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Effects of acetate, propionate, and pH on volatile fatty acid thermodynamics in continuous cultures of ruminal contents

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ABSTRACT

To investigate the effects of acetate, propionate, and pH on thermodynamics of volatile fatty acids (VFA) in the rumen, a dual-flow continuous culture study was conducted to quantify production of major VFA, interconversions among the VFA, and H_2 and CH_4 emissions in a 4 × 4 Latin square design. The 4 treatments were (1) control: pH buffered to an average of 6.75; (2) control plus 20 mmol/d of infused acetate (InfAc); (3) control plus 7 mmol/d of infused propionate (InfPr); and (4) a 0.5-unit decline in pH elicited by adjustment of the buffer (LowpH). All fermentors were fed 40 g of a pelleted diet containing whole alfalfa pellets and concentrate mix pellets (50:50) once daily. After 7 d of treatment, sequential, continuous infusions of [2-^{13}C] sodium acetate (3.5 mmol/d), [U-^{13}C] sodium propionate (2.9 mmol/d), and [1-^{13}C] sodium butyrate (0.22 mmol/d) were carried out from 12 h before feeding for 36 h. Filtered liquid effluent (4 mL) was sampled at 0, 2, 4, 6, 8, 12, 16, and 22 h after feeding, and assessed for VFA concentrations, with another filtered sample (20 mL) used to quantify aqueous concentrations of CH_4 and H_2. Headspace CH_4 and H_2 gases were monitored continuously. Ruminal microbes were isolated from the mixed effluent samples, and the microbial community structure was analyzed using the 16S rRNA amplicon sequencing technique. The digestibility of neutral detergent fiber, acid detergent fiber, and starch and microbial C sequestrated from VFA were not affected by treatments. The LowpH treatment increased net propionate production and decreased H_2 and CH_4 headspace emissions, primarily due to shifts in metabolic pathways of VFA formation, likely due to the observed changes in bacterial community structure. Significant interconversions occurred between acetate and butyrate, whereas interconversions of other VFA with propionate were relatively small. The InfAc and InfPr treatments increased net acetate and propionate production, respectively; however, interconversions among VFA were not affected by pH, acetate, or propionate treatments, suggesting that thermodynamics might not be a primary influencer of metabolic pathways used for VFA formation.

Key words: isotope dilution, low pH, methane, volatile fatty acid

INTRODUCTION

Acetate, propionate, and butyrate account for 95% of the total VFA produced in the rumen and provide up to 75% of ME for ruminants (Bergman, 1990; France and Dijkstra, 2005). As a co-product of ruminal fermentation, CH_4 produced from fermentation represents an energy loss because it cannot be used by the host animal (Hammond et al., 2016). Considering that the stoichiometry of production of the major VFA determines the amounts of CH_4 and CO_2 generated, the profile of VFA formed in the rumen reflects different fermentation pathways and partially determines the efficiency of energy utilization (Ungerfeld, 2015).

Production of acetate, propionate, and butyrate occurs via the common Embden-Meyerhof-Parnas pathway, with pyruvate serving as the branch point. Kohn and Boston (2000) indicated that thermodynamics control which pathway branches are available during ruminal fermentation and thus the final VFA produced. Ungerfeld and Kohn (2006) demonstrated that the change in Gibbs free energy ($\mathbf{\Delta G}$) of pyruvate's conversion to acetate, propionate, and butyrate is similar under normal ruminal conditions, suggesting that interconversions of the 3 major VFA should yield similar ΔG. Given common intermediates and roughly equal ΔG for interconversions implies that the VFA profile

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is thermodynamically controlled and that a thermodynamic equilibrium exists among the VFA.

