Test of conditions that affect in vitro production of volatile fatty acids and gases¹

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ABSTRACT: In vitro methods have been developed to measure digestibility, but such methods may not accurately reflect gas production or volatile fatty acid (VFA) profiles. The objective of this study was to determine the effect of different in vitro conditions on VFA and gas production. The experimental design was a $4 \times 2 \times 2$ factorial CRD with four replicates. Treatments were four ratios of medium to rumen fluid by volume (5:95, 25:75, 50:50, and 75:25), two concentrations (w/v) of added timothy hay (0.5% or 1%), with or without added sodium acetate (increased initial concentration by 50 mM). Total volume of medium and rumen fluid was 10 mL per tube. Measurements of gas production and VFA were recorded at 0, 4, 16, 24, and 48 h. Statistical analyses used a mixed model including all fixed effects and interactions with tube as a random effect, and time nested within tube. Total gas production increased

($P < 0.001$) with higher medium proportion. The final pH increased ($P < 0.0001$) as medium proportion increased. Medium proportion positively affected ($P < 0.05$) overall average concentration of both acetate production and propionate production. Higher hay concentration increased ($P < 0.0001$) total gas produced from 0 to 48 h, increased total acetate production ($P < 0.01$), propionate production ($P < 0.001$), and decreased pH between 24 and 48 h ($P < 0.0001$). Sodium acetate addition increased ($P < 0.0001$) pH between 24 and 48 h. Acetate:propionate (A:P) concentration decreased over time ($P < 0.0001$). Initial rumen fluid A:P ratio was 3.7 but average A:P ratio of produced VFA started at 2.2 and increased to 2.50 ($SE = \pm 0.51$). The A:P ratio differed for VFA produced in vitro compared to initial rumen fluid, but no tested treatments were found to change A:P ratio.

Key words: fermentation gases, in vitro procedures, methane, rumen fermentation, sodium acetate, volatile fatty acids

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INTRODUCTION

Increasing greenhouse gas emissions are a growing concern. Agriculture accounts for 7.7% of total U.S. greenhouse gas emissions (US EPA, 2014). Of all the greenhouse gas emissions, methane is the second leading source in the United States (Kebreab et al., 2008). Among ruminants in the United States, dairy cattle and beef cattle are

responsible for 25% and 71% of enteric methane emissions, respectively (US EPA, 2014). Nearly two-thirds (~60%) of the anthropogenic sources of methane in the world are derived from agriculture (Moss et al., 2000), with about 44% of global anthropogenic methane emissions coming from livestock (Gerber et al., 2013).

Although the most reliable measurements of enteric methane and carbon dioxide emissions are from animals placed in chambers, development of in vitro methods would facilitate replication of multiple treatments, and enable isolation of fermentation effects from animal interactions. In vitro methods have been developed to measure digestibility, but such methods may not accurately

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reflect gas and VFA production. Available in vitro methods focus on the digestibility of a sample (Goering and Van Soest, 1970), or cost-effective feed evaluation to determine nutritive quality (Dijkstra et al., 2005).

Rumen fermentation results in the production of three main volatile fatty acids (VFA): acetate, propionate, and butyrate, primarily via the conversion of glucose derived from plant biomass. Gas production is stoichiometrically linked with VFA profiles. For example, most glucose is fermented through a pathway that produces two acetates, two CO₂, and four H₂ molecules per molecule of glucose, and the H₂ is used to convert glucose into propionate, or CO₂ and H₂ are used to make CH₄. Although the fermentation pathways are limited in part by thermodynamics (Ungerfeld and Kohn, 2006), the profile of products (e.g., gases or VFA) could influence subsequent pathways.

The goal of this research was to develop an in vitro technique to study ruminal metabolism related to methane and VFA production. This first study compares different conditions of the fermentation on VFA and gas production. We hypothesize that the addition of different substrates or products (e.g., feed, acetate, and buffer) could bring about changes in the profile of products formed. For example, we hypothesize that addition of acetate into a rumen fermentation medium will shift fermentation away from acetate and toward propionate and butyrate in accordance with thermodynamic control.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) [398173-1].

Experimental Design and Treatments

The experimental design was a 4 × 2 × 2 factorial CRD; the treatments were ratios of medium:rumen fluid (calculated by volume at ratios of 5:95, 25:75, 50:50, and 75:25), Timothy hay amount (0.05 vs. 0.10 g), and addition of sodium acetate or not (NaOAc). Each treatment was replicated four times. Total volume of medium and inoculum was 10 mL per 20 mL Hungate tube.

Rumen Fluid Collection and Sample Preparation

Rumen fluid was collected from a permanently nonlactating rumen-cannulated cow consuming a timothy hay diet and was prepared

according to Goering and Van Soest (1970). Approximately 0.5–1 L of rumen contents (solids and liquid) were collected anaerobically in 50-mL centrifuge tubes. Rumen fluid was blended for 20 s under CO₂, and was strained through four layers of cheesecloth and glass wool into a 1 L flask infused with CO₂.

