Evaluation of a single-flow continuous culture fermentor system for determination of ruminal fermentation and enteric methane production

Continuous culture method evaluation

S. Leanne Dillard[1,2], Ana I. Roca-Fernández[1,3], Melissa D. Rubano[1], and Kathy J. Soder[1,4]

[1]USDA-Agricultural Research Service, Pasture Systems and Watershed Management Research Unit, University Park, PA 16802-3702

[2]Current Address: Dept. of Animal Sciences and Dept. of Crop, Soil, and Environmental Sciences, Auburn University, Auburn, AL 36849

[3] Depto. Producción Vegetal, Escuela Politécnica Superior, Universidad de Santiago de Compostela, Lugo, España 27002

[4]Corresponding author

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ABSTRACT

A 4-unit, single-flow continuous culture fermentor system was developed to assess *in vitro* nutrient digestibility, volatile fatty acid (VFA) concentration, and daily enteric methane (CH_4) production of ruminant diets. The objective was to develop a closed-vessel system that maintained protozoal populations and provided accurate predictions of total CH_4 production *in situ*. A diet of 50% orchardgrass (*Dactylis glomerata* L.) and 50% alfalfa (*Medicago sativa* L.) was fed during 4, 10-d periods (7-d adaptation and 3-d collection). Fermentors were fed 82 g of dry matter (DM)/d in 4 equal feedings. pH and temperature were taken every 2 min and CH_4 concentration was measured every 10 min. Samples for DM and protozoal counts were taken daily from vessels and daily effluent samples were collected for determination of DM, VFA, and NH_3-N concentrations. There was no effect ($P > 0.17$) of adaptation vs. collection days on vessel and effluent DM, temperature, or pH. Initial protozoal counts decreased ($P < 0.01$), but recovered to initial counts by the collection period. Total VFA, acetate, propionate, and isobutyrate concentrations did not differ ($P \geq 0.13$) among periods or days of the collection period. There was no difference ($P \geq 0.37$) among days or periods in total daily CH_4 production and CH_4 production per g of OM, NDF, digestible OM, or digestible NDF fed. Data collected throughout 4 experimental periods demonstrated that the system was able to reach a steady-state in fermentation well within the 7-d adaptation period and even typically variable data (i.e., CH_4 production) was stable within and across periods. This continuous culture fermentor system provides a valid comparison of *in vitro* ruminal fermentation and enteric CH_4 production of ruminant diets that can then be further validated with *in vivo* studies.

Keywords: continuous culture, digestibility, methane, protozoa, ruminal fermentation

INTRODUCTION

Researchers are increasingly interested in decreasing enteric greenhouse gas emissions through alterations in ruminant diets. Due to the ever increasing cost of animal research, *in vitro* techniques such as the continuous culture fermentation (CCF) system are considered useful tools that allow screening of a large number of treatments in an efficient manner, determination of optimal feed additive levels, and the ability for prolonged experiments at a lower cost compared to *in vivo* trials (Hristov, Leed, Hristova, Huhtanen, & Firkins, 2012). Some concerns about the extensive use of CCF systems are based on the large variability among CCF trials and between CCF and *in vivo* trials (Hristov et al., 2012). A disadvantage of using CCF systems is that they only simulate ruminal fermentation, not the entire ruminant digestive tract (Storm, Hellwing, Nielsen, & Madsen, 2012). Furthermore, the design and operation of the CCF system can alter the microbial community structure, and therefore fermentation end products might differ from *in vivo* conditions (Hristov et al., 2012). This is especially important when determining enteric methane (CH_4) production due to the close relationship between methanogenic arachaea and protozoa (Firkins &Yu, 2015).

While CCF systems are not able to fully model the complex *in vivo* ruminal fermentation or total tract digestion processes, they are useful tools that can be used to screen relative differences in diets on a small scale at lower expense, prior to testing in animal-level experiments. Many different artificial rumens have been described, including dual-flow (Hoover, Crooker, & Sniffen, 1976), single-flow (Miettinen & Setälä, 1989), and the semi-continuous-flow rumen simulation technique (Rusitec) system (Czerkawski & Breckenridge, 1977). These systems all differ in dilution rate, solids retention time, amount fed daily, frequency of feeding, and incubation period, all of which may have significant effects on fermentation parameters (Carro, Ranilla, Martín-Garíca, & Molina-Alcaide, 2009) and microbial dynamics. The system described herein is modeled after the dual-flow CCF system originally described by Hoover et al. (1976); however, liquid and solid flows were integrated in the current CCF system, resulting in a single-flow CCF system. The system of Hoover et al. (1976) has been used successfully for many years; however, in its original form, it is not capable of maintaining protozoal populations due to overflow rates that exceed the replication rate of protozoa, resulting in flushing of protozoa during effluent overflow (Teather & Sauer, 1988). Furthermore, determination of greenhouse gas output, such as CH_4, is difficult because traditional single- and dual-flow CCF systems are not closed systems. Others (Teather & Sauer, 1988; Muetzel, Lawrence, Hoffamn, & Becker, 2009; Karnati, Yu, & Firkins, 2009) have been able to modify the original system described by Hoover et al. (1976) in an attempt to overcome these shortfalls. However, these systems become labor intensive due to need to change protozoa filters to prevent clogging or the need to manually collect gas samples which do not capture true variations in diurnal patterns.

