Association of aqueous hydrogen concentration with methane production in continuous cultures modulated to vary pH and solids passage rate

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ABSTRACT

The objective of this study was to evaluate the effects of altering pH and solids passage rate (k_p) on concentration of aqueous H_2 [$H_2(aq)$], CH_4 production, volatile fatty acids (VFA) production, and fiber digestibility in a continuous culture fermentation system. The present study was conducted as a 2×2 factorial treatment arrangement in a Latin square design using continuous culture fermentors ($n = 4$). Our continuous culture system was converted to a closed system to measure CH_4 and H_2 emission while measuring $H_2(aq)$ concentration and VFA production for complete stoichiometric assessment of fermentation pattern. Treatments were control pH (CpH; ranging from 6.3 to 6.9) or low pH (LpH; 5.8 to 6.4) factorized with solids k_p that was adjusted to be either low (Lk_p; 2.5%/h) or high (Hk_p; 5.0%/h); liquid dilution was maintained at 7.0%/h. Fermentors were fed once daily (40 g of dry matter; 50:50 concentrate:forage diet). Four periods lasted 10 d each, with 3 d of sample collection. The main effect of LpH increased nonammonia nitrogen flow, and both LpH and Hk_p increased nonammonia nonbacterial N flow. We observed a tendency for Hk_p to increase bacterial N flow per unit of nonstructural carbohydrates and neutral detergent fiber degraded. The main effect of LpH decreased $H_2(aq)$ by 4.33 μM compared with CpH. The main effect of LpH decreased CH_4 production rate from 5 to 9 h postfeeding, and Hk_p decreased CH_4 production rate from 3 to 9 h postfeeding. We found no effect of LpH on daily CH_4 production or CH_4 produced per gram of neutral detergent fiber degraded, but Hk_p decreased daily CH_4 production by 33.2%. Both the main effects of LpH and Hk_p decreased acetate molar percentage compared with CpH and Lk_p, respectively. The main effect of both LpH and Hk_p increased propionate molar percentage, decreasing acetate-to-propionate

ratio from 2.62 to 2.34. We noted no treatment effects on butyrate molar percentage or total VFA production. The results indicate increasing k_p and decreasing pH decreased acetate-to-propionate ratio, but only increasing k_p decreased CH_4 production; lack of differences for LpH might be a result of compensatory methanogenesis during the second half of the day postfeeding.

Key words: methane, continuous culture, passage rate, pH, hydrogen

INTRODUCTION

Greenhouse gases originating from livestock production are a target of environmental emissions mitigation strategies, but estimates of the contribution of enteric CH_4 to global or national emissions vary based on assumptions inherent in the estimate approach (Hristov et al., 2014). Knapp et al. (2014) reported estimates of enteric CH_4 representing 17% of global CH_4 emissions. Enteric fermentation in dairy cattle is estimated to contribute approximately 3.9% of total anthropogenic CH_4 emissions in the United States (EPA, 2013).

A recent hypothesis proposed by Janssen (2010) expanded our perspective on the relationship between physiological changes instigated by dietary or feed additive treatment and their effect on the concentration of aqueous H_2 [$H_2(aq)$], shifting fermentation pathways. Increasing $H_2(aq)$ concentration was hypothesized to be required to maintain methanogen growth rates with increasing solids passage rate (k_p) based on steady-state microbial growth kinetics (Monod, 1949; Janssen, 2010). With methanogens limited by decreased pH, Janssen (2010) proposed that $H_2(aq)$ would also increase under these conditions because methanogens will lower their relative growth rate under current conditions given a decreased maximum growth rate (Janssen, 2010). Rather than assuming that decreased relative growth rate will simply decrease competitive assimilation of $H_2(aq)$ into methanogenesis and allow $H_2(aq)$ to increase, the kinetic model hypothesizes that low pH decreases the maximal growth rate, which will shift upward the $H_2(aq)$ concentration needed to main-

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tain relative growth rates. Increased concentrations of $H_2(aq)$ from either increased k_p or lowered pH were predicted to thermodynamically shift VFA production toward pathways that decrease net microbial production of H_2 , particularly the acetate pathways that would otherwise yield the most H_2 (Janssen, 2010). Decreasing total H_2 produced would then ultimately decrease total CH_4 production.

We proposed to use continuous culture fermentors as a model to measure concentration of $H_2(aq)$, CH_4 production, VFA production, and fiber digestibility as a screening and validation process for the model by Janssen (2010). We hypothesized that lower culture pH would inhibit methanogens, necessitating an increase in concentration of $H_2(aq)$, decreasing production of H_2 and CH_4 , and shifting VFA pathways toward a lower acetate-to-propionate ratio. Alternatively, increased solids k_p would increase methanogen relative growth rate (Monod, 1949; Janssen, 2010) and increase the concentration of $H_2(aq)$, thereby decreasing CH_4 production and again shifting VFA production pathways toward a lower acetate-to-propionate ratio. Because k_p and pH are presumed to affect methanogens via modes of action based on distinct growth or microbial activity inhibitions, we hypothesized that the combination of low pH and high k_p would not be additive (i.e., we would detect a significant statistical interaction).

