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Effect of mixtures of legume species on ruminal fermentation, methane and microbial nitrogen production in batch and continuous culture (RUSITEC) systems

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Short Title: Kelln et al. mixed legumes and fermentation in RUSITEC**Effect of mixtures of legume species on ruminal fermentation, methane and microbial nitrogen production in batch and continuous culture (RUSITEC) systems**

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ABSTRACT

The effect of cicer milkvetch (*Astragalus cicer* L.) and sainfoin (*Onobrychis viciifolia* Scop.) on ruminal fermentation, methane production, and microbial nitrogen synthesis was assessed in two experiments. Experiment 1 analyzed 2 legumes, cicer milkvetch and sainfoin at 2 stages (vegetative and late flower) incubated with alfalfa (*Medicago sativa*) at 5 inclusion rates 0:100; 25:75, 50:50, 75:25 and 100:0 (as DM) in batch culture. Experiment 2 analyzed vegetative cicer milkvetch and alfalfa incubated in ratios of 25:75, 50:50, 75:25 and 100:0 (as DM) in continuous culture systems (RUSITEC). In batch culture, increased dry matter disappearance (DMD), and propionate percentage (% total), and reduced methane (mg g⁻¹ DMD) occurred with vegetative cicer milkvetch inclusion. In RUSITEC, DMD linearly increased ($P < 0.01$), acetate:propionate ratio quadratically decreased, while ammonia (NH₃-N) concentration ($P < 0.01$) and butyrate percentage (% total) linearly decreased ($P < 0.05$) with increasing inclusion of cicer milkvetch. No differences were observed for methane (CH₄) production (mg g⁻¹ DMD), or short chain fatty acid (SCFA) production (mmol d⁻¹). Microbial nitrogen synthesis and efficiency of protein synthesis linearly increased ($P < 0.05$) with increased inclusion of cicer milkvetch. Results suggest cicer milkvetch may result in synchronicity of energy and nitrogen during rumen fermentation, which could enhance cattle production.

Key Words: sainfoin, RUSITEC, cicer milkvetch, legume, microbial nitrogen

INTRODUCTION

Hay production in western Canada has declined over the past 30-years as a result of producers converting upwards of 1 million hectares of tame perennial forage to arable land (Jefferson and Selles 2007; Statistics Canada 2017). Despite this cropping trend, including perennial forages in cropping rotations is well known to enhance the sustainability of cropping systems through improved soil fertility, reduced soil erosion, suppression of weeds, disruption of plant disease cycles and providing environmental benefits such as carbon sequestration, reduced NO₃ leaching, and provision of wildlife habitat (Entz et al. 1995). In addition, including perennial legumes, such as alfalfa, in pasture mixes can increase animal productivity (Popp et al. 2000). Thus, strategies to increase perennial forage hectares will benefit both the producer and the environment. It is well known that quantity and quality of forage provided can impact livestock performance (Popp et al. 2000; Petri et al. 2012). The microbial protein that flows from the rumen to the small intestine is the most important supply of essential amino acids and supplies the majority of the metabolizable protein needs to the host ruminant (NASEM 2016). Factors that influence the amount of microbial protein supply that leaves the rumen include the extent of ruminal degradation, nutrient availability, efficiency of the ruminal bacteria to utilize these nutrients, and rate of outflow from the rumen (Hespell and Bryant 1979; Mangan 1988; NASEM 2016).

Due to producer adoption of bloat safe legumes, there has been increased need to better understand their impact on rumen fermentation (McMahon et al. 1999; Williams et al. 2011; Stewart et al. 2019). Previous research has determined the effects of sainfoin inclusion in the diet on rumen fermentation and identified a recommended rate of inclusion to increase livestock performance and reduce bloat (McMahon et al. 1999). However, research surrounding the level of inclusion in the diet and effects of cicer milkvetch on ruminal fermentation and microbial nitrogen synthesis are negligible, leaving producers with limited information to make decisions regarding pasture mixtures. Hence, the objectives of these experiments were to assess the effects of differing proportions of cicer milkvetch (CMV; *Astragalus cicer* L) and sainfoin (SAIN; *Onobrychis viciifolia*), when displacing alfalfa (ALF; *Medicago sativa*) in forage mixtures on digestibility, ruminal fermentation, methane production, and microbial nitrogen synthesis in both batch and continuous fermentation systems.

MATERIALS AND METHODS

All animal procedures and protocols used in this experiment were reviewed and approved by the Lethbridge Research and Development Center Animal Care Committee (ACC number 1501) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

Plant Material and Forage Quality

Cicer milkvetch (AC Oxley), SAIN (Melrose), and ALF (AC Grazel) were harvested during the summer of 2018 (Vegetative SAIN, CMV & ALF on May 31, June 5 and May 25, respectively; Late flower SAIN, CMV, & ALF on July 5, July 13 and July 17) from plots grown at AAFC Saskatoon Research Center, Saskatoon, Canada. This was the first cut of the summer for these plants. To limit seasonal changes in forage composition (McGraw and Marten, 1986), samples were clipped to a 5-cm height at vegetative plant growth stage (16 to 30 cm stem length, no buds, flowers or seed pods) and at late flower plant growth stage (≥ 2 nodes with open flowers; no seed pods) (Kalu and Fick, 1981). Samples were dried in a forced air oven at 55°C for 72 h, then ground to pass through a 2-mm screen (AOAC 2000) and stored until initiation of the experiment. All samples were analyzed for CP (AOAC; method No. 990.03), ADF (AOAC; method No. 973.118), and ash-corrected NDF (aNDF) (Van Soest et al. 1991) at Cumberland Valley Analytical Services (Waynesboro, PA, USA) (Table 1).

The Batch Culture Procedure

The batch culture *in vitro* gas production technique as described by Mauricio et al. (1999) was used to study the effects of inclusion of legumes on ruminal digestibility and fermentation of forage mixtures. Results from the batch culture experiment were used to determine the ideal forage mixtures (legume inclusion level) for the subsequent RUSITEC experiment.

Experimental Design and Treatments

Each batch culture was arranged as 2 legumes (SAIN, CMV) $\times$ 2 plant growth stages of maturity (vegetative, late flower) $\times$ 5 inclusion rates for each species displacing ALF (0:25:50:75:100) $\times$ 3 incubation times (6, 24, 48 h) $\times$ 3 replicates for each time point (Table 2). Each run also included 3 bottles containing inoculum and medium without substrate for a

negative control. The two runs were completed on different days using inoculum from the same cows.