Timothy hay was pre-weighed (0.05 or 0.10 g) into labeled 20 mL Hungate glass tubes with rubber stoppers and screw caps. The Timothy hay was measured on a Mettler Toledo AE260 Delta Range (Columbus, OH) 4-place balance. Average weight for the 0.5 g Timothy hay and 0.10 g Timothy hay were 0.053 g (SD = 0.001) and 0.104 g (SD = 0.002), respectively. In vitro buffered medium was prepared, perfused with CO₂, and reduced with reducing agent as previously published (Goering and Van Soest, 1970). The Hungate tubes were perfused with CO₂ while the different amounts of buffered medium were added according to each treatment in random order. Each tube receiving added acetate treatment received 0.5 mL of 1 M sodium acetate (increasing starting concentration of acetate in these treatments by 50 mM), and those tubes without added acetate received an additional 0.5 mL in vitro medium. Processed rumen fluid (as described above) was added to each tube in random order, while infusing tubes with CO₂ and stirring the rumen fluid using a magnetic stir bar. Each tube was then sealed with a stopper and screw cap, and inverted. A 20 mL gas-tight syringe and needle were inserted into the rubber stopper at the top of each tube for measurement of gas production. The 20 mL syringes had tick marks at 0.2 mL intervals. Tubes were subsequently incubated at 39 °C.

Gas was removed and liquid in each tube was sampled before placing the samples into the incubator (39 °C). Liquid samples for volatile fatty acids (VFAs) were collected by inverting the in vitro tube, allowing the substrate to settle, and using a 27-gauge needle and syringe to withdraw 1 mL of sample. The sample was then expelled into a 1.5 mL microcentrifuge tube and frozen in a –20 °C freezer for further analysis. There was no correction for removal of fluids as this could impose a bias on the concentrations as existing VFA and some substrate would be removed. Nonlactating and lactating cows have mean retention times of approximately 20 to 24 h on high concentrate diets, and approximately 30 h on high forage diets, (Hartnell and Satter, 1979; Ishler et al., 1996); thus measurements of gas production and VFAs were recorded at 0, 4, 16, 24, and 48 h.

Gas Measurement

Gas production was measured at 39 °C in mL and converted to μmol by dividing the average gas produced for each treatment by 25.6 mL/mmol and multiplying by 1000 $\mu\text{mol}/\text{mmol}$ per the ideal gas law. Gas was recorded at each time point, the produced gas was then expelled from the syringe, and the syringe was screwed back onto the needle. The nonCO₂ gas was measured at 48 h by expelling produced gas from syringe into a Wheaton bottle containing 40 mL of 6 N NaOH. The bottle was vigorously shaken for 30 s and the remaining gas was measured by allowing the syringe to expand. The values were recorded as a ratio of nonCO₂ to total produced gas per sample and converted to μmol units. Previous experiments found using gas chromatography that nearly all fermentation gas was either CO₂ or methane.

VFA Analysis

The VFA samples were prepared using a modified Erwin et al. (1961) method. VFA samples were thawed at room temperature then spun in a centrifuge at 12,000 $\times g$ for 30 min at 4 °C. Phosphoric acid (10% H₃PO₄) was added to the supernatant of each sample. VFA concentrations were measured using gas chromatography (Hewlett-Packard model 6890) with a 4.6 m length $\times$ 0.318 cm outer diameter $\times$ 2.1 mm inner diameter packed GC column (60/80 Carboxen-1000 support, model 1-2390, Supelco, Inc, Bellefonte, PA) and flame ionization detector (FID). The split ratio of the injector port (220 °C) was 100:1. Helium was used as a carrier gas with a flow of 40 mL/min. The initial column temperature was 130 °C held for 10 min, then increased to 200 °C (ramp of 80 °C/min) for 1 min, and a post-run temperature of 120 °C. The detector temperature was 200 °C with a hydrogen and airflow of 40 mL/min and 200 mL/min, respectively. VFA production is reported as the change in concentration at each interval.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 11 (JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989–2007). Two mixed models were used. The first was a mixed model: $y_{ijklm} = \mu + H_i + B_j + A_k + T_l + \gamma_{ijkl(m)} + \varepsilon_{ijklm}$ for response variables measured over time within tubes. The second model was: $y_{ijkl} = \mu + H_i + B_j + A_k + \gamma_{ijkl(l)} + \varepsilon_{ijkl}$ where $y_{ijkl(l)}$ for

response variables measured only once. For each model, Y is the response, μ is the mean of the population, H_i is the effect of hay, 0.05 or 0.10 grams, B_j is the effect of the buffer/rumen fluid concentration at levels of 5:95, 25:75, 50:50, and 75:25, A_k is the effect of acetate, with or without 50 mM addition, T_l is time measured at 0, 4, 16, 24, and 48 h, $\gamma_{ijk(l)}$ is the random effect of the tube nested in treatment (hay, buffer, and acetate combinations or hay, buffer, acetate, and time), and $\varepsilon_{ijkl(m)}$ is the residual effect. All interactions were included in each model and time was continuous. This model measured the effect of treatment on total gas production, pH, and VFA production over time

RESULTS AND DISCUSSION

Effect of Buffered Media

Increasing ratio of medium:inoculum increased gas production after 4 h and resulted in higher total gas production (Table 1). Gas production is affected by fermentation and the presence of bicarbonate buffer (Getachew et al., 1998). Our results differed from a study in which the amount of gas produced increased as the concentration of rumen fluid increased (Rymer et al., 1999). We found the opposite effect. Rumen fluid in the cow is usually equilibrated with less than 1 atm CO₂ because of the presence of methane, but the in vitro buffer we used was equilibrated with 1 atm CO₂. Thus, having more bicarbonate buffer would allow for more CO₂ to be released (Kohn and Dunlap, 1998).

