and TUR having median values. The lower A:P ratio observed in ARG was the result of a greater proportion of propionate in ARG at the expense of butyrate. Sun et al. (2012) reported that total VFA concentrations after feeding were greater in rapeseed and turnip than perennial ryegrass (97.0 and 74.2 mol/100 mol, respectively). Keogh et al. (2009) found that the A:P ratio of pregnant, non-lactating dairy cows consuming 60% kale and 40% perennial ryegrass (*Lolium perenne* L.) silage was not different than cows consuming 100% perennial ryegrass silage. However, total VFA concentrations were greater in the 60% kale diet than the 100% perennial ryegrass diet.

Methane production (mg/d) was 84% greater ($P < 0.01$) in ARG than the brassica diets, while no differences ($P > 0.28$) were observed among brassicas (Table 4). Furthermore, CH_4 per gram of OM fed, per gram of NDF fed, per gram of digestible OM fed, and per gram of digestible NDF fed also followed a similar pattern with no differences ($P > 0.18$) observed among brassicas, but all brassicas being lower ($P < 0.01$) than ARG. A significant ($P < 0.01$) diet × hour of day interaction was observed, such that a diurnal pattern in CH_4 production was

observed in ARG (Figure 1). However, there was no difference ($P > 0.05$) in hourly CH_4 production throughout the day. This response was due to CH_4 production of each of the brassica diets being at least six times lower than the ARG diet resulting in a SEM (0.51 mg CH_4/h) that was similar to the hourly mean of the brassica diets. Sun et al. (2012) reported that gram of CH_4 per kilogram of DMI was 25% lower in lambs fed forage rapeseed than perennial ryegrass. However, the authors reported no differences in gram of CH_4 per kilogram of DMI between perennial ryegrass and turnip, disagreeing with the current study. These differences are due to the reduced DMI observed in the lambs fed turnip compared to those fed perennial ryegrass, while DMI was similar for lambs consuming rapeseed and perennial ryegrass (Sun et al., 2012). In a separate study, Sun et al. (2015) reported a 22% reduction in CH_4 production in lambs fed forage rapeseed compared with perennial ryegrass for 15 weeks. Methane reduction in the current study was greater than that reported in previous studies; however, this could be attributed to inherent differences between *in vivo* and *in vitro* studies (Hristov et al., 2012).

In the current study, differences in CH_4 production did not follow typical patterns for VFA ratios. This was also observed by Sun et al. (2012) who reported no correlation between CH_4 yield as g CH_4/kg DMI and total and individual VFA. This suggests that GLS directly affect methanogens or protozoa populations, but do not alter gram-positive bacteria populations. Ohene-Adjei et al. (2008) indicated that phylogenetic analysis of ruminal fluid from sheep fed a barley-based diet and supplemented with garlic oil (contains organosulphur compounds similar to GLS) inhibited *Methanobrevibacter ruminantium* when compared to non-supplemented sheep. Furthermore, Patra et al. (2010) reported no significant changes in protozoal populations in sheep supplemented with garlic oil compared to non-supplemented sheep. The authors then concluded that organosulphur compounds inhibited methanogenic arachaea without affecting other

328 microorganisms found in the rumen. Busquet et al. (2005) proposed that reduction in
329 methanogens was due to inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)
330 reductase, thus affecting the unique membrane lipids that contain ether linked glycerols and
331 long-chain isoprenoid alcohols, which are not present in other microorganisms found in the
332 rumen (De Rosa et al., 1986).
333 Correlation analyses between daily CH_4 production and the individual GLS that were
334 present in the diets revealed a significant ($P < 0.045$) negative relationship between daily CH_4
335 production and glucobrassicanapin, sinigrin, glucobrassicin, gluconapin, glucoraphanin,
336 glucoraphenin, progoitrin, and total GLS concentration (Table 5). Gluconasturtiin tended ($P =$
337 0.076) to be negatively correlated; however, glucoerucin was not significantly correlated ($P =$
338 0.207) with daily CH_4 production. Step-wise multiple regression analysis between CH_4
339 production and nutritive quality parameters, total GLS, and individual GLS concentrations
340 showed that NDF explained 75% of the variation in daily CH_4 production and CH_4 production
341 per OM fed, 73% of the variation in CH_4 per digestible OM fed, and 85% of the variation in CH_4
342 per digestible NDF fed (Table 6). Furthermore, when methane was expressed per gram of NDF
343 fed, NFC was the only independent variable to enter the model and explained 73% of the
344 variation in CH_4 production. Therefore, even though a significant negative correlation was
345 observed between CH_4 production and total GLS concentration, NDF still plays the most
346 important role in determining CH_4 production from forage-based diets. Previous studies (Brask
347 et al., 2013b; Storlien et al., 2017) showed that addition of rapeseed cake to dairy cows on
348 pasture or consuming a TMR also significantly reduced CH_4 per kg of concentrate and CH_4 as %
349 of GE intake, respectively. These studies were able to more closely control NDF and fat
350 concentrations of the diets, allowing the researchers to remove these confounding factors. In