Source of Inoculum

Rumen inoculum were obtained from 3 ruminally fistulated cattle fed an alfalfa-meadow brome grass hay. Mixed digesta contents were collected from four distinct sites within the rumen, 2 h prior to morning feeding and were filtered through 4 layers of cheesecloth to remove particulates. Strained fluid was pooled between the three cows and placed in a pre-warmed, air-tight, insulated vessel and transported at 39°C from collection site to the laboratory. The pH of the rumen inoculum (pH 6.43) was measured at the time of collection (B20PI,98 Symphony Benchtop Meters; VWR, Edmonton, AB, Canada). The rumen inoculum was then placed in a glass bottle, flushed with CO₂, fitted with a bottle-top dispenser, and stored in a water bath at 39°C until being added to incubation vials.

Sample Preparation and In vitro Procedure

Forage mixtures at the specified inclusion rates were created and then samples were prepared by placing a 0.5 g (DM basis) sample of each treatment substrate in a dried (55°C), pre-weighed, labelled, and acetone rinsed Ankom F57 filter bag (5.0 × 5.5 cm.; Ankom Technology Corporation, Fairport, NY). Bags were heat sealed prior to placement in Ankom serum vials (100 mL) and serum vials were loaded on a tray and placed in an incubator at 39°C for 48 h prior to incubation. In vitro medium (pH 6.71) was prepared according to Goering and Van Soest (1970) and it (45 mL) was mixed with rumen fluid (15 mL) prior to dispensing under CO₂ into serum vials warmed to 39°C. Serum vials were sealed with ANKOMRF Gas Production System rubber stoppers (Ankom Technology Corporation) and incubated in a rotary shaker (125 rpm) incubator at 39°C.

Measurements and Analyses

Gas was taken from each vial using a 20 mL syringe and injected into 6.8 mL exetainers (Labco Ltd., Wycombe, bucks, UK). Methane (CH₄) concentration was determined from gas samples obtained at 6, 24, 48 hr time points via gas chromatography (Agilent 7890B series GC custom, Agilent Technologies Canada Inc., Mississauga, ON, CA) and values were expressed as mg·g⁻¹ DMD. Headspace gas pressure in each vial was recorded at 3, 6, 9, 12, 24, and 48 h of

incubation by inserting a 23-gauge (0.6 mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada). The needle was left in the septum and the transducer removed to vent the gas. Gas pressure values, corrected for amount of substrate organic matter (OM) and gas produced by the negative control, were used to determine the gas volume produced using the equation of Mauricio et al. (1999).

$$\text{Gas volume} = 0.18 + (3.697 \times \text{gas pressure}) + (0.0824 \times \text{gas pressure}^2)$$

At the end of each incubation time, vials were removed from the incubator and placed in ice water for 5 min to inhibit fermentation. Three replicate vials were removed at each time point for each treatment and serum vials were opened, and pH was measured immediately. Filter bags were removed and washed with cold water until the water ran clear. After rinsing, filter bags were dried at 55°C for 48 h and subsequently weighed for DM calculation. Neutral detergent fibre of residue was determined as described by Van Soest et al. (1991) using heat-stable α -amylase and sodium sulfite and corrected for ash. *In vitro* aNDF (IVNDF) and dry matter (IVDMD) digestibility were calculated, and ash content was determined by combustion at 550°C for 5 h. The OM content was calculated as 100 minus the proportion of ash (AOAC, 2005; method 942.05).

Incubation liquid (5 mL) was mixed with 1 mL of metaphosphoric acid (0.25; w/v) for determination of short chain fatty acids (SCFA), and a second subsample (5 mL) was mixed with 1 mL of H₂SO₄ (0.01; v/v) for ammonia N(NH₃-N) analysis (Rhine et al. 1998). Concentration of SCFA was quantified using a gas chromatograph (Model 6890N, Agilent Technologies Canada Inc., Mississauga, ON) equipped with a capillary column (30 m × 0.32 mm i.d., 1- μ m phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA), and a flame ionization detector, with crotonic acid (trans-2-butenic acid) as an internal standard (Beauchemin et al. 2009).

The RUSITEC Procedure

Experimental Design and Treatments

Vegetative CMV was selected for assessment in the RUSITEC. The experiment was designed as a randomized complete block design (blocked for apparatus) with 4 treatments assigned to sixteen replicated (n=4) fermentation vessels. Vegetative CMV and ALF were

incubated in ratios of 25:75, 50:50, 75:25 and 100:0 (DM basis; Table 3). The RUSITEC experiment was 15-d in duration and included an 8-d adaptation and 7-d sampling period.

Source of Inoculum

Rumen inoculum were obtained from the 3 ruminally fistulated cattle, collected and stored in the same manner as described in the in vitro batch culture assay. Mixed digesta was squeezed through four layers of cheesecloth to partition into liquid and solid fractions. The pH of the fluid (pH 6.65) was measured at the time of collection (B20PI,98 Symphony Benchtop Meters; VWR, Edmonton, AB, Canada).

Experimental Apparatus

The experiment used two rumen simulation technique (RUSITEC) apparatuses (Czerkawski and Breckenridge, 1977) located at Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Center, each equipped with eight 920 mL anaerobic fermenters. An input and output port were attached to each fermenter to infuse buffer and collect effluent, respectively. Each fermenter was housed in a 39°C circulating water bath. To initiate fermentation, 200 mL of McDougall's buffer (McDougall 1948), 700 mL of strained rumen fluid, 20 g of mixed solid digesta and 5 g of diet (both digesta and diets were supplied in separate bags) were placed into each fermenter while they were flushed with CO₂. After 24 h, the bag containing 20 g of digesta was replaced with a bag containing 5 g of each specific diet. Thereafter, one bag was replaced daily so that each bag remained in the fermenter for 48 h. The buffer was modified according to McDougall (1948) with pH = 8.2, using NaH₂PO₄ • H₂O 3.69 g L⁻¹, and NaHCO₃ 9.83 g L⁻¹ containing 0.3 g L⁻¹ of (NH₄)₂SO₄ and was continuously infused into the fermenters via peristaltic pump to achieve a dilution of 2.9% h⁻¹. The effluent was collected daily into a 2.0 L Erlenmeyer flask, while gases were collected in 2.0 L gas tight collection bags (Covidien Dover Drainage Bag #30510).