The gas collected at the end of the fermentation comprised original CO₂ that was not flushed out and produced gases. We measured the fraction of the final gas that was CO₂ and attributed the remainder to the nonCO₂ fraction. In previous in vitro experiments, we have observed that nearly all the nonCO₂ gas in the fermentation is methane. Both the nonCO₂ gas and CO₂ gas numerically increased with higher medium percentage (Table 1). If the main reason for an increased gas production from the higher proportion of medium was merely the evolution of CO₂ from buffer, we would have expected the nonCO₂ fraction to have been diluted. The nonCO₂ fraction did not decrease in the treatments with more medium, and the increase in gas production occurred increasingly at later time points, suggesting that the evolution of CO₂ from buffer may not be a complete explanation for why gas production was higher in treatments with more media.

Table 1. Main effect of medium:inoculum on gas and VFA production, and pH over 48 h

Treatment						
Medium, % ¹						
Gas Production	5	25	50	75	SEM	P
Total Gas (μmol) ²	249 ^c	357 ^b	437 ^a	438 ^a	12.9	< 0.01
Total Gas/hay (μmol/g) ³	3418 ^c	4863 ^b	5944 ^a	5825 ^a	207.8	< 0.01
Final Gas (μmol) ⁴	31 ^c	62 ^b	90 ^a	77 ^{ab}	6.1	< 0.01
Fractional NonCO ₂ (μmol/μmol) ⁵	0.41	0.23	0.31	0.23	0.07	0.36
Total VFA Production and pH						
Acetate (mM)	14 ^b	20 ^{ab}	22 ^a	17 ^{ab}	1.0	< 0.05
Propionate (mM)	5.6	7.7	8.9	7.3	0.84	0.07
Butyrate (mM)	6.0	7.0	4.9	12.0	6.70	0.39
Total VFA (mM)	28	39	44	42	5.3	0.14
Acetate/Propionate ⁶ (mM/mM)	2.5	2.6	2.6	2.3	0.13	0.19
Acetate/Butyrate (mM/mM)	4.9	5.9	6.6	4.8	0.70	0.22
pH at 48 h	5.6 ^d	5.9 ^c	6.3 ^b	6.6 ^a	0.01	< 0.01

^{a-d}Within a row, means without a common superscript differ ($P < 0.05$)

¹Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

²Total Gas is the gas produced between 0 and 48 h

³Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)

⁴Final gas is the gas produced between 24 and 48 h

⁵NonCO₂ is a fractional value of nonCO₂ gas divided by total gas produced

⁶Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

The pH at 48 h increased as buffer concentration increased, resulting from greater buffering capacity of the treatment with higher ratio of medium:inoculum (Table 1). This increase in pH is in agreement with findings from other studies (Tripathi et al., 2004; González et al., 2008). Tripathi et al. (2004) found pH to range from 6.03 (0% bicarbonate) to 6.44 (2.25% bicarbonate), whereas in González et al. (2008), pH ranged from 5.91 (0% bicarbonate) to 6.38 (5% bicarbonate). The pH in this study is higher, compared to another study (Erdman, 1988), which may be due to the greater buffering capacity of treatments with higher ratios of medium:inoculum. It is possible in the present study that the treatments with higher ratios of medium:inoculum had higher pH as a result of the presence of the sodium bicarbonate and less rumen fluid. Studies have shown that when pH is lower than 6.0 the buffering capacity for bicarbonate is reduced due to having an effective pKa of 6.7 (Terry et al., 1969; Russell, 1998). The tubes with more rumen fluid had lower pH than tubes with less rumen fluid and this may have resulted from a slight reduction in buffering capacity for those.

VFA production was calculated as the change in concentration at each interval for each treatment. Acetate and propionate production (mM) increased ($P < 0.05$) as media concentration increased (Table 1). Initial acetate concentrations were: 59, 55, 47, and 38 mM (SE $\pm$ 0.8) for the 5,

25, 50, and 75% medium treatments, respectively. The initial propionate concentrations for the 5, 25, 50, and 75% medium treatments were: 13, 12, 10, and 8 mM (SE $\pm$ 0.2). These results are similar to findings that showed that increasing the ratio of medium:inoculum also increased the production of volatile fatty acids (González et al., 2008). Table 2 shows VFA production over time for the buffer treatment. There was an effect ($P < 0.01$) of buffer on acetate production between 4 and 16 h and between 24 and 48 h. Propionate production increased ($P < 0.01$) from 4 to 16 h, and between 16 and 24 h. There was no effect on the butyrate production, acetate:propionate (A:P) ratio, or acetate:butyrate (A:B) ratio over time by the buffer treatment.