A system that is both able to maintain protozoal populations similar to *in vivo* conditions and operate as a closed system allowing for CH_4 production data with minimal labor and fermentor disturbance has not yet been developed. Therefore, the objective of the current study was to develop and test a single-flow, closed CCF system that: 1) achieves a stable fermentation environment for at least 10 d; 2) limits flushing of protozoa to maintain protozoal populations similar to those in the in vivo rumen; 3) allows for natural stratification within the vessel to more precisely simulate *in vivo* conditions; and 4) maintains a closed system that would allow for measurement of total daily production of enteric CH_4.

MATERIALS AND METHODS

Continuous culture fermentor system

The fermentor system consisted of 4, 3.0-L closed glass vessels and accessory equipment for stirring, buffer input, temperature regulation, and pH and temperature logging (Figures 1 and 2). Each glass vessel (130 mm diameter × 250 mm height) was fitted with an electric heating blanket (115 VAC, heating blanket module, Applikon Biotechnology, B.V., Schideam, The Netherlands) and a temperature probe (Pt 100 temperature sensor, Applikon Biotechnology, B.V.) connected to a control unit (ezControl; Applikon Biotechnology, B.V.) for automatic control of vessel temperature. The pH probe (pH+, L=235mm pH sensor, Applikon Biotechnology, B.V.) was also connected to the control unit, and temperature and pH data were logged every 2 min during the experiment. Both the pH and temperature probes were located 6.4 cm from the bottom of each vessel, within the liquid layer. pH was monitored because it was not regulated during each 10-d period. Each vessel contained a stirrer assembly (magnetically coupled stirrer assembly, 3-L, Applikon Biotechnology, B.V.) with 2, 6-bladed turbine spinners (Rushton impeller, 45 mm diameter, Applikon Biotechnology, B.V.), located 5 and 9 cm from the bottom of the vessel and approximately 3 cm from the surface of the vessel contents. Stirrer speed was automatically controlled by each control unit. The control units also controlled CO_2 and buffer inputs via an internal regulator (mass flow controller assembly, 0.8 – 100 mL/min, Applikon Biotechnology, B.V.) and an externally mounted pump (standard, fixed-speed pump drive and assembly, 20 rpm, Applikon Biotechnology, B.V.), respectively. Each fermentor was fitted with a pressure relief valve (Applikon Biotechnology, B.V.) set to 50 kPa above atmospheric pressure. All probes and fittings on the vessel lid were sealed using 2 silicone gaskets to prevent any gas leakage from occurring. The lid of each fermentor had a 2-cm feeding port that was used for daily feedings.

Effluent was removed using tubing (Masterflex L/S-18, Tygon; Cole-Palmer Instrument Company, LLC., Vernon Hills, IL) placed 4 cm from the bottom of the vessel which aided in the maintenance of protozoal populations by reducing flushing of the protozoa out of the vessel (Teather & Sauer, 1988). To prevent interference with the spinners, tubing was inserted into a 22.9-cm long, 3.8-cm diameter polyvinyl chloride pipe. The space between the tubing and the pipe and the pipe and the vessel lid were sealed with Parafilm M® (Bemis Company, Inc., Neenah, WI) to prevent any gas escape. Furthermore, leak tests were conducted on d 7 using soapy water were used to verify the system was correctly sealed prior to CH_4 measurement. A peristaltic pump (Masterflex Model 7518-10; Cole-Palmer Instrument Company, LLC.) was used to pump effluent from the vessel into a 4-L, Nalgene bottle located in a 0.03-m^3 freezer (Whynter, LLC., Santa Fe Springs, CA). The door of the freezer was modified so the tubing from the vessels could be placed directly into the collection bottles. Throughout the day, the collected effluent was maintained between 0 and 4°C.

Herbage treatment

Total dry matter (DM) fed to all fermentors was maintained at a constant 82 g/d for the duration of each period. The feed amount was increased proportionally (36%) based on the increase in fermentor volume (1.1 to 1.5 L) from the previous studies by Dillard, Hafla, Roca-Fernández, Brito Rubano & Soder (2017), Soder, Brito, Rubano, & Dell (2012) and Soder, Brito, Hafla, & Rubano (2016). The fermentation substrate consisted of 50% orchardgrass (*Dactylis glomerata* L.) + 50% alfalfa (*Medicago sativa* L.). A forage-only diet was used because future studies with this system will focus on grass-fed dairy diets. Therefore, the researchers felt the most appropriate validation method was using a forage-only substrate. 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). All non-desirable plant species were removed from herbage by hand separation prior to freeze-drying. Freeze-dried herbage was ground to pass through a 1-mm sieve (Wiley Mill; Thomson Scientific Inc., Philadelphia, PA). 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 the experiment, and oven-drying forage can denature protein and decrease digestibility (Jones & Bailey, 1972). Representative samples of freeze-dried herbage were collected for nutrient analyses using wet chemistry at a commercial laboratory (Dairy One Laboratories, Ithaca, NY) prior to the start of the study. Specific analyses used is described below in Analytic Methods. Nutrient composition of herbage used in the experiment is in Table 1.