Measurements and Analyses

Gas volume, effluent volume and fermenter pH were measured daily at 0900 h during substrate bag exchange. Fermentation gases were collected daily into reusable urine collection bags. Total gas production was measured daily using a wet-type gas meter (Alexander-Wright and Co. London, UK). Gas was taken from each collection bag using a 20 mL syringe and

injected into 6.8 mL exetainers (Labco Ltd., Wycombe, bucks, UK). Methane (CH_4). Carbon dioxide (CO_2) and hydrogen (H_2) were analyzed using gas chromatography (Varian 4900 equipped with GS CarbonPLOT 30 m $\times$ 0.32 mm $\times$ 3 μm column and thermal conductivity detector) from Agilent Technologies Canada Inc (Santa Clara, CA, USA). The device was equipped with an isothermal oven at 35°C with helium as the carrier gas (27 cm s⁻¹).

Effluent was sampled on d 8 to 12 for measurement of SCFA and $\text{NH}_3\text{-N}$ concentration. Effluent subsamples of 5 mL were collected from the effluent flasks at the time of feed-bag exchange. The samples were then placed in screw cap vials, preserved in 1 mL of 25% (wt/wt) metaphosphoric acid and frozen in -20 °C until further analysis. A second subsample of the effluent (5mL) was collected and placed in screw cap vials preserved in 1 mL of H_2SO_4 (1% v/v) for determination of $\text{NH}_3\text{-N}$. Short chain fatty acid analysis was performed with same procedure as the batch culture assay. The concentrations of SCFA and $\text{NH}_3\text{-N}$ (mmol L⁻¹) were multiplied by daily effluent production (L d⁻¹) to determine SCFA and $\text{NH}_3\text{-N}$ production (mmol d⁻¹).

Dry matter, OM, CP, ADF, and aNDF disappearance were determined on sampling day 9 to 13 from feed bags incubated for 48 h. Feed bags were collected from each fermenter and were rinsed under cold running water until the rinsate was clear. The bags were then oven dried at 55°C for 48 h and hot-weighed to determine DM disappearance (DMD). Daily residues collected from each fermenter were pooled to yield a single sample for each fermenter over the 5 d measurement and analyzed for OM, total nitrogen (NA 2100 Carlo Erba Instruments, Milan, Italy), aNDF, and ADF content.

Effluent and feed residues were sampled on d 8 to determine the background ¹⁵N concentration. On day 9, the 0.3 g L⁻¹ $(\text{NH}_4)_2\text{SO}_4$ in the McDougall's buffer solution was replaced with 0.3 g L⁻¹ ¹⁵N-enriched $(\text{NH}_4)_2\text{SO}_4$ and infused until the end of the experiment. After day 9, daily effluents were collected and preserved with 3 mL of sodium azide at a final concentration of 0.1% wt/volume. On d 14 and 15, the daily effluents were measured from each fermenter and a 35 mL subsample was collected and centrifuged at 20,000 $\times$ g for 30 min, at 4° C, to isolate liquid associated bacteria (LAB). Collected microbial pellets were washed with deionized water, and centrifuged three more times (20,000 $\times$ g, 30 min, 4°C). Samples were then suspended in distilled water and lyophilized to determine N and ¹⁵N concentration.

Microbial fractions bound to feed particles were prepared from the 48 h feed bags. On d 14 and 15, bags were removed from the fermenter, gently squeezed, added to a stomacher bag filled with 20 mL of McDougall buffer and processed for 60s in a Stomacher 400 laboratory blender (Seward medical Ltd., London, UK). The processed liquid was exuded, poured off, and retained. The solid feed residues were washed twice with 10 mL of McDougall's buffer (1948) in each wash. The washed buffer was retained and pooled with the initially expressed fluid, to represent the feed particle associated bacteria (FPA), and the total volume recorded. Washed solid feed residues were considered to represent the feed particle bound (FPB) bacterial fraction. The FPA samples were centrifuged ($500 \times g$, 10 min, 4°C) to remove feed residues and the supernatant was centrifuged ($20,000 \times g$, 30 min, 4°C) to isolate a bacterial pellet which was washed three times as described above. Washed feed residues (FPB fraction) were oven dried for 55°C 48 h, weighed for DM determination, ball ground (MM400, RestchInc., Newtown, PA, USA), and analyzed for N and ^{15}N by combustion analysis using a mass spectrophotometer (NA1500, Carlo Erba instruments, Milan, Italy). All samples were frozen at -20°C until analyzed.

Total microbial nitrogen synthesis (mg d^{-1}) was calculated as (Ribeiro et al. 2015):

$$\text{Microbial Biomass} = \text{LAB} + \text{FPA} + \text{FPB}$$

Where,

LAB is the liquid associated bacteria

FPA is the feed particle associated bacteria

FPB is the feed particle bound bacteria

Efficiency of Microbial Nitrogen Synthesis (EMNS) ($\text{g bacteria N kg}^{-1} \text{ DMD}$) was calculated as (Refat et al. 2015):

$$\text{EMNS} = \text{g bacteria N} \div \text{kg DMD}$$

On d 14 and 15, fermentation liquid was sampled in duplicate (35 mL each) from each fermenter to determine the concentration of dissolved hydrogen (dH_2) and dissolved methane (dCH_4) as described in Wang et al. (2014) and Wang et al. (2016). In brief, a 50 mL syringe containing 35 mL of fermentation liquids was connected to a 20 mL syringe filled with 5 mL of N_2 gas. The N_2 was injected from the small syringe into the large syringe via a T tube and the apparatus was vigorously shaken by hand for 5 min. The entire gas phase was then transferred

from the large syringe into the small one to determine the gas volume. Finally, the small syringe was removed from the T tube and 6 mL of gas was sampled for both gas and dissolved gas analyses determined by gas chromatography as previously described.

Statistical Analysis

Data for the batch culture experiment was analyzed as a randomized complete block design using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) to test for the effect of percentage of legume species in the diet. Plant physiological stages of maturity were analysed separately. The model included treatment as fixed effects, and random effects of run (1 to 2) and run $\times$ treatment. The run was used as the experimental unit for statistical analysis. Polynomial contrasts were used to test for linear and quadratic components in the relationships with percentage of legume. Degrees of freedom were adjusted using the Kenward-Roger option. Results were considered significant when $P < 0.05$, and trends were discussed when $P \leq 0.10$.