Effect of Hay

As expected, gas production was greater ($P < 0.01$) with the higher concentration of hay in the tube (Table 3), but gas production (μmol/g) per unit hay was higher ($P < 0.05$) for the lower concentration of hay. Since gas is produced from both the hay substrate and additional substrate from the rumen fluid, and dividing by hay only corrects for the amount of hay, a higher gas/hay was expected for the lower hay concentration.

Between 24 and 48 h, pH decreased ($P < 0.01$) with increased hay concentration (Table 3) as

Table 2. Effect of medium:inoculum on the production of gas and VFAs by time¹

	Treatment					
	Medium, (%) ²					<i>P</i>
	5	25	50	75	SEM	
Gas (μmol)						
Initial Concentration (T = 0)	0	0	0	0	—	—
0–4 h	107 ^a	113 ^a	90 ^a	36 ^b	8.2	<0.01
4–16 h	104 ^d	155 ^c	222 ^b	282 ^a	8.4	<0.01
16–24 h	7 ^c	27 ^b	37 ^{ab}	42 ^a	3.9	<0.01
24–48 h	31 ^c	62 ^b	90 ^a	77 ^{ab}	6.1	<0.01
Acetate (mM)						
Initial Concentration (T = 0)	52 ^a	45 ^b	36 ^c	28 ^d	1.1	<0.01
0–4 h	3.9	4.7	3.8	2.4	0.69	0.14
4–16 h	3.8 ^b	7.4 ^{ab}	9.6 ^a	8.5 ^a	0.95	<0.01
16–24 h	3.2	2.2	2.6	4.1	1.30	0.74
24–48 h	4.0 ^{ab}	5.8 ^{ab}	6.4 ^a	1.8 ^b	1.07	<0.05
Propionate (mM)						
Initial Concentration (T = 0)	10.3 ^a	8.4 ^b	5.9 ^c	4.1 ^d	0.4	<0.01
0–4 h	1.4	1.7	1.6	1.1	0.17	0.06
4–16 h	1.1 ^b	2.3 ^a	3.4 ^a	3.2 ^a	0.31	<0.01
16–24 h	1.1	1.1	1.4	1.4	0.33	0.87
24–48 h	1.8 ^a	2.5 ^a	2.5 ^a	1.6 ^a	0.27	<0.05
Butyrate (mM)						
Initial Concentration (T = 0)	12.3	10.3	4.1	4.8	3.17	0.20
0–4 h	2.4	2.0	0.8	1.3	0.75	0.48
4–16 h	1.5	2.8	2.2	7.6	1.70	0.06
16–24 h	0.8	0.5	0.7	1.5	0.48	0.48
24–48 h	1.7	1.7	1.1	1.8	0.59	0.85
Total VFA(mM)						
Initial Concentration (T = 0)	80 ^a	68 ^a	49 ^b	40 ^b	3.8	<0.01
0–4 h	8.1	8.9	7.5	4.6	1.34	0.13
4–16 h	7 ^b	15 ^{ab}	20 ^a	24 ^a	2.7	<0.01
16–24 h	5.5	4.0	5.0	8.2	2.02	0.52
24–48 h	8	11	11	6	1.7	0.09
Acetate/Propionate (mM/mM)³						
0–4 h	2.0	2.2	2.4	2.1	0.52	0.97
4–16 h	3.3	2.8	2.9	2.7	0.28	0.40
16–24 h	2.9	1.7	1.9	2.7	0.80	0.66
24–48 h	2.1	1.5	2.6	4.5	1.11	0.26
Acetate/Butyrate (mM/mM)						
0–4 h	4.2	5.3	6.9	6.0	1.07	0.35
4–16 h	5.7	5.9	6.3	4.1	0.90	0.30
16–24 h	6.6	8.7	6.0	9.2	2.34	0.72
24–48 h	5.0	5.5	6.9	6.5	0.93	0.47

^{a–d}Within a row, means without a common superscript differ ($P < 0.05$)

¹VFA production is calculated as the change in concentration between each interval

²Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

³Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

expected since more acid would be produced from the greater amount of substrate.

Acetate production (mM; Table 3) and propionate production (mM; Table 3) increased ($P < 0.01$) as hay concentration increased. Initial acetate and propionate concentrations at 0.5 g and

0.10 g Timothy hay were 48 and 50 mM (acetate; SE $\pm$ 0.6) and 9.7 and 11.8 mM (propionate; SE $\pm$ 0.13). Table 4 illustrates the effect of hay on VFA production over time. Acetate production was higher ($P < 0.05$) with the higher hay concentration (Table 4) and propionate production