MATERIALS AND METHODS

Experimental Design

The present study was conducted as a 2×2 factorial treatment arrangement in a Latin square design using dual-flow continuous culture fermentors ($n = 4$). Fermentors were fed once daily [40 g of DM; 50:50 concentrate:forage diet, 37.5% NDF, 19.5% NSC (starch plus water-soluble carbohydrate), and 15.4% CP]. The pelleted forage was an alfalfa meal pellet and the pelleted concentrate was composed of ground corn (36.9%), soybean hulls (40.4%), dried distiller's grains (10.0%), 48% CP soybean meal (9.1%), corn oil (1.8%), MgO (0.2%), and trace-mineralized salt (0.9%). The latter contained Na (36.6%), Cl (56.4%), Zn (3500 mg/kg), Mn (2,800 mg/kg), Fe (1,750 mg/kg), Cu (350 mg/kg), I (70 mg/kg), and Co (70 mg/kg). The buffer also contained minerals that would support optimal microbial function. Treatments were control pH (CpH; diurnally ranging from 6.3 to 6.9) or low pH (LpH; 5.8 to 6.4) factorialized with solids k_p adjusted to be either low (Lk_p; 2.5%/h) or high (Hk_p; 5.0%/h). Liquid dilution was maintained at 7.0%/h to maintain constant infusion of buffer (for more accurate control of pH treatments) and to avoid a confounding dilution

factor in $H_2(aq)$ expectations. Four experimental periods lasted 10 d each, with 3 d of sample collection at the end of each period. Buffer pH for CpH treatments was made according to Weller and Pilgrim (1974), with the addition of 0.4 g/L of urea, and determined to be constant at pH 6.8 under continuous bubbling with CO_2 . For the LpH treatment, we added H_3PO_4 to a concentration of 12.5 mM to decrease buffer pH to 6.4. Both buffers allowed a parallel diurnal shift in pH (see Supplemental Figure S1; <https://doi.org/10.3168/jds.2016-12332>) to better reflect normal rumen function in vivo postfeeding (Cantalapiedra-Hijar et al., 2011). The CpH treatment allowed fluctuation of fermentor pH from 6.9 prefeeding to 6.3 approximately 8 h postfeeding, with a gradual return over the remaining 16 h of each experimental day. In a parallel response, the LpH treatment fluctuated between 6.4 prefeeding and 5.8 postfeeding. The fermentor pH was not allowed to drop below 5.8 to prevent long-term effects on protozoa (Dehority, 2010) that might confound treatment effects by decreasing protozoal metabolic activity and increasing bacterial abundance in replacement of lost protozoa.

Continuous Culture Operation

Dual-flow continuous culture fermentors ($n = 4$; Hoover et al., 1976), adapted for protozoa retention similar to Karnati et al. (2009), were used for this experiment. Average fermentor volume was 1.71 L (range: ± 0.09 L), and total buffer dilution rate was maintained at 7.0%/h. At the beginning of each period, rumen contents were manually sampled from 2 ruminally cannulated lactating Jersey cows fed a lactating diet, rapidly squeezed through 2 layers of cheesecloth, and then liquid was placed in insulated containers maintained at 39°C. Samples were pooled and inoculated into fermentors at 50% of fermentor volume. Sampling to inoculation interval averaged 30 min and did not exceed 40 min. Clarified rumen fluid (centrifuged at $15,000 \times g$, 4°C, 15 min, and autoclaved) was used in a 1:20 dilution of buffer for the first 3 d of each period to better adapt protozoa to fermentors; in addition, filters retained protozoa except for efflux with the solids as described previously (Karnati et al., 2009; Wenner, 2016). Fermentor buffer was made according to Weller and Pilgrim (1974); 40 mg/dL of urea was included to prevent depression of microbial protein synthesis because fermentors also remove the effect of N recycling observed in vivo. Buffer was continuously bubbled with CO_2 for at least 1 d before use to maintain anaerobic conditions in the fermentors. Treatments were imposed after 2 d of adaptation to the fermentors following each experimental period's inoculation.

Filters were replaced if clogged, typically in the first 4 d of each period while fibrous particulate content approached a semi-steady state dictated by once daily feeding. At the exit of the filtrate line from the lid of the fermentor, a y-shaped septum was attached to allow filtrate samples to be taken as liquid effluent was pumped from the fermentor. Fermentors were tested daily for pressure leaks using the Micro-Oxymax detection system (Columbus Instruments Inc., Columbus, OH). Detected leaks were repaired primarily during the adaptation period, and no samples were taken on days when repairs were made. Therefore, gas production measurement occurred during the last 3 d of each period, and the diurnal response was uninterrupted for any day recorded and used in data analysis. Ammonium sulfate that was enriched with 10% ^{15}N was dosed in a priming dose on d 5 at 50 mg and added to the buffer at 25 mg/L for a desired enrichment of 0.2% atom excess. Experimental periods lasted 10 d, including 7 d of adaptation and 3 d of sample collection.

Sample Collection and Analysis

Daily Samples. Daily fermentor effluent was collected over 24 h postfeeding in outflow containers on ice. A 20% subsample of daily effluent by weight was aspirated with a 63.5-mm diameter tube, to prevent sampling bias against larger particles, and pooled by fermentor within period. Effluent subsamples were lyophilized and stored for various analyses at -20°C until the end of the experiment. A subsample of lyophilized effluent was dried at 105°C overnight to determine DM, then ashed at 550°C overnight to determine OM (AOAC, 1990). Dried effluent N was determined by the Kjeldahl method (AOAC, 1990), and $\text{NH}_3\text{-N}$ remaining in samples was determined by dissolving approximately 0.2-g of sample in 1 mL of distilled H_2O before colorimetric analysis (Chaney and Marbach, 1962) to determine NAN flow by subtraction. Because of the small particle size of effluent inherent in continuous culture fermentors, NDF was determined by reflux units similar to those described by Goering and Van Soest (1970) with use of heat-stable amylase (Ankom Technology, Macedon, NY) and sodium sulfite (Van Soest et al., 1991). A 2-g subsample of all dried effluent and diet was commercially analyzed for starch and water-soluble carbohydrates, the sum of which is NSC (Dairy One Forage Lab, Ithaca, NY). A 50-mL aliquot of daily effluent was acidified with 3 mL of 6 N HCl before compositing by fermentor within period and storage at -20°C . Samples were later thawed, centrifuged ($15,000 \times g$, 4°C , 15 min), and filtered through Whatman No. 1 filter paper (Whatman International, Maidstone, UK) for analysis of $\text{NH}_3\text{-N}$ (Chaney and Marbach, 1962) and

VFA by GLC (Harvatine et al., 2002). A 10-mL aliquot of effluent and a 20-mL aliquot of agitated fermentor contents were taken and fixed in equal volume of 50% formalin for protozoal counts using the method of Dehority (1993). Microbial ^{15}N enrichment was analyzed similarly to Noftsger et al. (2003).