For the RUSITEC, data were analyzed as repeated measures according to a completely randomized block design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The MIXED model included treatment as fixed effects and random effects of fermenter, with sampling day as the repeated measure. The individual fermenter was used as the experimental unit for statistical analysis. Polynomial contrasts were used to test for linear and quadratic components in the relationships with percentage of legume. Degrees of freedom were adjusted using the Kenward-Roger option. Results were considered significant when $P < 0.05$, and trends were discussed when $P \leq 0.10$.

RESULTS

Batch Culture Experiment

With vegetative legumes, DMD increased linearly ($P < 0.01$) with increasing CMV inclusion, whereas DMD tended to decrease linearly ($P = 0.06$) with increasing SAIN inclusion (Table 4). At late flower, DMD remained unchanged by CMV or SAIN inclusion. The $\text{NH}_3\text{-N}$ concentration (mM) was unchanged in vegetative SAIN or CMV inclusion. Likewise, $\text{NH}_3\text{-N}$ was unchanged by late flower CMV or SAIN. Methane (CH_4 ; mg g⁻¹ DMD) decreased quadratically ($P = 0.05$) with increasing vegetative CMV inclusion, whereas no change was observed for vegetative SAIN, late flower CMV, or late flower SAIN.

At the vegetative stage, total short chain fatty acid production (mmol) and the percentage of acetate (% total) was unchanged by vegetative CMV and SAIN (Table 5). The percentage of propionate (% total) increased linearly ($P = 0.02$) with increasing vegetative CMV, with no difference observed for vegetative SAIN. Percentage of butyrate (% total) tended to decrease linearly ($P = 0.09$) with vegetative CMV inclusion and was reduced quadratically ($P = 0.05$) with SAIN. Percentage of minor SCFA (% total isobutyrate, valerate, isovalerate, caproate) were unchanged by vegetative CMV inclusion, and were reduced linearly ($P < 0.05$) by SAIN. The acetate to propionate ratio was unchanged by vegetative CMV or SAIN inclusion.

At late flower, no change was observed for total SCFA production (mmol) or percentage of acetate (% total) with either CMV or SAIN inclusion (Table 6). Percentage of propionate (% total) increased ($P = 0.04$) with increasing late flower CMV and was unchanged with SAIN. Percentage of butyrate (% total) was unchanged by late flower CMV but was decreased quadratically ($P = 0.04$) with increasing late flower SAIN. Percentage of isobutyrate (% total) tended to decrease linearly ($P = 0.07$) with increasing late flower CMV and decreased quadratically ($P < 0.01$) with increasing late flower SAIN. Percentages of valerate and isovalerate (% total) decreased linearly ($P < 0.01$) with increasing late flower CMV and decreased quadratically ($P < 0.05$) with increasing late flower SAIN. Percentage of caproate (% total) decreased linearly ($P < 0.01$) with late flower SAIN inclusion. Acetate to propionate ratio was unchanged by late flower CMV or SAIN inclusion.

The RUSITEC Experiment

Dry matter, ADF, and aNDF disappearance increased quadratically ($P < 0.01$) with increasing vegetative CMV (Table 7). Total gas production (L d^{-1}), CH_4 concentration (%), CH_4 production (mg g^{-1} DMD), and dH_2 ($\mu\text{mol L}^{-1}$) were unchanged by vegetative CMV; however, dCH_4 was reduced linearly ($P = 0.03$) by CMV. Total microbial nitrogen synthesis, LAB, FPB, and EMNS increased linearly ($P < 0.01$) with increasing vegetative CMV.

Media pH decreased linearly ($P < 0.01$) with increasing vegetative CMV (Table 8). Total ruminal SCFA production (mmol d^{-1}) was unchanged by increased vegetative CMV. Acetate production (% total) decreased quadratically ($P < 0.01$) with increasing vegetative CMV, averaging 64.96 % total. Propionate production (% total) increased quadratically ($P < 0.01$) and butyrate production (% total) decreased quadratically ($P < 0.01$) with increasing vegetative

CMV. Isobutyrate production (% total) decreased linearly ($P < 0.01$) with increasing inclusion of vegetative CMV. Isovalerate production (% total) was not affected by vegetative CMV, but valerate and caproate (% total) both increased quadratically ($P < 0.05$) with increasing vegetative CMV. Corresponding to the increase in propionate and decrease in acetate, the acetate to propionate ratio was reduced quadratically ($P < 0.01$) with increasing vegetative CMV. Ammonia nitrogen ($\text{NH}_3\text{-N}$) (mmol d^{-1}) decreased linearly ($P < 0.01$) with increasing vegetative CMV.

DISCUSSION

Alfalfa is the most widely grown perennial legume in western Canada, owing to its high yield, nutritive value, and winter hardiness (McMahon et al. 1999). Due to concerns with frothy bloat for cattle grazing monoculture ALF, research evaluating bloat-safe legumes, such as SAIN and CMV has increased (McMahon et al. 1999; Williams et al. 2011; Stewart et al. 2019). Cicer milkvetch is bloat safe due to its reticulate veined leaf pattern and thick epidermal layers which slows ruminal microbial digestion and has been reported to reduce ruminal $\text{NH}_3\text{-N}$ concentration, reduce CH_4 production, and shift N excretion from urine to faeces (Lees et al. 1982; Williams et al. 2011; Noviandi et al. 2014; Stewart et al. 2019). Conversely, legumes such as SAIN and birdsfoot trefoil, are bloat safe and modulate rumen fermentation due to condensed tannins (CT) in plant tissues, which can increase host protein utilization and confer anti-parasitic properties (Min and Solaiman 2018; Mueller-Harvey et al. 2019). Furthermore, feeding CT forages may offer environmental advantages, as they may reduce enteric CH_4 emission and urinary N excretion in ruminants, thus reducing greenhouse gas emissions (Mueller-Harvey et al. 2019; Stewart et al. 2019). In the present study, we evaluated how replacing ALF with CMV and SAIN and how inclusion rate affects in vitro digestion responses.