Continuous culture operation

Fermentors were fed equal portions of herbage 4 times daily (0730, 1030, 1400, and 1900 h). Solid mean retention time and liquid dilution rate of the fermentors were adjusted daily to 24 h and 10%/h, respectively, and were achieved by regulation of buffer input and effluent volume removal (Bargo, Varga, Muller, & Kolver, 2003; Rico, Chung, Martinez, Cassidy, Heyler, & Varga, 2012; Soder et al., 2016). A summary of all continuous culture fermentor parameters are summarized in Table 2. Mineral buffer was prepared using the method of Hoover et al. (1976), except that urea was added at a rate of 0.4 g/L to simulate recycled N (Weller & Pilgrim, 1974). To achieve the desired retention and dilution rates, the buffer pump was adjusted to 20.6 ± 0.2 mL buffer per 7.5 min. The buffer pumps were on (pulsing) for approximately 4.6 min, at which point they automatically stopped pulsing and did not deliver any buffer into the vessel for the remaining 2.9 min of the 7.5 min cycle. Adjustments to the pulse time were made daily to ensure that 20.6 mL of buffer were delivered every cycle. The daily estimated effluent volume was determined by the daily buffer and feed volume. The effluent pump was set to run for 6 s every 7.5 min and daily effluent volume was adjusted by altering the pump speed. The total volume of effluent that had to be removed from the system was calculated using the following equation: $V_{Fluid} = F_{LD} \times V_{Ferm} \times t_r$ where V_{Fluid} = total volume of fluid removed for given retention time (mL), F_{LD} = liquid dilution factor (%), V_{Ferm} = volume of the fermenter (mL), and t_r = retention time (hr). The resulting volume is equivalent to the daily effluent fluid volume needed to maintain proper turnover in the system. In order to determine the total volume of effluent, the fluid volume needed to be adjusted for the solids (feed) entering the system. Therefore, total volume removed per day is calculated as: $V_{Total} = V_{Fluid} + V_{Solids}$ where V_{Total} = total volume of effluent removed daily (mL) and V_{Solids} = total volume of feed added to the system per day (mL). The amount of effluent to be removed per sample can be determined using $V_{Sample} = V_{Total} / n_s$ where V_{Sample} = volume of effluent to be removed per sample (mL) and n_s = number of samples per day. The chosen number of daily samples for this set-up was 192 samples per day, correlating to a 7.5 min cycle running continuously for 24 h.

Ruminal fluid and solid digesta samples were collected from a ruminally-fistulated, non-lactating, non-pregnant 4-year old Holstein cow (*Bos taurus*; BW = 794 kg) cared for in accordance with the Pennsylvania State University Animal Care and Use (IACUC #46212) guidelines. The donor cow was group housed and fed daily 13.8 kg total DM of a diet consisting of (DM basis) 16.4% alfalfa haylage, 16.4% corn silage, 16.4% cool-season grass silage, 13.2% cool-season grass hay, 13.2% cereal straw, 9.9% cottonseed hull, 8.2% canola meal, 4.9% liquid molasses and 1.4% minerals at the Pennsylvania State University Dairy Research Farm

(University Park, PA, USA). The total mixed ration contained a vitamin/mineral premix in order to meet National Research Council (2001) nutrient recommendations for a non-lactating dairy cow. Approximately 3 h after feeding (1000 h), 7 L of ruminal fluid was collected with a hand pump into a pre-warmed (39°C), insulated container. Solid digesta was collected by hand from the ventral, central, and dorsal areas of the rumen. Liquid and solid 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 1.50 L was poured into each of the pre-warmed fermentation jars. Three samples of the filtered fluid (15 mL each) and three samples of digesta (15 g each) were taken for DM determination. Solid digesta was mixed by hand and 32 g was added to each fermentor. Each fermentor was purged with CO_2 gas at a rate of 20 mL/min for 1 h and then continuously purged with 1 mL/min for the remainder of the experiment to maintain anaerobiosis. The temperature was maintained at 39°C and contents were continually stirred at a rate of 255 rpm.