Hourly Gas and Filtrate Sampling. Headspace gas was collected using a Micro-Oxymax gas analyzer (Columbus Instruments Inc.). Gas production was calculated by pressure and programmed relationships according to gas laws embedded in the Micro-Oxymax system. The instrument sampled CH_4 and CO_2 by infrared absorption, but CO_2 evolving from buffer limits the application of this measurement and also further dilutes H_2 . Therefore, H_2 gas was determined by electrochemical sensor within the Micro-Oxymax system after the CO_2 had been scrubbed by passing it through a soda lime column (GFS Chemicals, Columbus, OH) that was replaced daily. The gas sensor was calibrated at the start of each experimental period to account for sensor baseline drift, which never exceeded 0.05% CH_4 . Fermentors were vented every 30 min, and gas production rates were calculated for CH_4 and H_2 and cumulative gas recorded per day.

Using the y-valve septum mentioned previously, 10 mL of filtrate was removed from the fermentors at 0, 1, 2, 3, 4, 6, and 8 h postfeeding on d 8, 9, and 10. Samples were acidified with 1 mL of 6 N HCl, pooled by fermentor within period, and analyzed for VFA by GLC (Harvatine et al., 2002). At the same time points (0, 1, 2, 3, 4, 6, and 8 h postfeeding) plus an additional 24-h sample on d 8, 9, and 10, we removed 20 mL of filtrate via the septum and injected filtrate into a 60-mL glass culture bottle (Wheaton Glass, Millville, NJ) containing 5 mL of 5 M H_3PO_4 , previously flushed with N_2 , and sealed with a butyl rubber stopper. To maintain fermentor volume, an equivalent volume of buffer was replaced at each time point to control k_p . The 25-mL sample of fermentor filtrate and H_3PO_4 was inverted and stored at 4°C until the end of the experiment (no more than 3 d) to quantify $\text{H}_2(\text{aq})$.

The $\text{H}_2(\text{aq})$ was measured based on a procedure similar to that of Robinson et al. (1981) because H_2 can be supersaturated (exceeding its solubility) and nucleation of gas bubbles is more likely to occur at decreased pH (Wattiaux et al., 1992) and with decreasing stirring (Pell and Schofield, 1993), such as with our slow stirring procedure used to retain protozoa. Supersaturation has previously influenced thermodynamic assumptions based on headspace derivations for measured $\text{H}_2(\text{aq})$ and aqueous CH_4 (Wang et al., 2016a). At the end of the experiment, the sample of filtrate combined with H_3PO_4 in the culture bottle was over-pressurized with 50 mL of N_2 and shaken to enable equilibrium, after

which a 50-mL headspace sample was removed and injected directly into the H_2 sensor of the Micro-Oxymax gas analyzer to derive $H_2(aq)$ as follows. Output was in percent gas as a total of injection and was used in an equation combining Henry's Law coefficient for H_2 , conservation of mass, and standard gas laws to estimate moles of $H_2(aq)$ originally in the liquid filtrate sample $\{n[H_2(aq)]_{\text{initial}}\}$. The equation used was:

$$n[H_2(aq)]_{\text{initial}} = \%H_2 \times (V_g + V_{N_2}) \times \left[\frac{K_H(H_2) \times V_g + R \times T \times V_l}{K_H(H_2) \times R \times V_g \times (100 - \%H_2)} \right]^{-1}, \quad [1]$$

where V_g is the volume (L) of the gas phase (bottle headspace), V_{N_2} is the volume (L) of N_2 injected into the headspace of the bottle, $K_H(H_2)$ is the Henry's law constant for H_2 (1,211.63 bar L mol⁻¹), R is the gas constant (0.08314 L bar K⁻¹ mol⁻¹; where bar = 100 kPa), T is the temperature (298.15°K), and V_l is the volume (L) of the liquid phase (filtrate and acid). We calculated $K_H(H_2)$ at pH = 7, ionic strength = 0.25 M, and $T = 298.15^\circ\text{K}$ according to Alberty (2003). Further information on the derivation of equation 1 is available in the Supplemental File (<https://doi.org/10.3168/jds.2016-12332>). The moles of measured H_2 were divided by the volume of the original sample to convert to $H_2(aq)$.

Statistical Analysis

Daily effluent data and ¹⁵N enrichment data were analyzed using the MIXED (mixed model) procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + H_i + K_j + (H \times K)_{ij} + p_k + f_l + \varepsilon_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall population mean, H_i is the fixed effect of i th pH treatment (i = control, low), K_j is the fixed effect of j th k_p (j = high, low), $(H \times K)_{ij}$ is the interaction of pH and k_p , p_k is the random effect of k th period (k = 1, 2, 3, 4), f_l is the random effect of l th fermentor (l = 1, 2, 3, 4), and ε_{ijkl} is the residual error, assumed independent and $\sim N(0, \sigma_e^2)$. For daily CH_4 production, the random effect of m th day (m = 1, 2, 3) was added to the model to account for large daily variations in CH_4 production between days. Hourly data were analyzed using the REPEATED (repeated measures) statement added to the previous model. The AR(1) covariate structure was selected for all measured variables based on best fit

using the lowest Bayesian information criterion. When a treatment $\times$ time interaction was significant, the effect of treatment was contrasted over time. A priori determination to test treatment effects by hour led us to use contrast statements for the main effect of pH, k_p , or pH $\times$ k_p for each time point.