Dry Matter Disappearance, Short Chain Fatty Acid Production, and Ammonia Production

In the current study, increasing the inclusion rate of vegetative CMV for ALF, linearly increased DMD in both experiments, and linearly increased ADF and aNDF digestibility in the RUSITEC. In batch culture incubations, increased inclusion of late flower CMV had no effect on DMD, and inclusion of vegetative SAIN tended to decrease DMD, whereas inclusion of late flower SAIN had no effect on DMD. Stewart et al. (2019) found cattle fed a diet of 100% vegetative CMV had greater nitrogen retention as compared to SAIN and ALF, which the

authors attributed to an increased supply of fermentable energy as a result of the higher aNDF digestibility of CMV. These results are similar to the study conducted by McGraw and Marten (1986) who reported greater IVDMD for vegetative CMV as compared to vegetative SAIN, birdsfoot trefoil and ALF. Increased DMD could potentially increase DMI (NASEM 2016); however, when compared to *in vivo* trials, the DMI potential of CMV and SAIN, have been reported to be similar to ALF regardless of plant maturity (Acharya, 2006; Stewart et al. 2019). In contrast, heifers fed early flower CMV, and SAIN hay were reported to have lower DMI than those fed early flower ALF hay (Stewart et al. 2019). In the current study, vegetative SAIN had the opposite impact of vegetative CMV as DMD linearly decreased as it was substituted for ALF. Similarly, Stewart et al. (2019) reported heifers fed individual SAIN and ALF hays experienced lower DMD than cows fed CMV hay, which the authors attributed to the greater aNDF and ADF digestibility of CMV hay as compared to SAIN or ALF (Stewart et al. 2019).

Effects of legume species on SCFA production were dependent on the level of inclusion. During batch culture experiment, although no difference was observed in the percentage of acetate or subsequent A:P ratio, the percentage of propionate was increased linearly as both vegetative and late flower CMV increased in the diet. This differed from vegetative and late flower SAIN in which no differences in propionate, acetate, or A:P ratios were observed. The results of the vegetative CMV incubations were echoed in the RUSITEC experiment, as the A:P ratio was linearly reduced, driven by greater propionate production and reduced acetate production with increasing CMV. These results are not unexpected as DMD was observed to increase with increasing CMV, and greater feed digestibility is associated with increases in proportion of propionate in fermentation end products (Janssen 2010). Other studies have reported similar responses in propionate production with CMV. For example, Noviandi et al. (2014), reported propionate concentration increased from 10.5 mM to 12.8 mM when CMV replaced 25 to 75% of tall fescue (*Schedonorus arundinaceus*) in a mixed forage diet in continuous cultures. Similarly, Williams et al. (2011) found that during continuous culture, fermenters fed a diet containing 70% CMV : 30% corn silage had higher propionate concentration (31.8 mmol) compared to those fed 70% ALF : 30% corn silage (27.3 mmol). Considering that propionate is the main precursor for gluconeogenesis, increased propionate production is beneficial as it supports host metabolism and energy production (NASEM 2016).

In the current study, butyrate concentrations were also reduced with increasing inclusion of SAIN and CMV in the batch culture and CMV in the RUSITEC. This is similar to Williams et al. (2011) who reported fermenters fed 70% CMV had lower butyrate concentration (5.13 %, total) compared to those fed 70% ALF or SAIN (8.16 and 7.75 %, total). Although metabolic hydrogen [H] is produced in the first step of butyrate production (oxidative decarboxylation of pyruvate to acetyl-CoA), its production also involves two [H] incorporating steps (conversion of acetoacetyl-CoA to β -hydroxybutyryl-CoA and crotonyl-CoA to butyryl-CoA) (Ungerfeld 2020). Therefore, although butyrate production results in a net release of [H], less [H] is produced when butyrate is formed in comparison to acetate (Janssen 2010). When H_2 is increased, carbon is diverted away from acetate and butyrate production in favour of propionate (Moss et al. 2000). Therefore, it is possible that butyrate production was decreased due to increased H_2 production with CMV.

As branched chain amino acids are deaminated, they are converted to branched chain SCFA, therefore both branched chain SCFA and NH_3 -N are indicators of ruminal crude protein and amino acid digestion (NASEM 2016). Concentration of branched chain SCFA (isovalerate and isobutyrate) were linearly reduced ($P < 0.01$) with increasing vegetative SAIN and quadratically reduced ($P < 0.05$) with increasing late flower SAIN within the batch culture experiment, reflective of a possible decrease in ruminal proteolysis (Dahlberg 1988). Similar results have been reported by other researchers, who observed a reduction in ruminal fluid branched chain SCFA concentration from cattle fed SAIN compared to CMV (Dahlberg et al. 1988). McMahon et al. (1999) reported linear reductions in branched chain SCFA production as SAIN replaced 0 to 50 and 100 % of ALF in the diet. Both studies attributed the decrease in branch chain SCFA to decreased proteolytic activity due to the formation of protein-CT complexes, which would limit the supply of branched chain amino acids available for deamination to branch chain SCFA (Dahlberg et al 1988; McMahon et al. 1999).

Ammonia nitrogen production (mM, NH_3 -N) remained unchanged by the inclusion of either CMV or SAIN in the batch culture experiment. McMahon et al. (1999), reported a decrease in NH_3 -N, when vegetative SAIN leaves were compared to diet that only contained 25% SAIN with ALF or only ALF, suggesting a decrease in ruminal proteolysis. In the current study, during the RUSITEC experiment, NH_3 -N (mMol d⁻¹) was linearly reduced ($P < 0.01$) with

inclusion of vegetative CMV. These results are similar to Williams et al. (2011) who reported $\text{NH}_3\text{-N}$ flow (g d^{-1}) was lower from continuous fermenters fed CMV (0.18 g d^{-1}) compared to ALF (0.21 g d^{-1}). Similar additive effects were observed by Noviandi et al. (2014) who reported no change to $\text{NH}_3\text{-N}$ production when fermenters were fed CMV at 25% of the diet along with ALF, but observed a reduction in $\text{NH}_3\text{-N}$ if CMV displaced more ALF in the diet (50% CMV = 16.8 and 18.7 mg 100ml^{-1} for CMV and ALF, respectively; 75% CMV = 19.5 and 20.6 mg 100ml^{-1} for CMV and ALF, respectively). Protein and amino acid degradation of CMV has been reported to be similar to ALF in continuous culture (Dalberg 1988). It is possible that low protozoal populations contributed to the decrease of $\text{NH}_3\text{-N}$ in fermenters vs what one would observe in the rumen. However, it is possible that reductions in $\text{NH}_3\text{-N}$ observed in the current study could indicate enhanced N utilization by ruminal microorganisms, as cattle fed CMV have been reported to have increased N retention due to synchronization of fermentable energy and nitrogen (Stewart et al. 2019).

Methane Production and Microbial Nitrogen Synthesis

In the current study, increased inclusion of vegetative CMV lowered CH_4 production, whereas increased inclusion of late flower CMV, vegetative SAIN or late flower SAIN had no effect on CH_4 production in batch culture incubations.