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 collection period. During all 10 d of the experiment, effluent was collected daily from the collection bottles, the weight and volume were recorded. Daily DM and protozoal samples were taken from each vessel using a 10-mL graduated pipette. The tip of the pipette was removed to allow large digesta particles to freely flow into the pipette (Dehority, 2005). Samples were taken just prior to the 1030 h feeding and 60 ml of sample was collected from each vessel. Samples were taken at a variety of depths to reduce variability due to the visual observation of vessel stratification. Vessel protozoal cell counts were completed using the Sedgewick-Rafter chamber following the procedure of Dehority (2005). During the collection period, a 50-mL sample of effluent was collected each day and strained through 8 layers of cheesecloth and a subsample taken for VFA and NH_3-N analyses (Erwin, Marco, & Emery, 1961; Chaney & Marback, 1962, respectively). An additional 1 L of effluent was collected each day of the collection period and composited at the end of each period. Composited effluent was freeze-dried, ground to pass through a 1-mm sieve, and analyzed for DM, organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein (CP) (AOAC, 2006), and total purines (Zinn & Owens, 1986). During the last day of each period, the contents of each fermentor vessel 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, Hoover, Miller, & Thayne, 1996). The final pellet was freeze-dried and analyses for DM, OM, CP (AOAC, 2006) and total purines (Zinn & Owens, 1986) were completed.

The target vessel volume was 1.50 ± 0.20 L throughout the experiment. The vessels were manually adjusted to 1.50 L each day just prior to the 1030 h feeding. Adjustments were made by either allowing the effluent pump to run continuously until the vessel volume decreased to 1.50 L, or if the vessel volume was below this value, buffer solution was added. The volume of buffer or the volume and weight of vessel contents removed were recorded. The volume of the contents removed or added were only -30 ± 31.3 mL across the experiment. Any vessel contents removed during the volume adjustment were not combined with daily effluent samples.

CH_4 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 vessel. 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 = Σ [CH_4 volume$_a$ – CH_4 volume$_b$], where CH_4 volume$_a$ equals CH_4 volume determined by multiplying the headspace volume by the measured CH_4 concentration and CH_4 volume$_b$ equals CH_4 volume 10 min prior to CH_4 volume$_a$ over each 24-h period during collection days.

Analytical methods

Samples of orchardgrass and alfalfa 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), rumen degradable protein [RDP (Cornell *Streptomyces griseus* enzymatic digestion; Coblentz, Abelgardir, Cochran, Fritz, Fick, Olson, & Turner, 1999], NDF, ADF, and lignin [Ankom model A200; Mertens (2002), with heat-stable alpha-amylase and sodium sulfite used in the NDF procedure (inclusive of ash)], minerals (Ca, P, Mg, K, Na; Thermo IRIS Advantage HX or intrepid inductively couple plasma radial spectrometer after microwave digestion; CEM Application Note for Acid Digestion, CEM Matthews, NC), and ether extract (method 2003.05; AOAC, 2006). The non-fiber carbohydrate (NFC) concentration was calculated using the equation NFC (g/kg) = (100% - (CP% + NDF% + ether extract% + ash%)/100%) × 1000 g/kg. Pectins were included in the NFC calculation but not included in water soluble carbohydrate or non-structural carbohydrate analyses.

Effluent samples were analyzed for DM and OM (methods 930.15 and 942.05, respectively; AOAC, 2006) and CP concentration (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 & 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. Apparent (DM, OM, NDF, and ADF) and true (DM, OM, and CP) digestibilities of nutrients were calculated using the equations of Soder et al. (2016).

Statistical analyses

Data were analyzed as a completely randomized block design with fermentor as the experimental unit using the PROC GLIMMIX procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC), fitted to the following model: $Y_{ijk} = \mu + P_i + F_j + D_k + e_{ijk}$ 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, D_k = mean effect of *k*th day, and e_{ijk} = residual error. Period was considered a fixed effect in order to determine the precision of the system across periods of the same experiment and day was also considered a fixed effect in order to determine the precision of the system within each period; fermentor was considered random. Pre-planned orthogonal contrasts were used to test the main effect of adaptation vs. collection periods.

Measures of CH_4 production were analyzed for temporal patterns using the following model: $Y_{ijk} = \mu + P_i + F_j + D_k + E1_{ijk} + H_l + E1_{ijkl}$ 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, D_k = mean effect of *k*th day, and $E1_{\mathrm{ijk}}$ = whole plot error, H_l = mean effect of *l*th hour analyzed as repeated measures, and $E1_{ijkl}$ = subplot residual error. Period, day, and hour sampled were considered fixed effects, and fermentor, whole plot error, and subplot error were considered random effects. Pre-planned orthogonal contrasts were used to test the main effect of adaptation vs. collection periods. For all statistical analyses, mean separation was achieved using the LSMeans statement of PROC GLIMMIX in SAS (SAS, Inc., Cary, NC). Significance was declared at $P \leq 0.05$.

RESULTS

pH, temperature, and vessel and effluent DM

Mean and minimum pH were lower ($P < 0.01$) during Period 2 than all other periods (6.63 and 6.50, respectively). The mean pH ranged from 6.37 to 6.75, while the maximum and minimum pH ranged between 6.98 and 6.33 across the 10 d, respectively (Figure 3a). Mean pH during the collection period was lower ($P < 0.01$) than that observed in the donor cow, but there was no difference between the adaptation and collection periods in mean, maximum, or minimum pH (Table 3). Temperature was not different among periods or days ($P > 0.11$).