RESULTS AND DISCUSSION

Controlling Fermentor pH

The pH in all 4 fermentors followed a diurnal range following feeding that consisted of a decline of 0.6 units over the first 8 h postfeeding followed by a gradual rise back to the maximum target range: for the CpH treatments, this range was 6.9 to 6.3; for the LpH treatments, this range was 6.4 to 5.8. The pH never exceeded this range during an experimental period. A primary limitation in traditional continuous culture fermentor experiments is the reliability of pH control. Total time at low pH (area under the curve) is a significant driver of pH effects in experiments (de Veth and Kolver, 2001), and pH control at intervals allows closer determination of physiological limits of continuous culture to low pH before sacrificing digestibility. However, magnitude of pH decline is also an important factor (Cerrato-Sánchez et al., 2008), highlighting the short-term resilience by microbes, especially bacteria, to inhibition by low pH. Cerrato-Sánchez et al. (2007a) attempted to account for this by dropping pH stepwise for 4-h increments, but we reasoned that repeated abrupt stepwise pH adjustments can shock the microbial population and could shift microbial populations more than a gradual pH change. Monitoring daily pH just before feeding (Goel et al., 2009) can be fairly predictive of fermentor pH throughout the day on a constant diet after the first 4 d of adaptation, allowing a diurnal shift in pH that is likely more reflective of ruminant biology (Cantalapiedra-Hijar et al., 2011).

Protozoal Counts and Generation Time

Fermentor counts of protozoa were decreased ($P = 0.03$) in our experiment by the main effect of LpH, but not by k_p or the interaction ($P > 0.10$) (Table 1). Protozoa counts in the present study are lower than the range reported in vivo ($\sim 1 \times 10^6$; Dehority, 2003) and in early continuous culture reports by Hoover et al. (1976), but the current counts are similar to those reported previously in fermentors fed a similar quantity of DM per day (Karnati et al., 2009), so part of the reason for the lower counts is a result of lower DM (substrate) concentration. Additionally, generation times of protozoa in excess of 20 or 40 h for Hk_p or Lk_p, re-

spectively, are indicative of protozoal sequestration on the bottom of fermentors to avoid outflow because we expected generation times to align with k_p (Sylvester et al., 2009). Low pH has previously been reported to decrease protozoal counts and increase generation time (Dehority, 2003). The shorter generation time with LpH could reflect a differentially greater inhibition of the sedimenting protozoa, especially those of the family *Isotrichidae*; however, another explanation seems more likely. All generation times in our study are still above the minima summarized for various entodiniomorphid protozoa (Dehority, 2010); thus, recovery after the nadir pH of 5.8 might have prevented a shift in populations resulting from the different k_p . We detected a relatively low number of cells in all treatments and a prevalence of primarily small (<50 μm length) *Entodinium* spp. (data not shown); therefore, only total cell counts (not differentiated by genera) were recorded. Considering that members of genus *Entodinium* are more resistant to inhibition by low pH (Williams and Coleman, 1992), the decrease ($P = 0.03$) in mean protozoal counts in fermentors by LpH in the present study combined with no difference in outflow of protozoal cells into the effluent might reflect a greater proportion of smaller protozoa that have a shorter ($P = 0.06$) generation time.

Nutrient Digestibility

Apparent NSC digestibility was high (average = 88.4%), but we found no effect of treatments or interaction ($P > 0.10$), and NSC digestibility is consistently high in continuous culture fermentation systems (Hristov et al., 2012). High NSC digestibility likely resulted from pelleted diets that sink to the bottom of the fermentor, increasing digestion time before fermentation buoyancy floats particles to potential outflow; however, pelleting is necessary to ensure correct diet delivery and prevent excessive overflow.

Apparent OM digestibility was not affected by k_p or the interaction of pH $\times$ k_p ($P > 0.10$), yet the main effect of LpH tended ($P = 0.07$) to have greater OM

digestibility compared with the main effect mean for CpH (Table 2). Despite the lack of treatment effects on NSC digestibility, LpH unexpectedly increased apparent OM digestibility without affecting the more pH-sensitive metric, NDF digestibility (Mouriño et al., 2001; Russell, 2002; Russell et al., 2009). The LpH treatment in the present study might not have been extreme enough to detect the decrease in apparent OM digestibility noted by others (de Veth and Kolver, 2001; Cerrato-Sánchez et al., 2007a,b) because we established a minimum pH of 5.8 to protect protozoa.

Digestibility of NDF did not differ by treatments ($P > 0.10$), which is surprising given a previously dramatic effect of increased k_p on NDF digestibility in our laboratory (Qiu et al., 2004). That study and most published literature had a much faster stirring rate, which would increase overflow of particles by disrupting digesta at the bottom of the fermentors, but also almost completely eliminates protozoa (Mansfield et al., 1995; Sharp et al., 1998; Ziemer et al., 2000). The lack of a pH effect on NDF digestibility is similar to previous work by Calsamiglia et al. (2002) but conflicts with results of de Veth and Kolver (2001), who noted that treatment of 4 h at suboptimal pH (<6.3) decreased NDF digestibility. The effect of low pH (<6.0) on cellulolytic bacteria probably is related to growth inhibition rather than activity of cellulase per se (Russell et al., 2009). Similarly, low pH is known to inhibit methanogenesis, but as pH returns to normal, so will methanogenic activity (Van Kessel and Russell, 1996). Methane production can uncouple from growth (Thauer et al., 2008), and therefore daily CH_4 production need not change. Based on the VFA and CH_4 concentration patterns reported in the present study (Table 3), we suggest that microorganisms inhibited during the first 4 to 8 h postfeeding by LpH were able to compensate in the last 16 h to overcome differences in digestibility, VFA, and CH_4 that occurred in the earlier hours. Future methane mitigation strategies must consider not only the capability of microbes to adapt to treatments, but also to compensate for short-term changes.