order to better determine the direct effect of GLS on CH_4 production when grazing forage brassicas on pasture, diets will need to be balanced for fiber and NFC components to prevent the effects of NDF masking any possible effects of GLS on CH_4 production.

Nitrogen Metabolism

Dietary N intake was greatest ($P < 0.01$) in CAN and TUR and least ($P < 0.01$) in RAP (Table 7). However, Luo et al. (2015) found that N intake was 10% greater in lambs fed forage rapeseed than perennial ryegrass. Diet selection by lambs in the Luo et al. (2015) study may have impacted total CP intake between diets, whereas this effect would have been lacking in the current *in vitro* study. Effluent NH_3-N concentration was 26% greater in ARG than the brassica diets and the same pattern was observed in NH_3-N flows. Kaur et al. (2010) found that daily ruminal NH_3-N concentrations were not different in lambs consuming 10, 25, or 40% forage rapeseed with a corn silage-based TMR (22.6 ± 1.4 mg/dL). Furthermore, the authors reported no differences in N intake among diets. In the current study, true CP digestibility was 25% greater ($P < 0.01$) in ARG and CAN than RAP and TUR. Sun et al. (2012) reported that the apparent CP digestibility of forage rapeseed and turnip in lambs was greater than perennial ryegrass (30 and 17%, respectively). Furthermore, Cassida et al. (1994) reported that apparent CP digestibility increased linearly with increasing amounts of tyfon (*B. campestrics* var *rapa* L. × *B. pekinensis* [Lour.] Rupr.) in lambs fed a diet of mixed-grass hay and tyfon. Total N flow was greater ($P = 0.04$) in ARG than CAN in the current study, while RAP and TUR had intermediate values. However, no difference ($P > 0.43$) was observed in NAN flows among all diets.

Bacterial N flows were 47% greater ($P \leq 0.01$) in ARG and CAN than RAP and TUR, while dietary N flows were 75% greater ($P < 0.01$) in RAP and TUR than ARG and CAN (Table

7). Furthermore, bacterial efficiency, measured as grams of N per kilogram of truly digestible DM and OM, was greater ($P \leq 0.01$) in ARG and CAN than RAP and TUR. Busquet et al. (2005) found no differences in dietary N flow, bacterial N flow, or bacterial efficiency per OM truly digested in a continuous culture system fed a 50:50 forage:concentrate diet and supplemented with or without garlic oil. In the current study, the greater bacterial efficiency observed in CAN compared to RAP and TUR could be a result of the greater forage starch concentration or apparent ADF digestibility of CAN.

CONCLUSIONS

Forage brassicas can provide a suitable alternative to cool-season annual grass pastures such as ARG during the fall months. In addition to being high in nutritive quality, all three brassica diets (CAN, RAP, and TUR) also significantly lowered CH_4 production compared to ARG. Furthermore, CAN provided greater ruminal bacterial efficiency than the other brassicas, making it the superior variety in this study. While other studies have established a link between organosulphur secondary plant metabolites and CH_4 reduction, in the current study we were unable to establish a causal relationship between individual or total GLS and CH_4 production even though a significant CH_4 reduction occurred. Use of brassicas in a ruminant grazing system could extend the fall grazing season and reduce winter feed costs while increasing animal efficiency and decreasing greenhouse gas emissions. However, more research is needed on the long-term effects of grazing brassicas to determine if these effects can be observed in pasture-based ruminants.

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Table 1. Chemical composition (% of DM) of individual forages and diets fed during continuous culture fermentation