Neither vessel nor effluent DM were different ($P = 0.81$ and 0.51, respectively) among periods. There was no difference ($P = 0.81$) in the vessel DM between the adaptation and collection periods in the current study, but the DM of the fluid taken from the donor cow was greater than the collection period (Figure 3b). In the current study, no differences ($P \geq 0.42$) in vessel or effluent DM among days were observed (Table 3).

Vessel, buffer, and effluent volumes

The vessel target volume was 1.50 ± 0.2 L, resulting in a target buffer volume of 3.96 L/d and effluent volume of 4.22 L/d (dependent on feed volume). Effluent and buffer volumes were similar ($P \geq 0.12$) among periods or between the collection and adaptation periods (Table 3). Buffer volume was different ($P < 0.01$) among days, ranging from 3.72 – 4.00 L/d throughout the experimental period (Figure 3c); however, no difference ($P > 0.41$) was observed among the days of the collection period (3.97 ± 0.031 L). Effluent volume was not different ($P = 0.53$) among days. Additionally, the largest variation in vessel volume was observed on d 1 (1.43 ± 0.05 L) and d 2 (1.38 ± 0.03 L); however by d 3, the volume only ranged from 1.48 L to 1.56 L. This did result in a significant difference in average vessel volume between the adaptation and collection periods (Table 3). However, the vessel volume was maintained at 1.53 ± 0.04 L within the collection period.

Protozoa

Protozoa were maintained above 5.0×10^4 cells/mL throughout the entire experimental period and slowly increased from d 2 to d 10. Protozoal numbers were greatest ($P < 0.01$) in the donor cow and least ($P < 0.01$) in the adaptation period (Table 3). Numbers initially declined after inoculation but then slowly rebounded throughout the experimental period reaching 6.0×10^4 cells/mL by d 10 (Figure 3d), which was approximately 50% of the protozoa observed in the initial inoculum.

VFA, Nutrient Digestibility, and N metabolism

When averaged across the 3-d collection period, there was no difference among periods for total VFA, acetate, propionate or isobutyrate concentrations (Table 4). Butyrate concentration was greater ($P \leq 0.03$) in Period 4 than Period 2 or 3. Valerate and isovalerate concentrations were greater ($P \leq 0.03$) in Period 1 than all other periods, which were not different ($P > 0.23$). However within each period, there was no difference ($P \geq 0.07$) among the 3 collection days for concentration of any individual VFA. Concentrations of NH_3-N in the effluent were not different ($P \geq 0.09$) among periods; however, a significant day effect was observed (Table 4). Day 10 had lower ($P \leq 0.04$) NH_3-N concentration than d 8 or 9 (27.29, 31.73, and 30.77 mg/dL, respectively; Table 4). Nutrient digestibility and N metabolism parameters are presented in Table 5. Samples were analyzed as composites taken across each 3-d collection period and therefore, no statistical analyses were conducted.

CH_4 production

There was no effect ($P \geq 0.33$) of period (Table 4) or day (Figure 4a) on total daily CH_4 production. Furthermore, there was no effect ($P \geq 0.27$) of period or adaptation vs. collection day on total daily CH_4 production per gram of OM fed, per gram of NDF fed, per gram of digestible OM fed, or per gram of digestible NDF fed. During the collection period, an hour effect was observed ($P < 0.01$; Figure 4b). Methane production per hour was greater ($P \leq 0.02$) 1 h after feeding than 1 h before feeding for the 0730 and the 1030 h feedings. However, there was no difference ($P \geq 0.30$) between the hours before and after feeding at the 1400 and 1900 h feedings. The hourly CH_4 production showed a minor peak after the 1000 h feeding and a major peak after the 1900 h feeding due to accumulative feed intakes during the day.

DISCUSSION

A major limitation of previous CCF systems is the buildup of DM within the vessel during the experimental period due to insufficient dilution/removal rates or clogging of the apparatus (Muetzel et al., 2009). In the current system, lack of difference in effluent and vessel DM reflects the stability of the CCF system throughout time. Additionally, it shows that both solid retention time (24 h) and the liquid dilution rate (10%/h) were achieved in the system. Difference in DM content between the donor cow and the collection period has been reported as a common problem among CCF systems. This can lead to buildup of fermentation end products and underestimation of digestibility (Muetzel et al., 2009) and impact CH_4 production. In the current system the donor cow and collection period effluent DM content was different, but only by 0.25%, therefore there is likely no effect of DM difference on digestion kinetics.