During the RUSITEC experiment, microbial nitrogen synthesis (mg d^{-1}) and EMNS ($\text{mg bacterial N g}^{-1} \text{ DM digested}$) increased linearly with inclusion of CMV. However, total gas production (L d^{-1}) and methane (CH_4 , % gas; CH_4 , $\text{mg g}^{-1} \text{ DMD}$) remained unchanged. Dissolved H_2 (dH_2) remained unchanged with increased CMV, but dissolved CH_4 (dCH_4) was linearly reduced with increasing vegetative CMV. Wang et al. (2014) determined that dH_2 correlated with ruminal CH_4 production and led to fermentation pathways that produce less H_2 such as propionate and butyrate. As H_2 is easily lost, the measurement of dH_2 concentrations can be challenging and differences in results may be negligible, as observed in the current study (Wang et al. 2014). However, the reduction of dCH_4 , may be indicative of reduced CH_4 production by methanogens, which would be similar to the reduction in CH_4 gas observed in the batch culture experiment with vegetative CMV. This could be explained by the decreased A:P ratio in CMV incubations, which was driven by increased propionate, as propionate acts as an electron acceptor lowering the availability of hydrogen for methanogenesis (NASEM 2016).

Gas production strongly correlates to digestibility of organic matter (Menke et al 1979), and microbial nitrogen synthesis is related to the availability of carbohydrate and N in the rumen (Chumpawadee et al., 2006). Rumen degradable crude protein is the portion of intake crude protein degraded to ammonia, amino acids, or dipeptides in the rumen, and varies in its susceptibility to ruminal proteolysis depending on ruminal dilution rate, dietary forage:concentrate ratio, ruminal pH, nutrient interactions, and feed processing (NASEM 2016; Lardner et al. 2019). Rumen microbes require degradable crude protein or sources of non-protein nitrogen (NPN), such as $\text{NH}_3\text{-N}$ and urea, for microbial nitrogen synthesis. In the current study, given the increased microbial nitrogen synthesis, EMNS, ADF and aNDF digestibility, combined with reduced $\text{NH}_3\text{-N}$ production with increasing dietary CMV, it is possible that N and energy release in the rumen allowed for synchronous fermentation and greater microbial nitrogen synthesis and energetic efficiency. The impact of SAIN and other CT sources on microbial nitrogen synthesis has been well documented (McMahon et al. 1999; Makkar et al. 1995; Dahlberg et al. 1988), but research analyzing the impact of tannin free legumes on microbial nitrogen synthesis is limited. Lees et al. (1982) observed a thick epidermal layer and reticulated secondary and tertiary veining pattern in CMV leaves providing greater structural strength and resistance to mechanical damage as compared to ALF and birdsfoot trefoil (Lees et al. 1982). Low digestibility of the thick epidermal layer, combined with epidermal layers that are firmly attached to mesophyll cells due to the leaf vein structure and pattern, results in reduced microbial digestion of mesophyll tissue (Lees et al. 1982). The leaf structure and epidermal layer did not appear to negatively impact digestibility during the current experiments, as DMD was increased with inclusion of vegetative CMV. This may reflect the destruction of structural barriers as a result of grinding the forage prior to incubation. Dahlberg et al (1988), reported decreased total bacterial N production (mg d^{-1}) when fermenters were fed CMV compared to SAIN. However, once substrate utilization was accounted for, CMV had greater efficiency of microbial nitrogen synthesis than both SAIN and ALF (Dahlberg et al. 1988). With respect to the current study, it is possible that increased dry matter digestibility, propionate production, and microbial nitrogen synthesis, could lead to greater energetic and production efficiency in CMV than ALF fed livestock.

CONCLUSION

Past studies have recommended the inclusion of SAIN in pasture blends at 20 or 35% (McMahon et al. 1999; Wang et al. 2006). The findings from our study would support this recommendation as increased inclusion of vegetative SAIN during batch culture incubations tended to linearly decrease dry matter disappearance, and branched SCFA production. In contrast, inclusion of vegetative CMV in RUSITEC linearly increased DMD, microbial nitrogen synthesis, efficiency of microbial nitrogen synthesis, and increased propionate production all of which may increase energetic efficiency of ruminal fermentation. Results suggest dietary inclusion of CMV may supply synchronous sources of energy and nitrogen, which could enhance efficiency of production. Therefore, these results suggest CMV could benefit cattle production when substituted for ALF in mixed pasture blends and grazed at the vegetative stage. However, although CMV appears to have nutritional benefit to the ruminant, mixed pasture systems are dynamic and therefore more research is needed to determine agronomic best management practices for CMV, its persistence in mixed species pastures, and grazing management strategies which will allow for its successful adoption by producers.

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TABLES

Table 1. Chemical composition of legumes evaluated in forage mixtures incubated in batch cultures and the RUSITEC						
Treatment	Stage of Maturity	Chemical composition (% DM)				
		CP	ADF	aNDF	Ca	P
Alfalfa	Vegetative	28.3	23.9	37.8	1.1	0.3
	Late flower	20.5	33.1	41.1	1.1	0.3
Sainfoin	Vegetative	21.4	25.1	39.7	1.0	0.3
	Late flower	16.3	35.2	42.4	1.1	0.2
Cicer milkvetch	Vegetative	27.2	22.9	40.3	0.86	0.4
	Late flower	16.9	30.9	46.6	0.93	0.3

Note: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; aNDF, ash corrected neutral detergent fiber.