Table 3. Main effects of hay on gas and VFA production, and pH over 48 h

	Treatment			
	Hay (g) ¹		SEM	P
Gas Production	0.05	0.1		
Total Gas (μmol) ²	262 ^b	478 ^a	9.1	< 0.01
Total Gas/hay ($\mu\text{mol/g}$) ³	5244 ^a	4781 ^b	146.9	< 0.05
Final Gas (μmol) ⁴	34 ^b	96 ^a	4.3	< 0.01
Fractional Non-CO ₂ ($\mu\text{mol}/\mu\text{mol}$) ⁵	0.28	0.31	0.05	0.66
Total VFA Production and pH				
Acetate (mM)	14.6 ^b	21.9 ^a	1.53	< 0.01
Propionate (mM)	5.7 ^b	9.1 ^a	0.59	< 0.01
Butyrate (mM)	4	4.1	1.21	0.95
Total VFA (mM)	18	22	1.96	0.13
Acetate/Propionate (mM/mM) ⁶	2.6	2.4	0.09	0.17
Acetate/Butyrate (mM/mM)	5.0	6.1	0.47	0.10
pH at 48 h	6.2 ^a	6.0 ^b	0.01	< 0.01

^{a,b}Within a row, means without a common superscript differ ($P < 0.05$)

¹Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

²Total Gas is the gas produced between 0 and 48 hours

³Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)

⁴Final gas is the gas produced between 24 and 48 h

⁵Non-CO₂ is a fractional value of non-CO₂ gas divided by total gas produced

⁶Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

(Table 4) also increased ($P < 0.05$) with increasing time interval and was highest with the higher concentration of hay. There was no effect of the concentration of hay on total VFA, A:P, or A:B ratio over time. Studies have shown that the form of digestible energy can affect the volatile fatty acid (VFA) concentrations in the rumen (Sutton et al., 2003).

Effect of Acetate

The addition of acetate was hypothesized to shift fermentation away from acetate, which might have decreased acetate production and concomitant gas production. Acetate addition did not affect total gas production (Table 5), and surprisingly increased gas production between 24 to 48 h ($P < 0.05$).

The addition of 50 mM sodium acetate (Table 5) increased ($P < 0.01$) pH from 24 to 48 h, also as expected the acetate salt ($\text{pKb} = 9.25$) acts as an additional buffer. At lower pH, the use of hydrogen for propionate production could decrease the availability of hydrogen for methane production (Johnson and Johnson, 1995; Janssen, 2010; Zijderfeld et al., 2010). The acetate added can decrease acetate production by thermodynamics. Additionally, the acetate itself could be interconverted to other VFAs or methane (Ungerfeld and Kohn, 2006).

Increased medium (%) and acetate increased total gas production between 24 and 48-h

Figure 1. The effect of increasing ratio of medium:inoculum (% by volume) and sodium acetate addition (50 mM NaOAc) on gas production (μmol) between 24 and 48 h. Medium:inoculum values are reported as percentage of sodium bicarbonate buffered medium by volume in relation to rumen fluid: 5% medium and 95% rumen fluid, 25% medium and 75% rumen fluid, 50% medium and 50% rumen fluid, and 75% medium and 25% rumen fluid. Gas production increased as medium increased with acetate vs. (33, 46, 76, 76 μmol) without acetate; SE = ± 10.2 μmol . Significance was determined at $P < 0.05$ and a trend at $P < 0.10$. Multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean $\pm$ S.E. and means with different letters (a, b, c, and d) are significantly different.

Table 4. Effect of hay on the production of gas and VFAs by time¹

	Treatment			
	Hay (g) ²		SEM	P value
	0.05	0.1		
Gas (μmol)				
Initial Concentration (T = 0)	0	0	—	—
0–4 h	65 ^b	108 ^a	6	< 0.01
4–16 h	149 ^b	233 ^a	6	< 0.01
16–24 h	14 ^b	43 ^a	2.8	< 0.01
24–48 h	34 ^b	96 ^a	4.3	< 0.01
Acetate (mM)				
Initial Concentration (T = 0)	48 ^b	51 ^a	0.56	< 0.01
0–4 h	3.0 ^b	4.4 ^a	0.49	< 0.05
4–16 h	6.3 ^b	8.3 ^a	0.69	< 0.05
16–24 h	2.7	3.3	0.90	0.65
24–48 h	3.0 ^b	6.0 ^a	0.74	< 0.01
Propionate (mM)				
Initial Concentration (T = 0)	10 ^b	12 ^a	0.13	< 0.01
0–4 h	1.1 ^b	1.8 ^a	0.12	< 0.01
4–16 h	2.0 ^b	3.1 ^a	0.22	< 0.01
16–24 h	1.0	1.5	0.23	0.15
24–48 h	1.5 ^b	2.8 ^a	0.19	< 0.01
Butyrate (mM)				
Initial Concentration (T = 0)	14	10	3.2	0.49
0–4 h	1.6	1.6	0.53	0.94
4–16 h	3.2	3.9	1.20	0.71
16–24 h	1.3	0.5	0.34	0.08
24–48 h	1.3	1.8	0.42	0.39
Total VFA(mM)				
Initial Concentration (T = 0)	78	79	3.5	0.87
0–4 h	6.5	8.0	0.95	0.26
4–16 h	15	18	1.9	0.21
16–24 h	5.7	5.6	1.39	0.96
24–48 h	6.8 ^b	11.5 ^a	1.17	< 0.01
Acetate/Propionate (mM/mM)²				
0–4 h	2.0	2.4	0.37	0.52
4–16 h	3.3 ^a	2.6 ^b	0.20	< 0.05
16–24 h	3.1 ^a	1.4 ^b	0.58	< 0.05
24–48 h	3.3	2.1	0.77	0.26
Acetate/Butyrate (mM/mM)				
0–4 h	4.8	6.5	0.76	0.12
4–16 h	5.5	5.5	0.65	0.95
16–24 h	6.3	8.9	1.70	0.31
24–48 h	6.2	5.8	0.65	0.67