The pH was not regulated as in previous CCF systems (Dahlberg, Stern, & Ehle, 1988; Bach, Yoon, Stern, Jung, & Chester-Jones, 1999). Other studies reported that the mean pH of herbage-based diets in CCF systems without regulated pH ranged between 6.67 and 6.75 (de Veth & Kolver, 2001; Soder, Brito, Rubano, & Dell, 2012), slightly higher than that observed in the current study. Given the inherent variability in ruminal pH, the difference between the donor cow and collection period is assumed to be a combination of changing diet, changing environment of ruminal microbial community, or the feed to rumen volume ratio which can vary tremendously among CCF apparatus. For example, Rusitec systems use 10 – 30 g substrate/d per 0.7 L = 29 g/L (Hristov et al., 2012), while the Dillard et al. (2017) system used 60 g substrate/d per 1.1L = 55 g/L. This results in differences in dilution rates and retention times within each system, causing large discrepancies among different systems even when using the same diet ingredients. This also changes the effluent removal rate and buffer dilution rate based on the chosen digesta retention time. A large difference between the CCF system described in the current study and previous systems (Hoover et al., 1976; Teather and Sauer, 1988; Karnati et al., 2009; Soder et al., 2016) is the lack of an overflow spout. When this CCF system was initially developed, the overarching objective was to develop a system to measure total enteric CH_4 production. In order to successfully meet this objective, the overflow spout was removed so that the system could be fully sealed and have no leakage of gases in or out. However, as a result the volume of the system was not automatically maintained via passive overflow when it reached a specific volume. Therefore, it became necessary to adjust the internal vessel volume daily in order to maintain 1.50 L internal volume. The daily adjustments averaged -31 mL ± 31.3 mL throughout each period, and the vessel volume was maintained at 1.50 L ± 0.01 L.

Protozoa play an important role in the maintenance of ruminal microbial communities by predation of bacteria, and also allow methanogens to attach to their cell surface (McAllister, Meale, Valle, Guan, Zhou, Kelly, Henderson, Attwood, & Janssen, 2015). However, many CCF systems are unable to maintain protozoal communities within the vessel due to vessel turnover time being faster than protozoal generation time and lack of natural stratification (Hoover et al., 1976; Teather & Sauer, 1988). More recently, fermentors have been modified with multistage filters to aid in the retention of protozoa (Karnati et al., 2009). Using a filtered effluent removal apparatus, Karnati et al. (2009) maintained protozoal cell counts at 1.9×10^4/mL during a 23-d experiment. This system, however, required the routine changing of the filters to prevent clogging and influenced solid and liquid removal rates. This disturbs the vessel contents and also prevents the use of a closed system throughout duration of the experiment. Teather & Sauer (1988) observed that using an effluent removal tube similar to the system described herein, protozoal counts were maintained at 6×10^4 for 11 d. Others (Muetzel et al., 2009; Hristov et al., 2012) reported values ranging from $7.8 \times 10^3 – 1.5 \times 10^5$ cells/mL. Cabeza-Luna, Carro, Fernández-Yepes, & Molina-Alcaide (2018) developed a filter system that did not need to be changed, however, while protozoa number were higher in the system compared with the control, they were not significantly different. In the current system no filter was used, therefore there was no need to change filters and open the system. Furthermore, vessel contents were not disturbed by changing apparatus multiple times during the experiment. While our initial protozoal counts did decrease and rebounded to only 50% of the inoculum values, they were still well within the range reported by Muetzel et al. (2009) and Hristov et al. (2012).

Another factor in protozoal survival is the ability to replicate the natural substrate stratification of the rumen. Teather & Sauer (1988) were able to successfully maintain a natural stratification of solids into floating, suspended and sediment components in the modified system. This was achieved by decreasing the stirrer speed. Muetzel et al. (2009) further adapted this system by using a variable stirrer speed that increased speed for short periods before returning to the slower speed. The authors reported that this reduced problems with clogging and helped decrease sampling variability. During development of the current system, spinner speed was initially decreased to approximately 150 rpm to facilitate stratification, but this created effluent clogging problems. After performing multiple tests, the speed was then increased back to 255 rpm to alleviate this problem. It was initially assumed that at this speed no natural stratification would occur due to the fast spinner speed. However, not only was a forage mat easily visible (2 – 3 cm thick), closer inspection clearly showed both suspended solids and sediment. It was determined that due to the spinner placement, the rounded bottom of the vessel, and the spinner impeller diameter to vessel diameter ratio, areas of differential spinning speed within the fluid column were created. This was caused by changes to the net vorticity and recirculatory flow of the fluid inside the vessel (Kirby, 2010) which appear to have resulted in natural stratification of the vessel contents. While vessel stratification was not directly measured, presence of a forage mat and protozoal counts well within the range of *in vivo* counts described by others (Muetzel et al., 2009; Hristov et al., 2012) is evidence that natural stratification did occur.

Total VFA, acetate, propionate, butyrate, and NH_3-N concentrations in the current system were greater than those reported by Noviandi, Neal, Eun, Peel, Waldron, ZoBell, & Min (2014) for a 50:50 tall fescue (*Schedonorus arundinaceus* (Schreb.) and alfalfa diet fed in continuous culture (50.0 m*M*, 31.5 m*M*, 9.91 m*M*, 5.87 m*M*, 3.20 m*M*, and 18.7 mg/dL, respectively). This is likely due to the greater quality of orchardgrass used in the current study compared with tall fescue used in Noviandi et al. (2014). Apparent DM, OM, and NDF digestibilities and true DM and OM digestibilities were comparable to Bargo et al. (2003) and Soder, Brito, & Rubano (2013) who reported digestibilities of herbage diets in a traditional, dual-flow CCF system; yet, CP digestibility and effluent N-flows were greater in the current study, a result of a greater RDP and CP digestibilities of the orchardgrass/alfalfa diet used. The concentration of NH_3-N was similar to that reported by Dahlberg et al. (1988) and Bargo et al. (2003) for a grass/legume diet in CCF systems (40.6 and 32.2 mg/dL, respectively).