Table 1. Counts of protozoa populations within fermentors, calculated effluent flow, and generation time for continuous cultures treated with control or low pH and either low or high solids passage rate (k_p)¹

Item	Control pH		Low pH		SEM	P-value ²		
	Low k_p	High k_p	Low k_p	High k_p		pH	k_p	pH $\times$ k_p
Fermentor counts (cells $\times 10^3/\text{mL}$)	14.0	11.2	9.0	7.4	2.5	0.03	NS	NS
Effluent flow (cells $\times 10^6/\text{d}$)	11.2	11.2	11.2	11.2	2.1	NS	NS	NS
Generation time ³ (h)	51.4	39.2	33.3	28.9	6.5	0.06	NS	NS

¹Treatments are Control pH: range 6.9 to 6.3, Low pH: range 6.4 to 5.8, Low k_p : 2.5%/h, High k_p : 5.0%/h.

²P-values are reported for the main effects of pH, k_p , and their interaction. NS = $P > 0.10$.

³Generation time = total pool size of cells: [(fermentor counts $\times$ fermentor volume)/effluent flow of cells/d] $\times$ 24 h/d.

Table 2. Nutrient digestibilities and N effluent flows in continuous cultures that were treated with either control or low pH and either low or high solids passage rate (k_p)¹

Item	Control pH		Low pH		SEM	<i>P</i> -value ²		
	Low k_p	High k_p	Low k_p	High k_p		pH	k_p	pH $\times$ k_p
Apparent OM digestibility (%)	43.4	38.1	48.7	47.2	3.4	0.07	NS	NS
Apparent NSC digestibility (%)	88.5	87.9	89.3	88.0	1.6	NS	NS	NS
NDF digestibility (%)	66.8	60.2	63.4	62.4	3.3	NS	NS	NS
NH ₃ -N (mg/dL)	16.7	17.3	18.8	17.3	1.1	NS	NS	NS
Effluent N flows ³								
Total N (g/d)	1.29	1.37	1.53	1.54	0.08	0.01	NS	NS
Bacterial N (g/d)	0.440	0.471	0.428	0.492	0.031	NS	0.09	NS
Bacterial N per OMD (g/kg)	30.4	36.7	24.5	31.3	3.3	0.08	0.05	NS
Bacterial N per NSCD (g/kg)	64.0	69.0	61.2	72.0	5.2	NS	0.08	NS
Bacterial N per NDFD (g/kg)	44.6	52.5	42.8	53.7	5.1	NS	0.10	NS
NAN (g/d)	0.825	0.896	1.000	1.050	0.068	0.03	NS	NS
NAN ⁴ (% of N intake)	83.8	91.0	101.9	106.5	6.9	0.03	NS	NS
NANBN ⁵ (g/d)	0.385	0.426	0.482	0.557	0.030	<0.01	0.04	NS
NANBN ⁴ (% of N intake)	39.1	43.2	48.9	56.5	3.1	<0.01	0.04	NS

¹Treatments are Control pH: range 6.9 to 6.3, Low pH: range 6.4 to 5.8, Low k_p : 2.5%/h, High k_p : 5.0%/h.²*P*-values reported for the main effects of pH, k_p , and their interaction. NS = *P* > 0.10.³OMD = OM apparently degraded; NSCD = NSC (starch + water-soluble carbohydrates) apparently degraded; NDFD = NDF degraded.⁴Percent of dietary N intake only, does not include N from buffer.⁵NANBN = nonammonia, nonbacterial N.

Nitrogen Flows and NH₃-N Concentration

Concentration of NH₃-N in daily effluent (average = 17.5 mg/dL) was not affected by treatment or interaction (*P* > 0.10) (Table 2). However, LpH increased (*P* < 0.01) total N flow and tended (*P* = 0.08) to increase bacterial N flow per kilogram of OM degraded, with no effect of k_p or the interaction of pH $\times$ k_p (*P* > 0.10). Bacterial N flow was not affected by pH or the interac-

tion of pH $\times$ k_p (*P* > 0.10), but the main effect mean of H k_p trended (*P* = 0.09) toward an increase compared with the main effect mean of L k_p (Table 2). This is congruent with the hypothesis of Janssen (2010), that increased k_p would increase microbial growth rate in response, and is supported by previous reviews that predicted increased k_p should improve the efficiency of microbial protein synthesis (Dijkstra et al., 1998; Firkins et al., 2007). The main effect of H k_p tended (*P* <

Table 3. Effluent flow, VFA and methane production, and dihydrogen (H₂) emission into headspace or aqueous (aq) concentration for continuous cultures that were treated with either control or low pH and either low or high solids passage rate (k_p)¹

Item	Control pH		Low pH		SEM	<i>P</i> -value ²		
	Low k_p	High k_p	Low k_p	High k_p		pH	k_p	pH $\times$ k_p
Effluent (L/d)	2.86	2.89	2.89	2.84	0.06	NS	NS	0.09
VFA (mmol/d)	147	153	163	161	12	NS	NS	NS
Individual VFA (mol/100 mol)								
Acetate	61.8	59.6	58.2	57.4	1.0	<0.01	<0.01	NS
Propionate	22.5	24.4	23.9	26.2	1.2	0.02	<0.01	NS
Isobutyrate	0.775	0.725	1.000	0.625	0.158	NS	NS	NS
Butyrate	10.6	11.0	11.2	9.75	0.67	NS	NS	NS
Isovalerate ³	2.53	2.55	3.18	3.58	0.28	<0.01	NS	NS
Valerate	1.78	1.78	2.50	2.48	0.27	0.04	NS	NS
Acetate:propionate	2.77	2.46	2.47	2.21	0.16	<0.01	<0.01	NS
H ₂ emission (μmol/d)	110	181	13	17	52	<0.01	NS	NS
H ₂ (aq) (μM)	6.90	5.66	1.99	1.91	3.69	<0.01	NS	NS
Methane (mmol/d)	37.8	30.1	42.3	23.4	6.2	NS	<0.01	NS
Methane per NDF (mmol/g degraded)	3.84	3.56	4.66	2.74	0.97	NS	NS	NS

¹Treatments are Control pH: range 6.9 to 6.3, Low pH: range 6.4 to 5.8, Low k_p : 2.5%/h, High k_p : 5.0%/h.²*P*-values reported for the main effects of pH, k_p , and their interaction. NS = *P* > 0.10.³Isovalerate co-elutes with 2-methylbutyrate, and the 2 could not be distinguished in the present study.