^{a,b}Values within a row with different superscripts are statistically different ($P < 0.05$)

¹VFA production is calculated as the change in concentration between each interval

²Hay values represent the levels of substrate used; 0.5 or 0.10 g of Timothy hay

³Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Although A:P concentrations decreased ($P < 0.01$) over time (data not shown), there was no effect of acetate addition on the production ratio of A:P, or A:B over time (Table 6). These findings were contrary to our hypothesis that addition of acetate would lead to a shift in fermentation away from acetate and towards propionate and butyrate and

that fermentation conditions will affect the ratio of produced VFA and gas profiles. One might expect in the presence of sodium propionate or sodium butyrate that VFA profiles (overall concentrations) would differ from these findings. Sodium propionate may increase the A:P ratio by decreasing propionate production and sodium butyrate may

Table 5. Main effect of sodium acetate on gas production, VFA production, and pH

	Treatment			
	Acetate (mM) ¹		SEM	P
Gas Production	No	Yes		
Total Gas (μmol) ²	375	366	9.1	0.49
Total Gas/hay (μmol/g) ³	5120	4905	146.9	0.3
Final Gas (μmol) ⁴	58 ^b	72 ^a	4.3	< 0.05
Fractional NonCO ₂ (μmol/μmol) ⁵	0.35	0.23	0.05	0.12
Total VFA Production and pH				
Acetate (mM)	18.3	18.2	1.53	0.94
Propionate (mM)	7.3	7.4	0.59	0.90
Butyrate (mM)	7.1	7.9	2.24	0.81
Total VFA (mM)	39	38	3.8	0.91
Acetate/Propionate (mM/mM) ⁶	2.6	2.4	0.09	0.36
Acetate/Butyrate (mM/mM)	6.1	5.1	0.47	0.14
pH at 48 h	6.08 ^b	6.13 ^a	0.01	< 0.01

^{a,b}Within a row, means without a common superscript differ ($P < 0.05$)

¹Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

²Total Gas is the gas produced between 0 and 48 h

³Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)

⁴Final gas is the gas produced between 24 and 48 h

⁵NonCO₂ is a fractional value of non-CO₂ gas divided by total gas produced

⁶Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

increase the A:B ratio by decreasing butyrate production. The initial rumen fluid A:P ratio was 3.7 but the A:P ratio of VFA produced averaged 2.5 (SE = ±0.51).

Effect of Treatment Interactions

There was a tendency ($P < 0.10$) for an interaction between acetate treatment by ratio of medium:inoculum treatment on gas production between the 24 to 48 h interval (Figure 1). Gas production between 24 to 48 h increased with acetate addition.

There was an interaction ($P < 0.01$) of hay with the ratio of medium:inoculum on total gas production (Figure 2A) and a tendency ($P < 0.10$) on gas production between 24 to 48 h (Figure 2B). As the concentration of timothy hay doubled and medium proportion increased, total gas production and gas production between 24 and 48 h increased. Total gas increased ($P < 0.01$) as ratio of medium:inoculum increased in the higher hay concentration compared to the lower hay concentration.

There was an acetate treatment by hay concentration interaction on pH (Figure 3A). The lower hay concentration had higher average pH with added acetate than without. There also was an interaction of the ratio of medium:inoculum with acetate addition (Figure 3B) on pH ($P < 0.01$).

The pH was lower without acetate addition for the low ratio of medium:inoculum. The pH was also affected by the buffer by hay interaction (Figure 3C) with ratio of medium:inoculum and was depressed more for the high concentration of hay when the ratio of medium:inoculum was low. There would be a greater effect of bicarbonate buffering in the treatment with higher ratio of medium:inoculum when there was a higher concentration of hay and more need for buffering.

Fermentation may be regulated by kinetic control when concentrations of products are limited and activities of substrates and enzymes determine rates of individual reactions and profile of products (Ungerfeld and Kohn, 2006; Kohn, 2007). Most fermentation systems, however, are often near thermodynamic equilibrium in which the product accumulation regulates which pathways are available (Kohn and Kim, 2015). Focus in biology has often been on the kinetic regulation of fermentation, though recent studies have shown that thermodynamic regulation of rumen fermentation is also important (Kohn, 2014). For example, in the present study, products like CO₂ and acetate could affect the thermodynamic feasibility of reaction pathways producing these products in the fermentation system. When flooding the system with sodium acetate, we are perturbing the in vitro system away from thermodynamic control (equilibrium); therefore,