When using a closed system, buildup of headspace pressure is a concern. Multiple studies (Theodorou, Williams, Dhanoa, McAllan, & France, 1994; Pell, Pitt, Doane, & Schofield, 1998; Tagliapietra, Cattani, Bailoni, & Schiavon, 2010) reported that pressures in excess of 48 kPa retarded microbial growth and altered fermentation kinetics. This can then result in underestimation of digestibility and CH_4 production, in part due to the dissolving of gaseous CO_2 into the vessel liquid and leading to possible overestimation of CH_4 concentration (Maccarana, Cattani, Tagliapietra, Schiavon, Bailoni, & Mantovani, 2016). For that reason, each fermentor was fitted with a release valve that opened at pressures greater than 50 kPa. The venting of gas can then result in an underestimation of CH_4 production due to gas escape. In order to determine if any venting occurred, a gas-impermeable bag was placed on each release valve for 24 h during the collection period. It was determined that no venting occurred and thus the headspace pressure never exceeded 50 kPa nor was gas lost through venting.

Differences in apparatus and diet can have a large impact on CH_4 production and accuracy of measurements, thus limiting the ability to compare data from multiple studies (Getachew, DePeters, Robinson, & Fadel, 2005). While it is difficult to compare the current results to previous studies due to differences in apparatus and procedures, lack of difference among periods or days demonstrates the lack of variability within our system when measuring CH_4 production. Variability in CH_4 measurements can also be high within each experiment. For example, Dillard et al. (2017) observed a coefficient of variation of 41% in CH_4 output across 4 forage diets fed in a dual-flow CCF system. In the current study, the coefficient of variation in CH_4 production was only 23%. Furthermore, other studies have reported a wide range in coefficient of variation of CH_4 emissions measured *in vivo* (2 to 67%), and these authors also suggest the variability is largely dependent on method of measurement and individual cow variability (Garnsworthy, Craigon, Hernandez-Medrano, & Saunders, 2012; de Hass, van Riel, Veerkemp, Liansun, & Ogink, 2013; Huhtanen, Cabezas-Garcia, Utsumi, & Zimmerman, 2015).

The CCF system proposed herein allowed for improved and more frequent CH_4 measurements, natural stratification, and maintained protozoal populations throughout the duration of the experimental period. Data collected throughout 4 experimental periods demonstrated that the system was able to reach a steady-state in fermentation well within the 7-d adaptation period and even typically variable data (i.e., CH_4 production) were stable within and across periods. Further research is still needed to determine the response of this system to concentrate-based diets and the relationship between this system and *in vivo* data. However, this CCF system provides more robust data on ruminal fermentation due to the retention of protozoa and vessel stratification compared with previous CCF systems. Additionally, the ability to determine daily enteric CH_4 production will allow researchers to better characterize the effects of differing diets and feed additives on total enteric CH_4 production.

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Table 1. Chemical composition (g/kg of DM) of 50% orchardgrass and 50% alfalfa mixture fed during continuous culture fermentation.

Item	Orchardgrass	Alfalfa	50:50 Mixture[†]
	-------------------g/kg of DM----------------		
OM[‡]	926	907	917
CP	230	266	248
RDP	181.7	218.1	199.6
NDF	554	347	451
ADF	324	267	296
Lignin	63	100	82
NFC[§]	108	272	190
CP:NFC	2.13	0.98	1.55
NE_L[¶], Mcal/kg	1.28	1.39	1.34
Ca	3.8	11.4	7.6
P	2.6	3.8	3.2
Mg	2.9	3.5	3.2
K	25.5	28.9	27.2
Na	1.3	0.3	0.8

[†] Calculated using actual nutrient composition and proportion of individual ingredients (DM basis)

[‡] OM, organic matter; CP, crude protein; RDP, rumen degradable protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrate; NE_L, net energy of lactation

[§] Calculated as NFC = ((100% - (CP% + NDF% + ether extract% + ash%)) / 100%) × 1000 g/kg

[¶] Estimated by the NRC (2001) model

Table 2. Summary of continuous culture fermentor parameters.

Parameter	Setting
Vessel Content Volume	1.5 L
Daily Feed DM	82 g[†]
CO_2 Rate	1 mL/min
Temperature	39.0 °C
Buffer Rate	2.75 mL/min
Retention Time	24 h
Liquid Dilution Rate	10%/h
Effluent Removal	Every 7.5 min
CH_4 Measurement	Every 10 min

[†] Divided into 4 equal feedings per day.

Table 3. pH, temperature, DM, volume, and protozoa from the donor cow and the vessel contents throughout the experimental period during continuous culture fermentation.