Current mechanistic models estimate net ruminal VFA production rates based on static sets of stoichiometric parameters for soluble carbohydrate, starch, hemicellulose, cellulose, and protein (Dijkstra et al., 1992; Baldwin, 1995; Bannink et al., 2006). This approach assumes that interconversions among VFA are insignificant, constant, or scale with total production (Murphy et al., 1982; Baldwin, 1995). Ghimire et al. (2014) reported that VFA production rates were poorly predicted by these mechanistic models with 50 to 60% prediction errors. However, adding thermodynamically driven interconversions among major VFA did not significantly improve prediction accuracy for VFA production rates (Ghimire et al., 2017). Janssen (2010) demonstrated that H_2 could affect CH_4 formation and fermentation balances through microbial growth kinetics and thermodynamics. van Lingen et al. (2016) indicated that H_2 partial pressure, controlled by the NAD^+-to-NADH ratio, could be a key controller of VFA fermentation and CH_4 emissions in the rumen. They developed a dynamic mechanistic model that considered the effects of the NAD^+:NADH on VFA production, and predicted that as much as 90% of the observed variation in VFA proportions and 86% of the variation in daily CH_4 emissions may be explained by NAD^+:NADH (van Lingen et al., 2019). However, the work was based on net predictions of non-steady-state concentrations of substrates and products, which are functions of production, absorption, and passage or emissions, and thus not definitive in an open system. They also did not consider the potential for the aqueous concentration of H_2 [$\mathbf{H_2(aq)}$] to be supersaturated (Wenner et al., 2020) and, because the model did not predict bidirectional exchange among VFA, they could not test for model consistency in predicting those exchanges that are thermodynamically driven.

If thermodynamic control is an important factor in the rumen under normal feeding conditions, then ruminal pH and VFA concentrations will be the primary controllers of thermodynamic state and thus ruminal fermentation (Ungerfeld and Kohn, 2006; Ungerfeld, 2015). Ruminal pH is dependent on the concentration of HCO_3^- and H^+ and the partial pressure of CO_2 in the rumen, which could affect H_2 and CO_2 supply for methanogenesis (Kohn and Boston, 2000). Wang et al. (2016) indicated that ruminal dissolved H was associated with changes in fermentation pathways from acetate to propionate and microbial populations. Seal and Parker (1994) reported that intraruminal propionic acid infusion changed interconversion rates between acetate and propionate, suggesting that the interconversions were thermodynamically regulated under normal ruminal conditions. However, it remains unclear to what extent this control may affect overall VFA production rates. The objective of this study was to extend those observations by investigating the effects pH and acetate and propionate infusions on thermodynamics of ruminal fermentation using a stable isotope technique. We hypothesized that varying pH, acetate, and propionate concentrations within normal ranges in continuous culture fermentors would alter production rates of major VFA, interconversions among VFA, and H_2 and CH_4 emissions in a simulated ruminal environment.

MATERIALS AND METHODS

Experimental Design

This study was conducted using dual-flow continuous culture fermentors, which were initially described by Hoover et al. (1976) and modified for protozoal retention (Karnati et al., 2009) and simultaneous liquid sampling and gas emission quantification (Wenner et al., 2017). Four fermentors were randomly assigned to 1 of 4 treatments in a 4 × 4 Latin square design (n = 4). All fermentors were fed 40 g/d of a common pelleted diet containing equal proportions of whole alfalfa pellets and concentrate mix pellets (50:50). The diet was provided once daily at 0800 h as described in Wenner et al. (2017). All feed ingredients were ground to pass a 2-mm screen before pelleting. The ingredient composition and nutrient content of the diet are listed in Table 1. Fermentors were given 7 d of adaptation to treatments, and the treatments were (1) control treatment with pH buffered to an average of 6.75 (**Con**); (2) Con with 20 mmol/d acetate infused into the fermentor (**InfAc**); (3) Con with 7 mmol/d propionate infused (**InfPr**); and (4) altered buffer composition to cause a 0.5-unit pH reduction compared with Con (**LowpH**). Based on past experiments, the infusion rates of acetate and propionate were projected to increase VFA supplies by 20 to 25% on a net VFA basis (Wenner et al., 2017), which was predicted to elicit a significant shift in interconversions if our hypothesis was correct.

Isotopically labeled VFA were infused sequentially in a manner described below from d 8 to 14 in each experimental period. Sample collection of liquid occurred from d 8 to 14, and sample collection of effluent overflows, from the last 24 h, was on d 10, 12, and 14.

Continuous Culture Operation

At the beginning of each experimental period, inoculum was obtained from 2 ruminally cannulated Jersey

Table 1. Ingredients and nutrient composition of the experimental diet

Composition	Value
Ingredient (% of DM)	
Pelleted alfalfa hay	50.0
Concentrate pellet	
Ground corn	18.4
Soybean hulls	20.2
Distillers dried grains with solubles	5.0
Soybean meal, 47%	4.5
Corn oil	0.9
Dicalcium phosphate, 18.5%	0.4
Magnesium oxide, 56%	0.1
Salt, trace mineralized	0.5
Nutrient[1] (% of DM)	
OM	91.2
CP	17.6
NDF	32.6
ADF	26.5
Starch	13.6
Soluble carbohydrates	4.5
Ether extract	3.8
NE_L (Mcal/kg)	1.6