Table 6. Effect of acetate on the production of gas and VFAs by time¹

	Treatment			
	Acetate (mM) ²		SEM	<i>P</i>
	N	Y		
Gas (μmol)				
Initial Concentration (T = 0)	0	0	—	—
0–4 h	103 ^a	70 ^b	6	<0.05
4–16 h	190	192	6	0.89
16–24 h	24 ^b	33 ^a	2.8	<0.05
24–48 h	58 ^b	72 ^a	4.3	<0.05
Acetate (mM)				
Initial Concentration (T = 0)	36 ^b	63 ^a	0.6	<0.01
0–4 h	3.5	3.9	0.49	0.52
4–16 h	7.0	7.6	0.70	0.51
16–24 h	2.5	3.5	0.90	0.46
24–48 h	5.4	3.7	0.74	0.10
Propionate (mM)				
Initial Concentration (T = 0)	10.9	10.6	0.13	0.07
0–4 h	1.5	1.4	0.12	0.70
4–16 h	2.4	2.6	0.22	0.49
16–24 h	1.1	1.4	0.23	0.27
24–48 h	2.2	2.1	0.19	0.63
Butyrate (mM)				
Initial Concentration (T = 0)	11	13	3.2	0.76
0–4 h	1.7	1.5	0.53	0.83
4–16 h	3.3	3.8	1.23	0.76
16–24 h	0.6	1.2	0.34	0.23
24–48 h	1.6	1.5	0.43	0.84
Total VFA (mM)				
Initial Concentration (T = 0)	65 ^b	92 ^a	3.5	<0.01
0–4 h	7.2	7.3	0.95	0.91
4–16 h	16	17	1.9	0.87
16–24 h	4.7	6.6	1.39	0.32
24–48 h	10	8	1.2	0.16
Acetate/Propionate (mM/mM)²				
0–4 h	2.3	2.1	0.37	0.70
4–16 h	3.0	2.9	0.20	0.68
16–24 h	2.6	2.0	0.58	0.47
24–48 h	2.5	2.9	0.77	0.71
Acetate/Butyrate (mM/mM)				
0–4 h	5.7	5.5	0.76	0.86
4–16 h	6.1	4.9	0.60	0.17
16–24 h	8.5	6.7	1.70	0.46
24–48 h	6.3	5.6	0.66	0.45

^{a,b}Values within a row with different superscripts are statistically different (P < 0.05)

¹VFA production is calculated as the change in concentration between each interval

²Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc. Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

allowing us to evaluate the kinetics for the return to equilibrium.

Summary

This study evaluated the effect of starting conditions on VFA and gas production in vitro. We

looked at the effects of different ratios of medium:inoculum, substrate (Timothy hay) concentrations, with or without 50 mM sodium acetate addition. This study found that differing ratios of medium:inoculum affect gas production and VFA profile. Higher ratios of medium:inoculum produced more gas. The higher concentration of

Figure 2. The effect of increasing ratio of medium:inoculum (% by volume) and Timothy hay (g) on: (A) total gas production (186, 258, 314, and 290 μmol average per treatment) for lower hay concentration versus (312, 456, 560, and 585 μmol average per treatment) for higher hay concentration; $\text{SE} = \pm 21.0$; and (B) gas production (μmol) between 24 and 48 h (10, 34, 46, and 46 μmol average per treatment) for lower hay concentration versus (53, 89, 133, and 108 μmol average per treatment) for higher hay concentration, $\text{SE} = \pm 10.2$ μmol . Significance was determined at $P < 0.05$ and a trend at $P < 0.10$. Multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean $\pm$ S.E. and means with different letters (a, b, c, and d) are significantly different.

substrate also produced more gas and increased acetate and propionate production. Most surprising was that the addition of sodium acetate did not affect gas or VFA production.

To effectively develop a method to measure VFA and gases, future studies need to further elucidate the in vitro system environment. Other factors that can potentially affect VFA and gas production may include headspace gas composition.

Figure 3. The effect of (A) sodium acetate addition (NaOAc, 50mM) and hay on pH (6.3 vs. 6.0) with acetate and (6.2 vs. 6.0) without acetate, $\text{SE} = \pm 0.01$; (B) ratio of medium:inoculum and acetate addition on pH (5.7, 6.0, 6.3, and 6.6) with acetate vs. (5.5, 5.9, 6.3, and 6.6) without acetate, $\text{SE} = \pm 0.02$; and (C) ratio of medium:inoculum and hay (5.8, 6.1, 6.4, and 6.6) for lower concentration of hay vs. (5.5, 5.7, 6.2, and 6.5) for higher hay concentration, $\text{SE} = \pm 0.02$. Significance was determined at $P < 0.05$ and multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean $\pm$ S.E. and means with different letters (a, b, c, and d) are significantly different.