0.10) to increase grams of bacterial N per kilogram of OM, NSC, or NDF degraded compared with the main effect mean of Lk_p (Table 2).

The main effect of LpH increased ($P = 0.03$) NAN flow and NAN flow as a percentage of N intake, with no effects ($P > 0.10$) of k_p or the interaction of pH $\times$ k_p. Because of the removal of protozoa before ¹⁵N analysis, nonammonia, nonmicrobial N is termed nonammonia, nonbacterial N (NANBN) in the present study. The main effect of LpH increased ($P < 0.01$) NANBN flow and NANBN flow as a percentage of N intake, and the main effect of Hk_p also increased ($P = 0.04$) NANBN flow and flow as a percentage of N intake. We found no interaction of pH $\times$ k_p ($P > 0.10$). The main effect of LpH shifted N flow in the current experiment, increasing both NAN and NANBN flows when calculated directly or as percentages of N intake. The increase in NANBN flow by LpH in the present study is similar to that reported previously by Calsamiglia et al. (2008), and the exact cause is not yet elucidated.

Unfortunately, protozoal N could not be measured because small contaminating particles could not be removed by separation of the protozoal fraction based on centrifugation or filtration. Using the estimate of 5.46×10^{-10} g of N/cell (Sylvester et al., 2005), protozoal N can be estimated at approximately 1.3% of total microbial N, but this estimate depends on cell size and potential for treatment differences. Fermentation activity inside small particles in continuous culture is not rapid enough for typical flocculation to separate protozoa; without flocculation, small feed particles that are contaminated with adherent bacterial N are similar in size and density to protozoal cells and are not removed during harvesting of protozoa (Ye, 2013). A method by Ye (2013), which used sonication and boiling to destroy protozoa and subtracted lysed protozoal N from a control sample containing both protozoal N and contaminating feed N, was also insufficient to determine N with acceptably low error.

VFA

We noted no interactions ($P > 0.10$) of pH $\times$ k_p for total VFA production or molar percentage of individual VFA (Table 3). Total concentration of VFA measured in daily effluent was not affected by pH or k_p, nor was net production of VFA (calculated by effluent VFA concentration $\times$ effluent outflow per day). However, the molar percentage of acetate was decreased ($P < 0.01$) by lowering pH and decreased ($P < 0.01$) by increasing k_p. The lower acetate was mirrored by a higher ($P < 0.05$) propionate molar percentage for main effects means of both LpH and Hk_p when compared with

main effect means for CpH and Lk_p, respectively. We observed no treatment effects ($P > 0.10$) on isobutyrate or butyrate. The main effect of LpH increased ($P < 0.05$) both isovalerate and valerate, with no effect of Hk_p ($P > 0.10$, Table 3). The acetate-to-propionate ratio was decreased by both the main effect of LpH and Hk_p ($P < 0.01$). Increased isovalerate might be a result of decreased *iso* long-chain fatty acid synthesis into bacterial membranes with decreasing pH (Vlaeminck et al., 2006). This effect cannot be verified because, under our GLC conditions, isovalerate coelutes with 2-methylbutyrate (the primer for *anteiso* long-chain fatty acids); isovalerate production should not have increased from leucine deamination because LpH also increased NANBN (an index of undegraded dietary protein; Table 2).

When time had a main effect difference or an interaction with treatment, the VFA were presented over time (Figure 1). Hourly measurements indicated that VFA total concentration was increased by LpH ($P < 0.05$) for 1 to 4 h postfeeding (Figure 1A). Although we noted a pH $\times$ time interaction ($P < 0.05$) for molar percentage of acetate (Figure 1B), acetate was consistently decreased by the main effects of LpH ($P < 0.01$) at every time point compared with CpH, reflecting the main effect differences (Table 3). For molar percentage of propionate, we observed interactions with time for both the pH ($P = 0.09$) and the k_p ($P = 0.02$) treatments (Figure 1C). Respective differences for both main effects ($P < 0.01$) were in the first 8 h after feeding. Butyrate molar percentage (Figure 1D) had a main effect of time ($P < 0.01$) and an interaction of pH $\times$ time ($P < 0.01$). The main effect of LpH was lower ($P < 0.05$) from 3 to 8 h postfeeding compared with the main effect of CpH, again confirming most of the responses before 8 h.

Continuous culture studies have demonstrated the decline of acetate-to-propionate ratio with decreasing pH (Cerrato-Sánchez et al., 2007a,b; Cantalapiedra-Hijar et al., 2011). A constant low pH (5.8) also decreased acetate-to-propionate ratio in our laboratory previously (Qiu et al., 2004); however, their low pH treatment was much lower than the diurnal average of the present study. Most importantly, the effect of pH has changed fermentation conditions independent of changes in diet (Calsamiglia et al., 2008; Fuentes et al., 2009), but these studies have previously been performed using immediate changes to the fermentor pH rather than the gradual shift in pH implemented in the present study. In the present study, acetate-to-propionate ratio was decreased by LpH and also by Hk_p. This shifted ratio was observed throughout the first 8 h postfeeding, indicating potential for microbial

adaptation as the root cause rather than an increase in concentration of $H_2(aq)$ (Janssen, 2010) during heaviest fermentative activity postfeeding (Hungate, 1966). Previously, doubled k_p (4.0 to 8.0%/h) in continuous culture actually increased acetate-to-propionate ratio (Qiu et al., 2004), in contrast to the present study. However, values in that study represent unusually high acetate-to-propionate ratio for a continuous culture experiment, perhaps because of its constant feeding. The response of total VFA concentration and acetate-to-propionate ratio possibly is not linear across a wider variation in k_p , as in other previous work in single-flow fermentors (Isaacson et al., 1975). Despite the shift in acetate-to-propionate ratio in the present study, we

found no change in total VFA production; thus, more carbon and hydrogen were incorporated into VFA as acetate production decreased and propionate production increased.