	Ingredient					Diet[1]			
Item	Annual Ryegrass	Canola	Rapeseed	Turnip	Orchard-grass	ARG	CAN	RAP	TUR
OM	88.1	89.3	88.6	85.8	90.2	89.2	89.7	89.4	88.0
CP	30.2	28.2	23.2	22.2	34.0	32.1	31.1	28.6	28.1
RDP, % of CP	83	87	89	87	80	82	84	85	84
NDF	29.7	16.1	16.6	17.2	41.2	35.5	28.7	28.9	29.2
ADF	21.2	10.8	11.8	12.0	22.8	21.2	16.8	17.3	17.4
Lignin	5.3	0.8	1.3	1.3	2.5	3.9	1.7	1.9	1.9
NFC[2]	21.9	39.2	44.3	42.1	10.5	16.2	24.9	27.4	26.3
WSC[3]	21.6	31.4	34.1	33.2	11.9	16.8	21.7	23.0	22.6
ESC[4]	19.6	24.7	24.6	26.9	7.9	13.8	16.3	16.3	17.4
Starch	0.2	0.5	1.7	0.2	0.3	0.3	0.4	1.0	0.3
Ether extract	6.2	5.7	4.5	4.3	4.5	5.4	5.1	4.5	4.4
NE_M[5], Mcal/kg	1.63	1.96	1.83	1.74	1.61	1.62	1.79	1.72	1.68
NE_G[5], Mcal/kg	1.03	1.30	1.21	1.12	0.99	1.01	1.15	1.10	1.06
NE_L[5], Mcal/kg	1.68	1.98	1.87	1.79	1.61	1.65	1.81	1.74	1.70
Ca	0.64	1.78	1.98	2.47	0.42	0.53	1.10	1.20	1.45
P	0.28	0.40	0.36	0.35	0.43	0.36	0.42	0.40	0.39
Mg	0.18	0.25	0.22	0.24	0.28	0.23	0.42	0.25	0.26
K	3.62	2.63	2.64	3.44	3.03	3.33	2.83	2.84	3.24
Na	0.04	0.08	0.10	0.06	0.19	0.11	0.13	0.14	0.12
S	0.40	0.72	0.73	0.88	0.43	0.42	0.58	0.58	0.66
Cu, ppm	7	4	4	5	9	8	7	7	7

[1]Diets calculated using actual nutrient composition and proportion of individual forages (DM basis); ARG = 50% orchardgrass + 50% annual ryegrass; CAN = 50% orchardgrass + 50% canola; RAP = 50% orchardgrass + 50% rapeseed; TUR = 50% orchardgrass + 50% turnip.

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

[3]WSC = water-soluble carbohydrate.

[4]ESC = ethanol-soluble carbohydrate.

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

Table 2. Total and individual glucosinolate concentrations (mg/g DM) of individual forages and diets fed during continuous culture fermentation

	Ingredient					Diet[1]			
Glucosinolate[2]	Annual Ryegrass	Canola	Rapeseed	Turnip	Orchard-grass	ARG	CAN	RAP	TUR
Glucobrassicanapin	0.00	5.72	5.13	17.29	0.00	0.00	2.86	2.57	8.65
Progoitrin	0.00	3.04	9.66	15.26	0.00	0.00	1.52	4.83	7.63
Gluconapin	0.00	1.00	1.42	4.15	0.00	0.00	0.50	0.71	2.08
Glucobrassicin	0.00	0.95	1.25	1.96	0.00	0.00	0.48	0.63	0.98
Gluconasturtiin	0.00	0.68	1.16	3.95	0.00	0.00	0.34	0.58	1.98
Glucoraphanin	0.00	0.16	0.63	0.42	0.00	0.00	0.08	0.32	0.21
Glucoerucin	0.00	0.01	0.05	0.41	0.00	0.00	0.01	0.03	0.21
Sinigrin	0.00	0.08	0.23	0.31	0.00	0.00	0.04	0.12	0.16
Glucoraphenin	0.00	0.04	0.04	0.05	0.00	0.00	0.02	0.02	0.03
Total	0.00	11.68	19.51	43.80	0.00	0.00	5.84	9.76	21.90

[1]Diets calculated using actual nutrient composition and proportion of individual forages (DM basis); ARG = 50% orchardgrass + 50% annual ryegrass; CAN = 50% orchardgrass + 50% canola; RAP = 50% orchardgrass + 50% rapeseed; TUR = 50% orchardgrass + 50% turnip.

[2]No detectable levels of gluoiberin, glucoiberverin, gluochlearin, or sinalbin were found in any of the forages or diets tested.