[1]Nutrient contents were analyzed using the wet chemistry methods described in Van Soest et al. (1991) and AOAC International (2005); NE_L was estimated according to NRC (2001).

cows fed a lactating cow diet similar to that described in Wenner et al. (2017). The animal use protocol was approved by the Institutional Animal Care and Use Committee at The Ohio State University. Ruminal content samples were quickly squeezed through 2 layers of cheesecloth, placed into a thermos maintained at 39°C, and taken to the laboratory, where the inocula were pooled, blended, and then distributed to all fermentors at 50% of their working volume. The agitation rate of each fermentor was maintained at 50 rpm and the temperature at 39°C. Clarified ruminal fluid (centrifuged at 15,000 × *g* and 4°C for 15 min and then autoclaved) was added to buffer in a 1:20 dilution rate for the first 2 d of each period to help the microbial community, particularly protozoa, adapt to the continuous culture fermentors (Karnati et al., 2009). The average working liquid volume of the fermentors was 1.71 ± 0.09 L, total buffer dilution rate was maintained at 7.0%/h (average of 2.87 L/d outflow), solids passage rate was maintained at 4.5%/h, temperature was set to 39°C, and fermentors were mixed at 50 rpm for the duration of the experiment. The buffer was prepared as described by Weller and Pilgrim (1974) with the addition of urea (0.4 g/L) to prevent depressions in microbial protein synthesis. Buffer was anaerobically maintained for at least 1 d before use by constant bubbling of CO_2 in the buffer tank. Buffer pH was maintained at 6.75 and allowed fermentor pH to fluctuate freely. In the LowpH treatment, phosphoric acid was added to the buffer to decrease pH by 0.5 unit, as previously done in Wenner et al. (2017).

Isotope Infusion and Sampling Procedures

Starting on 2000 h on d 8, 10, and 12, each of the 3 ^{13}C-labeled VFA was sequentially infused continuously into the fermentors for 36 h followed by 12 h of water infusion. Infusion rates were as follows: 3.5 mmol/d [2-^{13}C] sodium acetate, 2.9 mmol/d [U-^{13}C] sodium propionate, and 0.22 mmol/d [1-^{13}C] sodium butyrate. The sequence order for the [^{13}C]-VFA infusions was randomly assigned at the beginning of the experiment. Ideally, butyrate labeled in positions other than the carboxyl C would have been used because it is known to be more labile than that labeled in positions 2 through 4. However, the relative loss rate of the carboxyl C was found to be only marginally greater than for the 2 position in acetate (He et al., 2018), and [2-^{13}C] butyrate was only available through a custom order at a very substantial cost that was not financially possible.

Fermentor H_2 and CH_4 emissions from d 8 to 14 were measured using a Micro-Oxymax detection system (Columbus Instruments Inc.) by sampling headspace emissions at 30-min intervals. On d 8, 20-mL liquid samples were collected from a y-valve in the liquid filtrate line before feeding at 0800 h (0 h) and 2, 4, 8, 12, 16, and 22 h after feeding for measurement of aqueous H_2 and CH_4, as previously described by Wenner et al. (2017). Additional 4-mL samples were collected in a similar manner on d 9, 11, and 13, transferred into a vial, and immediately stored at −20°C until analysis for VFA concentrations and isotope enrichment. Additionally, for pH measurement, 2 mL of the liquid filtrate sample was taken at intervening times from d 9 through d 14 in such a way that yielded samples at 0, 2, 4, 6, 8, 12, 16, and 22 h after feeding. The samples for pH measurement were immediately dispensed into a 10-mL measuring cylinder, and a portable pH meter was used to obtain the pH readings. The pH readings obtained from this method matched those obtained by directly dipping the pH meter probe into the fermentors. At the end of each [^{13}C] VFA infusion, 650 mL of mixed effluent samples was collected for microbial ^{13}C enrichment analysis, microbial DNA analysis, and determination of VFA concentrations.

Before feeding on d 10, 12, and 14, 7.5% of mixed liquid and solid effluents from the previous 24-h period after feeding was collected for DM and nutrient analysis. During these collection days, the mixed liquid effluents were collected on ice to stop microbial fermentation. The effluent samples from each treatment were later pooled separately by period and freeze-dried. Pelleted diet samples were also freeze-dried, and both dried feed and effluent samples were ground and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for nutrient analysis.