The ideal ratio of acetate-to-propionate ratio for milk production or ruminal fermentative efficiency is not known (Ungerfeld, 2013). We must be careful not to interpret shifts in acetate-to-propionate ratio as indicative of greater or lesser fermentative efficiency, but only as a descriptive variable for the VFA pathway balance in the culture measured. After our study was conducted, a theoretical publication provided evidence that VFA may not be shifted by thermodynamic control under normal rumen conditions (van Lingen et al., 2016).

Figure 1. Total concentration or molar percentages of VFA over time postfeeding for continuous cultures that had treatments of either control (CpH) or low (LpH) pH and either low (Lk_p) or high (Hk_p) solids passage rate (k_p). We found no pH $\times$ k_p $\times$ time interactions ($P > 0.10$), but interactions of pH and k_p are noted when $P \leq 0.10$. The main effects and interactions of treatments were tested at each time point and are reported when they were $P \leq 0.10$. (A) Total VFA concentration had the main effect of time ($P < 0.01$). Total VFA tended ($P = 0.07$) to be increased for the main effect of LpH at 0 h and was increased ($P \leq 0.05$) from 1 to 4 h postfeeding compared with the main effect of CpH. High k_p tended ($P = 0.07$) to decrease total VFA at 4 h postfeeding. Pooled standard error was 2.75 mM. (B) Acetate molar percentage had a pH $\times$ time interaction ($P = 0.02$). The main effect of LpH decreased ($P \leq 0.05$) acetate molar percentage for all time points compared with the main effect of CpH. Pooled standard error was 1.22 mol/100 mol. (C) Molar percentage of propionate had main effects of time ($P = 0.02$), and we found interactions for pH $\times$ time ($P = 0.09$) and k_p $\times$ time ($P = 0.02$). The main effect of LpH was greater ($P < 0.01$) from 1 to 8 h postfeeding compared with the main effect of CpH. The main effect of Hk_p was greater ($P < 0.01$) from 0 to 8 h postfeeding compared with Lk_p. Pooled standard error was 1.14 mol/100 mol. (D) Molar percentage of butyrate had a main effect of time ($P < 0.01$) and an interaction of pH $\times$ time ($P < 0.01$). The main effect of LpH was lower ($P < 0.05$) from 3 to 8 h postfeeding compared with the main effect of CpH. Pooled standard error was 0.499 mol/100 mol.

Further, VFA absorption *in vivo* complicates potential thermodynamic feedback that was not accounted for in the current experiment.

Aqueous Hydrogen

Concentration of $H_2(aq)$ (average for all time points and treatments = $4.11 \mu M$) had an interaction ($pH \times k_p$, $P = 0.02$) at 1 h postfeeding: CpH/Lk_p was greatest, followed by CpH/Hk_p and LpH/Hk_p, and LpH/Lk_p was lowest (Figure 2A). Over all time points, the main effect of LpH decreased ($P < 0.01$) $H_2(aq)$ by $4.33 \mu M$ compared with CpH. Although not the hypothesized result, the decreased acetate-to-propionate ratio (and therefore H_2 production) might have limited accumulation of $H_2(aq)$ because methanogens readily consumed available substrate. Our hypothesized role of $H_2(aq)$ was unlikely to shift VFA production in the LpH treatments because concentration of $H_2(aq)$ was actually decreased by LpH (Figure 2A). The decrease in CH_4 production by LpH might not have been mediated by $H_2(aq)$ via LpH in the present study because the concentration never shifted enough ($>10 \mu M$) to see the dramatic pathway shifts proposed by Janssen (2010).

Aqueous hydrogen concentrations were within a similar range to previously published measurements for rumen fluid (Robinson et al., 1981; Morgavi et al., 2012; Guyader et al., 2015). Our data also demonstrated day-to-day and within-day variability similar to that previously seen by Robinson et al. (1981) and represents biological variation in addition to experimental error. Measurement of concentrations of $H_2(aq)$ in excess of equilibrium predictions accentuates the necessity of measuring $H_2(aq)$ rather than the simpler headspace H_2 or calculations for $H_2(aq)$ derived from headspace H_2 because of supersaturation (Wang et al., 2016a). Although only different at 1 h postfeeding, the $H_2(aq)$ landscape probably is more dynamic than the current sampling points allowed us to measure. The production of H_2 probably occurred more rapidly than it could be consumed by methanogens or other bacteria, which is supported by *in vitro* results from Czerkawski et al. (1972) and Wang et al. (2016b) and by *in vivo* results (Rooke et al., 2014). Headspace gas merely represents H_2 escape; once escaped, H_2 models predict a relatively low proportion of gaseous H_2 returning to $H_2(aq)$ for contribution to methanogenesis within the rumen fluid culture unless methanogenesis has been interrupted by an inhibitor (Wang et al., 2013). Moreover, when H_2 production exceeds its usage, supersaturation is likely *in vivo* (Wang et al., 2016a) and is expected in our slow-stirring fermentors especially during its peak production; during these times when H_2 is accumulating in the headspace, we expect limited redissolving into