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REFERENCES

- Dijkstra, J., E. Kebreab, A. Bannink, J. France, and S. López. 2005. Application of the gas production technique to feed evaluation systems for ruminants. *Anim. Feed Sci. Technol.* 123–124(Part 1):561–578. doi:10.1016/j.anifeedsci.2005.04.048
- Erdman, R.A. 1988. Dietary buffering requirements of the lactating dairy cow: a review. *J. Dairy Sci.* 71:3246–3266. doi:10.3168/jds.S0022-0302(88)79930-0. Available from <http://www.sciencedirect.com/science/article/pii/S0022030288799300>
- Erwin, E. S., G. J. Marco, and E. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44:1768–1771.
- Gerber, P.J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013. Tackling climate change through livestock- A global assessment of emissions and mitigation. Rome: Food and Agriculture Organization of the United Nations (FAO).
- Getachew, G., M. Blümmel, H.P. S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. *Anim. Feed Sci. Technol.* 72:261–281. doi:10.1016/S0377-8401(97)00189-2
- Goering, H.K., and P.J. Van Soest. 1970. Forage fiber analysis (apparatus reagents, and some applications). Washington, D.C.: United States Department of Agriculture.
- González, L., A. Ferret, X. Manteca, and S. Calsamiglia. 2008. Increasing sodium bicarbonate level in high-concentrate diets for heifers. II. Effects on chewing and feeding behaviors. *Animal.* 2:713–22. doi:10.1017/S1751731108001663. Available from <http://www.ncbi.nlm.nih.gov/pubmed/22443596>
- Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time and ruminal turnover rates of ingesta at different stages of lactation in dairy cows. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 48:381–392.
- Ishler, V., J. Heinrichs, and G. Varga. 1996. From feed to milk: understanding rumen function. *Ext. Circ.* 422:1–32.
- Janssen, P.H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160:1–22. doi:10.1016/j.anifeedsci.2010.07.002. Available from <http://dx.doi.org/10.1016/j.anifeedsci.2010.07.002>
- Johnson, K.A., and D.E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73:2483–2492.
- Kebreab, E., K.A. Johnson, S.L. Archibeque, D. Pape, and T. Wirth. 2008. Model for estimating enteric methane emissions from United States dairy and feedlot cattle. *J. Anim. Sci.* 86:2738–2748. doi:10.2527/jas.2008-0960
- Kohn, R.A. 2007. Thermodynamics and kinetics of metabolism, gene regulation, and evolution. In: Hanigan, M.D., J.A. Novotny, and C.L. Marsteller, editors. *Mathematical Modeling in Nutrition and Agriculture*. Blacksburg, VA: Virginia Tech University; p. 293–312.
- Kohn, R.A. 2014. Thermodynamic and kinetic control of methane emissions from ruminants. In: Malik, P.K., R. Bhatta, J. Takahashi, R.A. Kohn, and C.S. Prasad, editors. *Livestock production and climate change*. Boston, MA: CAB International; p. 245–262.
- Kohn, R.A., and T.F. Dunlap. 1998. Calculation of the buffering capacity of bicarbonate in the rumen and in vitro. *J. Anim. Sci.* 76:1702–1709.
- Kohn, R.A., and S.-W. Kim. 2015. Using the second law of thermodynamics for enrichment and isolation of microorganisms to produce fuel alcohols or hydrocarbons. *J. Theor. Biol.* 1–7. doi:10.1016/j.jtbi.2015.07.019. Available from <http://linking-hub.elsevier.com/retrieve/pii/S0022519315003562>
- Moss, A.R., J.P. Jouany, and J. Newbold. 2000. Methane production by ruminants : its contribution to global warming. *Ann. Zootech.* 49:231–253.
- Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *J. Dairy Sci.* 81:3222–3230. doi:10.3168/jds.S0022-0302(98)75886-2
- Rymer, C., J.A. Huntington, and D.I. Givens. 1999. Effects of inoculum preparation method and concentration, method of inoculation and pre-soaking the substrate on the gas production profile of high temperature dried grass. *Anim. Feed Sci. Technol.* 78:199–213. doi:10.1016/S0377-8401(99)00006-1
- Sutton, J.D., M.S. Dhanoa, S. V Morant, J. France, D.J. Napper, and E. Schuller. 2003. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *J. Dairy Sci.* 86:3620–3633. doi:10.3168/jds.S0022-0302(03)73968-X
- Terry, R.A., J.M. A. Tilley, and G.E. Outen. 1969. Effect of pH on cellulose digestion under in vitro conditions. *J. Sci. Food Agric.* 20:317–320.
- Tripathi, M.K., A. Santra, O.H. Chaturvedi, and S.A. Karim. 2004. Effect of sodium bicarbonate supplementation on ruminal fluid pH, feed intake, nutrient utilization and growth of lambs fed high concentrate diets. *Anim. Feed Sci. Technol.* 111:27–39. doi:10.1016/j.anifeedsci.2003.07.004
- Ungerfeld, E.M., and R.A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: Sejrsen, K., T. Hvelplund, and M.O. Nielsen, editors. *Ruminant Physiology*. Wageningen, The Netherlands: Wageningen Academic Publishers; p. 55–86.
- US Environmental Protection Agency. 2014. Inventory of U.S. Greenhouse Gas Emissions and Sinks : 1990–2012.
- Zijderfeld, S.M. Van, W.J. J. Gerrits, J.A. Apajalahti, J.R. Newbold, J. Dijkstra, and R.A. Leng. 2010. Nitrate and sulfate : Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93:5856–5866. doi:10.3168/jds.2010-3281. Available from <http://dx.doi.org/10.3168/jds.2010-3281>