Sample Analysis

Pelleted diet samples and effluent were analyzed for OM, CP, starch, soluble carbohydrates, and ether extract using AOAC International (2005) methods, and NDF and ADF were analyzed according to Van Soest et al. (1991). Volatile fatty acid concentrations in the fluid samples were determined by isotope dilution as described by Kristensen (2000). Briefly, ruminal liquid samples collected from fermentors were centrifuged for 30 min at 2,500 × *g* at room temperature to remove solid particles, external VFA tracers were added (deuterated and of a different mass than the infused ^{13}C-tracers), and VFA in the supernatant were derivatized using 2-chloroethyl chloroformate in a water, acetonitrile, and 2-chloroethanol solution. The derivatized samples were analyzed for isotopic enrichment using a Thermo Polaris Q MS in tandem with a Thermo Focus GC (Thermo Electron Corp.). Concentrations were derived from a gravimetric standard curve constructed with the same external tracer mix and varying amounts of unlabeled standards.

The ^{13}C enrichment of VFA was determined using a solid-phase microextraction (**SPME**) method with a Trace GC linked to a Delta V-advantage isotope ratio mass spectrometer (**IRMS**; Thermo Electron Corp.) via a combustion oven. The supernatants from centrifuged effluent samples (450 μL) were placed into 20-mL glass vials and acidified with 50 μL of phosphoric acid. The vial was capped with butyl rubber stopper with crimp seal and heated to 40°C for 12 min followed by exposure of the SPME fiber in the headspace gas for 1 min, transfer to the GC injection port, and exposure within the port at 250°C for 5 min with splitless flow for 1 min followed by a split flow of 50 mL/min. A Zebron capillary GC column (30 m × 0.25 mm × 0.25 μm; Phenomenex) was used for separation with constant helium flow of 1.5 mL/min. The column oven was set to an initial temperature of 32°C for 5 min followed by 7°C/min ramp up to 200°C, and 15°C/min ramp up to 240°C. Because eluting compounds are combusted, IRMS measures the ratio of ^{13}C/^{12}C within the entire molecule, which was then converted to an atom fraction [^{13}C/(^{12}C + ^{13}C)]. Ruminal microbes were isolated from the mixed effluent samples according to the procedure described by Larsen et al. (2000), and stable isotope analyses were conducted on an Isoprime 100 IRMS coupled to an Vario Isotope Cube elemental analyzer (Elementar Americas). Microbial ^{13}C enrichment was analyzed and calculated as described by Metges et al. (1990).

Microbial samples frozen at −80°C were extracted for DNA using the repeated bead beating plus column (RBB+C) method of Yu and Morrison (2004) followed by purification with a QIAamp DNA Stool Mini Kit (Qiagen Inc.). Extractions were initially screened for DNA concentration using a Nanodrop 1000 spectrophotometer (NanoDrop) before submission for 16S rRNA gene sequencing (MiSeq, Illumina Inc.) at the Molecular and Cellular Imaging Center (The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH). DNA was amplified with a universal primer targeting the V4-V5 hypervariable region using 2 × 300-bp paired end reads, similar to the method described in Faulkner et al. (2017). Data were processed using QIIME 1.9.0 (Caporaso et al., 2010), with a minimum Phred score of 20 yielding 843,254 quality reads. Operational taxonomic units (**OTU**) were assigned based on clustering sequences at 97% and sequences matched to taxonomy with the Silva database (v123; Edgar, 2013; Quast et al., 2013). Only OTU with relative abundance >0.5% were analyzed to condense the most relevant results for the present study.

Mathematical Modeling and C Exchange Calculation

An a priori expectation was that steady-state enrichment would be achieved within 12 h, allowing sample collection to begin with feeding. However, when [^{13}C] acetate or [^{13}C] propionate was infused, results from samples taken after feeding indicated that the concentration-corrected enrichment continued to increase for the entire 16-h postfeeding period. These changes are partially due to changes in VFA production rates after the single meal each day rather than failure to truly achieve isotopic equilibration in the system. Therefore, the isotopic calculations were based on the mass balance of infused and recovered isotope during the 24-h effluent sample collection period. This method accounts for the disequilibrium in the isotope state and is preferred to the conventional approach of using isotopic ratios with a steady-state assumption, which was originally planned. However, not all of the [^{13}C] acetate and [^{13}C] propionate was flushed from the system during the 12-h postinfusion period, indicating that more than 12 h is required to reach true steady-state enrichment in the fermentors. Therefore, in addition to conducting the calculations on a mass basis, the residual isotope remaining at the start of the next infusion was subtracted from the respective pools. To better estimate this residual, isotope disappearance rates were derived from enrichments at 0, 2, 6, and 12 h following isotope infusion.