				P-value	
Item	Donor Cow	Adaptation Period[†]	Collection Period[‡]	Adaptation vs. Collection	Donor cow vs. Collection
pH					
Mean	6.74 ± 0.042[§]	6.53 ± 0.016	6.50 ± 0.024	0.215	< 0.001
Maximum	n.d.[¶]	6.75 ± 0.023	6.69 ± 0.035	0.221	------------
Minimum	n.d.	6.37 ± 0.012	6.37 ± 0.018	0.931	------------
Temperature, °C	n.d.	39.0 ± 0.00	39.1 ± 0.01	0.314	------------
DM, g/kg					
Vessel	23.1 ± 0.65	20.7 ± 0.27	20.6 ± 0.38	0.805	0.002
Effluent	n.d.	20.7 ± 0.23	21.2 ± 0.34	0.180	-----------
Volume, L/d					
Vessel	n.d.	1.49 ± 0.020	1.54 ± 0.012	0.035	-----------
Buffer	n.d.	3.94 ± 0.016	3.97 ± 0.031	0.294	-----------
Effluent	n.d.	4.23 ± 0.035	4.27 ± 0.043	0.365	-----------
Protozoa, cells × 10^4/mL	12.8 ± 0.22	5.2 ± 0.22	5.6 ± 0.22	< 0.001	< 0.0001

[†] Average of samples taken from d 0 through d 6

[‡] Average of samples taken from d 7 through d 9

[§] Mean ± SE

[¶] Not determined

Table 4. Effluent VFA and NH_3-N concentration and total daily CH_4 production of an orchardgrass-alfalfa mixture during fed in continuous culture.

		P-value	
Item[†]	Mean ± SE	Period[‡]	Collection[§]
VFA, m*M*			
Total	66.7 ± 3.01	0.217	0.736
Acetate	43.0 ± 1.96	0.214	0.685
Propionate	16.4 ± 0.83	0.127	0.643
Butyrate	6.2 ± 0.32	0.052	0.338
Isobutyrate	0.4 ± 0.08	0.542	0.361
Valerate	0.7 ± 0.23	0.014	0.068
Isovalerate	0.1 ± 0.06	0.070	0.422
NH_3-N, mg/dL	29.9 ± 0.89	0.085	0.037
CH_4 Production			
Total, mg/d	183.5 ± 8.27	0.269	0.367
mg CH_4/g of OM fed	2.4 ± 0.11	------	0.567
mg CH_4/g NDF fed	5.0 ± 0.22	------	0.567
mg CH_4/g digestible OM fed	3.8 ± 0.17	------	0.565
mg CH_4/g digestible NDF fed	3.2 ± 0.60	------	0.663

[†] VFA, volatile fatty acids; OM, organic matter; NDF, neutral detergent fiber

[‡] Comparison of the 3-d average for samples taken during each period

[§] Comparison of samples taken daily from d 7 through d 9

Table 5. Digestibility, N use efficiency, and bacterial efficiency of an orchardgrass-alfalfa mixture fed in continuous culture.

Item	Mean	SE[†]
Apparent digestibility, g/kg		
DM[‡]	457	6.6
OM	619	6.7
NDF	529	14.1
ADF	534	16.6
True Digestibility, g/kg		
DM	691	13.6
OM	774	9.4
CP	908	44.8
N intake, g/d[§]	4.04	0.000
N flow, g/d		
Total N	2.65	0.073
NH_3-N	1.05	0.013
NAN	1.60	0.079
Bacterial N	1.32	0.070
Dietary N	0.30	0.109
Bacterial efficiency		
g N/kg DM truly digested	23.32	0.854
g N/kg OM truly digested	22.70	1.096

[†] Statistical comparisons were not completed because samples were composited across days within period.

[‡] DM, dry matter; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein; NAN, non-ammonia nitrogen

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

FIGURE CAPTIONS

Figure 1. Diagram of each continuous culture fermentor. 1) control unit, 2) buffer pump, 3) buffer reservoir, 4) gas analyzer, 5) effluent pump, 6) freezer unit, 7) effluent bottle, 8) motor for stirrer, 9) pH probe, 10) effluent removal tubing, 11) stirrer rod with dual spinners, 12) temperature probe, 13) heat-jacketed 3.0-L glass vessel, 14) CO_2 input, 15) buffer input, 16) headspace gas output, 17) feeding hole. Hash marks indicate a long break line. Diagram not to scale.

Figure 2. A 2-unit fermentor system with gas analyzer, controller unit, and freezer for effluent storage.

Figure 3. Daily a) mean (pH), minimum (Min pH), and maximum (Max pH) pH; b) vessel and effluent DM; c) effluent and buffer volumes; and d) protozoal counts in continuous culture fermentors fed an orchardgrass-alfalfa mixture. Error bars indicate standard errors.

Figure 4. a) Mean total daily CH_4 production (mg/d) during the 10-d experimental period and b) hourly CH_4 production (mg/h) during the 3-d collection period (average of all 3 d) in continuous culture fermentation. Error bars indicate standard errors and arrows indicate time of feeding.