587 **Table 3.** Nutrient digestibility of annual ryegrass, canola, rapeseed, and turnip fed with
588 orchardgrass during continuous culture fermentation
589

	Diet[1]				
Item	ARG	CAN	RAP	TUR	SEM
Apparent Digestibility					
DM, %	44.5	44.7	45.3	46.0	2.77
OM, %	61.6	62.5	63.6	65.0	2.84
NDF, %	38.1	52.8	40.9	44.5	5.57
ADF, %	52.1[a]	64.0[b]	48.8[a]	53.9[a]	3.15
True Digestibility					
DM, %	70.0	66.4	69.5	62.4	2.86
OM, %	89.7	86.4	90.1	82.6	3.13

590 [1]ARG = 50% orchardgrass + 50% annual ryegrass; CAN = 50% orchardgrass + 50% canola;
591 RAP = 50% orchardgrass + 50% rapeseed; TUR = 50% orchardgrass + 50% turnip.
592 [a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 4. Fermentor pH, VFA concentration and molar proportion, and CH_4 output of annual ryegrass, canola, rapeseed, and turnip fed with orchardgrass during continuous culture fermentation

	Diet[1]				
Item	ARG	CAN	RAP	TUR	SEM
pH					
Mean	6.61	6.29	6.41	6.57	0.142
Minimum	6.40[a]	6.08[b]	6.14[a,b]	6.16[a,b]	0.078
Maximum	6.95	6.73	7.04	6.69	0.201
Total VFA, m*M*	90.2	84.8	88.9	84.9	4.68
Individual VFA, mol/100 mol					
Acetate (A)	48.10	42.30	45.14	42.72	2.353
Propionate (P)	25.82[a]	22.28[b]	21.80[b]	23.66[a,b]	1.092
Butyrate (B)	11.44[a]	15.53[a,b]	16.80[b]	14.21[a,b]	1.478
Isobutyrate	0.84[a]	0.39[b]	0.42[b]	0.43[b]	0.044
Valerate (V)	3.22[a]	3.98[a,b]	4.37[b]	3.55[a,b]	0.256
Isovalerate	0.74[a]	0.30[b]	0.33[b]	0.32[b]	0.047
A:P	1.86[a]	1.90[a]	2.08[b]	1.80[a]	0.049
A+B:P+V	2.05[a]	2.20[a,b]	2.36[b]	2.09[a]	0.057
CH_4					
mg CH_4/d	68.9[a]	13.1[b]	7.4[b]	13.1[b]	5.77
mg of CH_4/g of OM fed	0.94[a]	0.18[b]	0.18[b]	0.10[b]	0.079
mg of CH_4/g of NDF fed	2.37[a]	0.56[b]	0.31[b]	0.55[b]	0.204
mg of CH_4/g of digestible OM fed	2.22[a]	0.30[b]	0.22[b]	0.37[b]	0.139
mg of CH_4/g of digestible NDF fed	1.37[a]	0.25[b]	0.14[b]	0.24[b]	0.119

[1]ARG = 50% orchardgrass + 50% annual ryegrass; CAN = 50% orchardgrass + 50% canola; RAP = 50% orchardgrass + 50% rapeseed; TUR = 50% orchardgrass + 50% turnip.

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

Table 5. Correlation coefficients (r) between individual and total glucosinolate concentrations (mg/g DM) and daily CH_4 production (mg/d) of fermentors fed annual ryegrass, canola, rapeseed, or turnip with orchardgrass

Glucosinolate[1]	r	*P*-value
Glucobrassicanapin	-0.523	0.038
Sinigrin	-0.643	0.007
Glucobrassicin	-0.732	0.001
Glucoerucin	-0.333	0.207
Gluconapin	-0.509	0.044
Gluconasturtiin	-0.456	0.076
Glucoraphanin	-0.670	0.005
Glucoraphenin	-0.787	< 0.001
Progoitrin	-0.593	0.015
Total	-0.567	0.022

[1]No correlation was tested between daily CH_4 production and sinalbin, glucohlearin, glucoiberin, or glucoibeverin due to lack of measurable concentrations in any forage analyzed.

Table 6. Multiple regression of nutritive quality parameters and glucosinolate concentrations on daily CH_4 production, CH_4 production per OM fed, CH_4 production per NDF fed, CH_4 production per digestible OM fed, and CH_4 production per digestible NDF fed of fermentors fed annual ryegrass, canola, rapeseed, and turnip with orchardgrass

	Independent Variable	Partial r^2	*P*-value
Daily CH_4 production, mg/d	NDF	0.75	< 0.0001
CH_4 production per OM fed, mg/g	NDF	0.75	< 0.0001
CH_4 production per NDF fed, mg/g	NFC	0.73	< 0.0001
CH_4 production per digestible OM fed, mg/g	NDF	0.73	< 0.0001
CH_4 production per digestible NDF fed, mg/g	NDF	0.85	< 0.0001

610 **Table 7**. Nitrogen metabolism of annual ryegrass, canola, rapeseed, and turnip fed
611 withorchardgrass during continuous culture fermentation