Figure 2. Emission of dihydrogen (H_2) or aqueous (aq) concentration and emission (production) of methane (CH_4) over time post-feeding for continuous cultures that had treatments of either control (CpH) or low (LpH) pH and either low (Lk_p) or high (Hk_p) solids passage rate (k_p). We found no $pH \times k_p \times$ time interactions ($P > 0.10$), but interactions of pH and k_p are noted when $P \leq 0.10$. The main effects and interactions of treatments were tested at each time point and are reported when they were $P \leq 0.10$. (A) For $H_2(aq)$, we found an interaction of $pH \times k_p$ ($P = 0.02$) at 1 h postfeeding with CpH/Lk_p being greatest, CpH/Hk_p and LpH/Hk_p being intermediate, and LpH/Lk_p being least. We also noted a trend ($P = 0.08$) for LpH to be lower at 2 h than CpH. Pooled standard error was $3.68 \mu M$. (B) For H_2 emission, we found a main effect of time ($P < 0.01$) and a $pH \times$ time interaction ($P < 0.01$). The main effect of LpH was lower than CpH from 2 to 8 h ($P < 0.05$) and 9 h ($P = 0.06$) postfeeding. Pooled standard error was $13.8 \mu mol/h$. (C) For CH_4 production rate, we observed a main effect of time ($P < 0.01$). The main effect of LpH was lower ($P < 0.05$) from 5 to 9 h postfeeding compared with the main effect of CpH. The main effect of Hk_p was lower ($P < 0.05$) from 3 to 9 h compared with the main effect of Lk_p. Pooled standard error was $326 \mu mol/h$.

liquid before it is vented by our gas collection system. Eructation and absorption of H_2 into blood for expiration also would remove gaseous H_2 in vivo.

Gas Production

We found no interactions ($P > 0.10$) of $pH \times k_p$ for H_2 production. Hydrogen escape into headspace gas was decreased ($P < 0.05$) by LpH for 2 to 8 h postfeeding, and we observed no effect of k_p ($P > 0.10$, Figure 2B). This result is similar to the decrease in $H_2(aq)$ caused by LpH treatment. The rate of H_2 escape into headspace probably was not decreased by lower culture pH; if this were true, $H_2(aq)$ measurements—from which H_2 is equilibrated for a standard sample pH across all treatments—would have increased detected quantities of $H_2(aq)$. More likely, the lower $H_2(aq)$ in LpH could be due to H_2 diversion away from escape resulting from reducing equivalent disposal into increasing chain length of fermentation end-products, such as valerate (as noted in Table 3), with more negative redox potential of bacteria if methanogenesis is disrupted (Nerdahl and Weimer, 2015).

Methane production rate was decreased by the main effect of LpH ($P \leq 0.05$) for 5 to 9 h postfeeding and was also decreased by the main effect of Hk_p ($P \leq 0.05$) for 3 to 9 h post-feeding (Figure 2C). With trends ($P \leq 0.10$) to decrease CH_4 production rate for both LpH and Hk_p at 10 h, CH_4 production rates were not different ($P > 0.10$) from 11 to 24 h postfeeding. In the present study, methanogenesis did occur below pH 6.0, which is contrary to that previously reported after abrupt treatments (Van Kessel and Russell, 1996). However, CH_4 production rate was decreased by both LpH and Hk_p during the time of most active fermentation postfeeding. In combination with observed shifts in the current study of VFA production toward a lower acetate-to-propionate ratio, decreased CH_4 production rate would support the hypothesis by Janssen (2010), that increased $H_2(aq)$ shifts VFA production away from H_2 -producing pathways. This may be accomplished by redirecting pyruvate toward propionate or by disposal of reducing equivalents when acetyl coA is elongated to butyrate and valerate (Ungerfeld and Kohn, 2006). In support, valerate increased when LpH suppressed methanogenesis (Table 3); however, the H_2 emission and $H_2(aq)$ themselves did not respond as hypothesized according to Janssen (2010) for the LpH treatments.

The net result on a daily basis from minor hourly differences for CH_4 production rates between treatments was no effect of LpH or interaction of $pH \times k_p$ ($P > 0.10$) for daily CH_4 production (Table 3); however, Hk_p decreased daily CH_4 production ($P < 0.01$) by 13.3 mmol/d. When considering millimoles of CH_4 pro-

duced per gram of NDF degraded, we found no effect of treatment ($P > 0.10$, Table 3). Although the daily CH_4 production was not significant for LpH, the shift in the present study in CH_4 production rate and VFA ratio was accomplished without any change in the diet fed. Furthermore, the mitigation of CH_4 by increased k_p without decreases in NDF digestibility or changes in dietary composition support the hypothesis by Janssen (2010), whereby increased methanogen growth rate and requirement for substrate (H_2) at an increased growth rate would increase $H_2(aq)$ concentration and decrease net methanogenesis by decreasing H_2 -producing VFA pathways.

CONCLUSIONS

We aimed to determine whether decreased pH or increased k_p would increase $H_2(aq)$, shifting VFA pathways away from acetate production and decreasing net H_2 production available for methanogenesis. Although we did not observe the hypothesized increase of $H_2(aq)$, CH_4 production rate was decreased during early fermentation by both LpH and Hk_p , in support of the hypothesis of Janssen (2010). However, the thermodynamic control by $H_2(aq)$ might be less influential in standard dietary conditions for dairy cattle. The decrease in CH_4 produced with increased k_p without a decline in NDF digestibility provides strong in vitro evidence that methanogenesis can be mitigated by changing conditions influencing archaeal growth rate. Responses in VFA production, primarily a decline in acetate-to-propionate ratio, were also observed independent of any dietary changes. Increasing k_p also tended to increase grams of bacterial N per kilogram of NSC and NDF degraded. The microbial community appears to be dynamically adaptive, such that VFA production shifted in response to inhibition of methanogenesis inhibition, yet detection of biologically significant differences in $H_2(aq)$ eluded us due to limited time points measured and high variability in its measurement. Although challenging, $H_2(aq)$ can be measured experimentally and offers valuable information for future modeling of the relationship between VFA production and methanogenesis.

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