Table 2. Ingredient and chemical composition of experimental treatments incubated in batch culture

Nutrient (% DM)	Cicer milkvetch:alfalfa combination (DM) (CMV)				
	0:100	25:75	50:50	75:25	100:0
	<i>Vegetative</i>				
DM	92.4	92.6	92.9	94.4	94.5
CP	28.3	26.9	26.0	27.2	27.2
ADF	23.9	23.9	23.2	23.0	22.9
aNDF	37.8	40.0	41.3	39.7	40.3
	<i>Late Flower</i>				
DM	93.4	93.7	93.4	93.3	93.9
CP	20.5	19.4	18.4	18.1	16.9
ADF	33.1	31.9	31.4	31.6	30.9
aNDF	41.1	43.3	44.9	44.9	46.6
	Sainfoin:alfalfa combination (DM) (SAIN)				
	0:100	25:75	50:50	75:25	100:0
	<i>Vegetative</i>				
DM	92.4	92.8	92.1	91.9	92.7
CP	28.3	26.4	24.8	23.5	21.4
ADF	23.9	23.9	25.3	23.7	25.1
aNDF	37.8	38.2	38.2	37.8	39.7
	<i>Late Flower</i>				
DM	93.4	92.4	92.7	92.8	92.4
CP	20.5	17.2	18.0	16.7	16.3
ADF	33.1	35.4	33.3	34.4	35.2
aNDF	41.1	42.6	42.8	43.3	42.4

Note: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; aNDF, ash corrected neutral detergent fiber

Table 3. Diet composition and forage quality of vegetative cicer milkvetch and alfalfa diets incubated in the RUSITEC				
Nutrient (% DM)	Cicer milkvetch:alfalfa combination (DM) (CMV)			
	25:75	50:50	75:25	100:0
DM	92.2	94.0	94.7	95.4
CP	27.5	28.5	26.3	26.9
ADF	23.7	24.2	22.9	23.3
aNDF	43.8	43.1	46.6	44.9

NOTE: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; aNDF, ash corrected neutral detergent fiber

Table 4. Effect of stage of maturity, legume species and diet inclusion on dry matter disappearance (DMD), NH₃-N and methane production after 48 h *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)					SEM	P-value	Contrasts	
	0:100	25:75	50:50	75:25	100:0			L	Q
	<i>Vegetative</i>								
DMD (%)	62.68b	67.00ab	66.75ab	71.03a	68.34a	1.188	<0.01	<0.01	0.68
NH ₃ -N (mM)	34.76	35.10	35.8	35.74	35.03	0.627	0.72	0.23	0.99
CH ₄ (mg g ⁻¹ DMD)	48.74a	43.79b	41.31bc	37.27cd	35.34d	1.915	<0.01	<0.01	0.05
	<i>Late Flower</i>								
DMD (%)	54.13	54.41	57.26	58.17	63.24	1.429	0.08	0.65	0.39
NH ₃ -N (mM)	30.92	32.17	31.79	31.14	30.31	0.938	0.52	0.47	0.47
CH ₄ (mg g ⁻¹ DMD)	49.47a	49.09a	44.42ab	44.16ab	41.12b	1.887	<0.01	0.14	0.54
	Sainfoin:alfalfa combination (DM) (SAIN)								
	0:100	25:75	50:50	75:25	100:0				
	<i>Vegetative</i>								
DMD (%)	62.68a	62.48a	61.10a	56.96b	54.10b	1.574	<0.01	0.06	0.75
NH ₃ -N (mM)	34.76a	33.45a	31.83ab	31.30ab	27.96b	0.996	0.02	0.29	0.14
CH ₄ (mg g ⁻¹ DMD)	48.74	47.52	44.38	45.91	45.26	2.092	0.17	0.13	0.36
	<i>Late Flower</i>								
DMD (%)	54.13a	51.70abc	52.40ab	50.03bc	48.78c	1.238	<0.01	0.11	0.26
NH ₃ -N (mM)	30.92	29.12	31.02	29.83	29.38	0.810	0.22	0.78	0.26
CH ₄ (mg g ⁻¹ DMD)	49.47	51.61	53.48	50.21	50.16	1.565	0.21	0.24	0.12

NOTE: Means within the same row with different letters differ at the $P < 0.05$. L, linear, Q, quadratic

Table 5. Effect of inclusion level of vegetative legume species with alfalfa on short chain fatty acid (SCFA) profile after 48 h of *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)					SEM	<i>P</i> -value	Contrasts	
	0:100	25:75	50:50	75:25	100:0			L	Q
Total SCFA (mmol)	94.94	91.63	93.88	93.67	88.40	5.461	0.78	0.82	0.47
Acetate ^a	59.49	58.34	59.37	59.12	57.94	0.929	0.59	0.79	0.32
Propionate	18.45c	19.11bc	19.43abc	19.20ab	20.43a	0.415	<0.01	0.02	0.14
Butyrate	19.62a	19.04ab	17.83ab	17.36ab	16.48b	0.979	0.03	0.09	0.39
Isobutyrate	3.43	3.40	3.37	3.34	3.24	0.115	0.56	0.90	0.71
Valerate	5.35	5.39	5.33	5.28	5.30	0.113	0.80	0.64	0.60
Isovalerate	6.32	6.40	6.37	6.35	6.34	0.106	0.97	0.75	0.66
Caproate	0.53b	0.56ab	0.57ab	0.56ab	0.63a	0.016	0.06	0.75	0.11
A:P	3.23a	3.05ab	3.06ab	2.96ab	2.84b	0.104	0.06	0.16	0.17
Sainfoin:alfalfa combination (DM) (SAIN)									
	0:100	25:75	50:50	75:25	100:0				
Total SCFA (mmol)	94.94	85.29	87.60	83.02	84.18	4.235	0.30	0.15	0.37
Acetate ^a	59.49	57.52	59.37	59.58	61.56	1.140	0.26	0.56	0.60
Propionate	18.45	19.10	18.79	18.78	18.15	0.470	0.35	0.23	0.56
Butyrate	19.62a	18.49ab	17.71bc	16.6cd	15.62d	0.521	<0.01	<0.01	0.05
Isobutyrate	3.42a	3.24ab	3.09bc	2.87cd	2.76d	0.063	<0.01	<0.01	0.20
Valerate	5.36a	5.18ab	4.98bc	4.73cd	4.62d	0.070	<0.01	<0.01	0.34
Isovalerate	6.32a	6.12ab	5.81b	5.42c	5.26c	0.067	<0.01	<0.01	0.53
Caproate	0.53a	0.52a	0.51ab	0.47b	0.46b	0.028	<0.01	0.03	0.77
A:P	3.23	3.01	3.17	3.19	3.39	0.138	0.31	0.39	0.59

Note: Means in the same row with different letters differ at the *P* < 0.05. L, linear, Q, quadratic
^aindividual SCFA concentrations are reported as percentage of total SCFA.