	Diet[1]				
Item	ARG	CAN	RAP	TUR	SEM
N intake, g/d[2]	4.33[b]	4.83[a]	4.27[c]	4.83[a]	0.004
NH_3-N, mg/dL	28.4[a]	21.1[b]	22.2[b]	20.1[b]	0.91
True CP digestibility, %	94.3[a]	91.0[a]	69.6[b]	70.4[b]	2.84
N flows, g/d					
Total N	2.95[a]	2.67[b]	2.70[ab]	2.70[ab]	0.100
NH_3-N	1.14[a]	0.85[b]	0.90[b]	0.80[b]	0.038
NAN	1.80	1.83	1.80	1.90	0.092
Bacterial N	1.60[a]	1.46[a]	0.73[b]	0.89[b]	0.159
Dietary N	0.20[a]	0.37[a]	1.07[b]	1.21[b]	0.115
Bacterial efficiency					
g N/kg DM truly digested	29.9[a]	27.5[a]	16.6[b]	15.4[b]	2.63
g N/kg OM truly digested	27.5[a]	24.7[a]	14.4[b]	13.2[b]	2.40

612 [1]ARG = 50% orchardgrass + 50% annual ryegrass; CAN = 50% orchardgrass + 50% canola;
613 RAP = 50% orchardgrass + 50% rapeseed; TUR = 50% orchardgrass + 50% turnip.
614 [2]N intake (g/d) = dietary N (g/d) + urea-N from buffer (g/d).
615 [a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

Figure Captions

Figure 1. Temporal CH_4 production of orchardgrass fed with annual ryegrass (ARG), canola (CAN), rapeseed (RAP), and turnip (TUR) during continuous culture fermentation. Error bars indicate standard errors. Vertical arrows indicate times of feeding.

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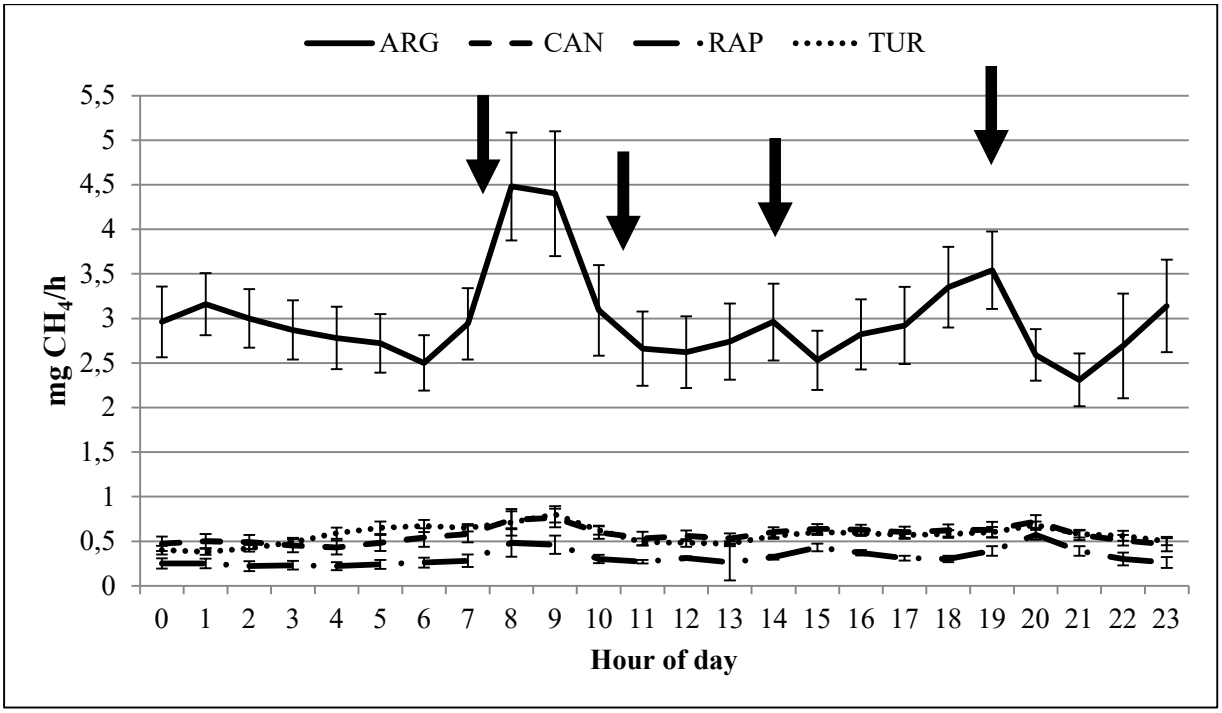
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Hour of day

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