Table 6. Effect of inclusion level of late flowering legume species with alfalfa on short chain fatty acid (SCFA) profile after 48 h of *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)					SEM	<i>P</i> -value	Contrasts	
	0:100	25:75	50:50	75:25	100:0			L	Q
Total SCFA (mmol)	88.86	87.86	85.12	93.63	94.08	5.591	0.27	0.95	0.39
Acetate ^a	60.48	60.10	59.54	61.63	61.46	0.757	0.27	0.86	0.27
Propionate	17.87b	18.19ab	18.64ab	18.79ab	19.08a	0.364	0.02	0.04	0.22
Butyrate	18.16	18.05	17.55	17.37	17.18	1.119	0.27	0.28	0.86
Isobutyrate	3.02	2.93	2.87	2.88	2.89	0.139	0.22	0.07	0.31
Valerate	4.96a	4.91ab	4.80bc	4.74c	4.75c	0.185	<0.01	<0.01	0.70
Isovalerate	5.69a	5.55ab	5.43b	5.39b	5.47ab	0.162	0.02	<0.01	0.32
Caproate	0.55ab	0.56a	0.53abc	0.51bc	0.51c	0.007	0.01	0.10	0.35
A:P	3.38	3.31	3.20	3.28	3.22	0.099	0.29	0.26	0.23
	Sainfoin:alfalfa combination (DM) (SAIN)					SEM	<i>P</i> -value	Contrasts	
	0:100	25:75	50:50	75:25	100:0			L	Q
Total SCFA (mmol)	88.86	82.27	83.66	85.39	82.70	3.428	0.27	0.38	0.07
Acetate ^a	60.48	60.48	59.74	61.45	60.18	0.708	0.58	0.53	0.31
Propionate	17.87	18.05	18.14	17.88	18.26	0.321	0.77	0.88	0.35
Butyrate	18.16a	16.91b	17.60ab	17.01b	17.11b	0.699	0.02	0.02	0.04
Isobutyrate	3.02a	2.69bc	2.86ab	2.67c	2.72bc	0.077	<0.01	<0.01	0.03
Valerate	4.96a	4.55b	4.72ab	4.48b	4.56b	0.139	<0.01	<0.01	0.05
Isovalerate	5.69a	5.09b	5.39ab	5.02b	5.14b	0.090	<0.01	<0.01	0.04
Caproate	0.55a	0.49ab	0.53a	0.46b	0.49ab	0.010	0.02	<0.01	0.52
A:P	3.38	3.35	3.30	3.44	3.30	0.096	0.72	0.68	0.32

Note: Means in the same row with different letters differ at the $P < 0.05$. L, linear, Q, quadratic

^aindividual SCFA concentrations are reported as percentage of total SCFA.

Table7. Effect of cicer milkvetch inclusion on nutrient disappearance, gas production, and microbial nitrogen synthesis in the RUSITEC

	Cicer milkvetch:alfalfa combination (DM) (CMV)				SEM	P-value	Contrasts	
	25:75	50:50	75:25	100:0			L	Q
<i>Nutrient disappearance (%)</i>								
Dry matter	74.44d	77.78c	80.65b	82.54a	0.391	<0.01	<0.01	<0.01
Acid detergent fiber	53.85c	59.24bc	62.13ab	66.86a	1.452	<0.01	<0.01	<0.01
aNDF	68.67c	72.08b	78.21a	79.34a	0.469	<0.01	<0.01	<0.01
<i>Gas production</i>								
Total (L d ⁻¹)	0.97	1.10	1.10	1.13	0.066	0.36	0.14	0.16
CH ₄ , % of gas	3.89	4.15	3.59	3.71	0.332	0.66	0.45	0.58
CH ₄ , (mg g ⁻¹ DM digested)	6.89	7.74	6.02	6.71	0.861	0.58	0.56	0.38
Total dissolved hydrogen (μmol L ⁻¹)	0.35	0.10	0.23	0.15	0.079	0.16	0.19	0.28
Total dissolved CH ₄ (mmol L ⁻¹)	0.32	0.30	0.30	0.27	0.015	0.14	0.03	0.62
<i>Bacterial nitrogen</i>								
Total (mg d ⁻¹)	35.89b	42.24ab	48.63ab	52.48a	3.463	<0.01	<0.01	0.71
LAB (mg d ⁻¹)	26.54b	32.88ab	39.21ab	41.86a	3.493	0.02	<0.01	0.61
FPA (mg d ⁻¹)	5.07	4.62	4.52	4.79	0.434	0.87	0.63	0.41
FPB (mg d ⁻¹)	4.28	4.80	4.91	5.82	0.526	0.51	0.05	0.71
EMNS (g bacteria N kg ⁻¹ DM digested)	9.22	10.38	11.53	12.14	0.826	0.08	0.01	0.75

NOTE: Means within the same row with different letters differ at the $P<0.05$. L, linear, Q, quadratic; aNDF, ash corrected neutral detergent fiber; CH₄, methane; LAB, liquid associated bacteria; FPA, feed particle associated bacteria; FPB, feed particle bound bacteria; EMNS, efficiency of bacterial nitrogen synthesis.

Table 8. Effect of cicer milkvetch inclusion on ruminal pH, short chain fatty acid (SCFA) and ammonia (NH₃-N) production in the RUSITEC

	Cicer milkvetch:alfalfa combination (DM) (CMV)				SEM	P-value	Contrasts	
	25:75	50:50	75:25	100:0			L	Q
pH	7.03a	7.02a	7.01a	6.99b	0.007	<0.01	<0.01	0.25
Total SCFA (mmol d ⁻¹)	27.54	28.70	29.46	28.17	1.117	0.66	0.59	0.28
Acetate (A) ^a	64.48ab	65.89a	65.32ab	64.148b	0.355	0.02	0.37	<0.01
Propionate (P)	20.54b	20.26b	21.44ab	22.53a	0.356	<0.01	<0.01	0.08
Butyrate	8.33a	7.40b	6.72c	6.39c	0.150	<0.01	<0.01	0.07
Isobutyrate	1.51a	1.43ab	1.39b	1.37b	0.026	0.01	<0.01	0.32
Valerate	2.10ab	2.06b	2.19ab	2.48a	0.094	0.03	<0.01	0.10
Isovalerate	2.68	2.57	2.54	2.56	0.06	0.27	0.10	0.24
Caproate	0.37b	0.38b	0.41ab	0.49a	0.025	0.02	<0.01	0.19
A:P	3.14ab	3.27a	3.05ab	2.85b	0.069	<0.01	<0.01	0.04
NH ₃ -N (mmol d ⁻¹)	7.34a	6.81ab	6.40b	6.26b	0.217	<0.01	<0.01	0.38

NOTE: Means within the same row with different letters differ at the $P < 0.05$. L, linear; Q, quadratic; C, cubic.

^aindividual SCFA concentrations are reported as percentage of total SCFA.