Net VFA production (mmol/d) was calculated by summation of the individual measurement periods to

yield a 24-h estimate using an effluent outflow (F_{liquid}) of 120 mL/h, and measured VFA concentrations ($C_{VFA,i}$, mmol/mL) at 0, 2, 4, 6, 8, 12, 16, and 22 h after feeding:

$$NetVFA = \sum \left[\left(\frac{C_{VFA(tend)} + C_{VFA(tstart)}}{2}\right) \times F_{liquid} \times (tstart - tend)\right] + C_{VFAend} \times V_{end} - C_{VFAstart} \times V_{start},$$

where $C_{VFA(tstart)}$ and $C_{VFA(tend)}$ were the initial VFA and final concentrations for each time interval ($tstart - tend$, h); $C_{VFA(start)}$ and $C_{VFA(start)}$ were the initial and final VFA concentrations in the fermentors; and V_{start} and V_{end} were the initial and final volumes in the fermentors.

Labeled VFA output ($Net\ VFA^*$; mmol/d) over the 24-h period was calculated as follows:

$$Net\ VFA^* = \frac{VFA^E_{ef}}{100} \times Net\ VFA + \frac{VFA^E_{end}}{100} \times VFA_{end} - \frac{VFA^E_{start}}{100} \times VFA_{start},$$

where VFA^E is the excess atom fraction percent of the 24-h pooled effluent $\left(VFA^E_{ef}, \%\right)$, VFA^E_{end} is the excess atom fraction percent in the fermentor VFA pool at the end of the 24-h collection, and VFA^E_{start} is the excess atom fraction percent in the fermentor VFA pool at the start of the 24-h collection. The excess atom fraction was calculated as $AF_s - AF_b$, where AF is the atom fraction [^{13}C/(^{12}C+^{13}C) × 100] for the samples (s) and the background (b). VFA_{net} is net VFA production (mmol/d), VFA_{end} (mmol) and VFA_{start} (mmol) are the VFA pools at the end and start of each 24-h sampling period, the latter being calculated as the scalar product of VFA concentration and volume of the fermentor.

Total VFA production (mmol/d) was estimated from the infused [^{13}C]VFA ($Inf\ VFA^*$, (mmol/d) corrected for the number of labeled C, $Net\ VFA^*$, and net VFA production (mmol/d) as follows:

$$Total\ VFA = \frac{Inf_{VFA^*} \times \frac{labeled\ carbons\ in\ VFA\ isotope}{total\ carbons\ in\ VFA}}{Net\ VFA^*} \times Net\ VFA.$$

Conversions of one VFA to the other VFA were calculated based on the ratio of recipient VFA^* to donor VFA^*, the net production rate of the donor VFA, and the number of carbons (C) in each VFA. For example, when [^{13}C] acetate was infused, conversion of acetate to propionate (VFA_{ap}, mmol/d) was estimated as follows:

$$VFA_{ap} = Net\ \ VFA_a \times \left(\frac{NetVFA^*_p \times C\ \ in\ \ propionate}{NetVFA^*_a \times C\ \ in\ \ acetate}\right),$$

where $Net\ \ VFA_a$ was the net acetate production rate (mmol/d), and $NetVFA^*_p$ and $NetVFA^*_a$ were the isotopic outputs (mmol/d) of propionate and acetate, respectively. This approach avoids the effects of violating the conservation of mass assumptions of the France et al. (1991) model, but it underestimates the total exchange because it is assumed the reverse reactions are not affected by isotopic transfer during each of the infusions.

The proportion of ruminal microbial C sequestrated from each VFA ($Microbial\ C_{VFA}$, %) was calculated as follows:

$$Microbial\ \ C_{VFA} = \frac{DM_{ef} \times Microbial\ \ C \times (AF_S - AF_B)}{DM_{ef} \times Microbial\ \ C \times AF_S} \times 100,$$

where DM_{ef} is the microbial DM outflow (g/d) of each fermentor; $Microbial\ C$ is the C proportion of microbial DM (%); AF_S is the atomic fraction [^{13}C/(^{12}C+^{13}C)] of ruminal microbes at the end of each [^{13}C] VFA infusion; AF_B is the atomic fraction [^{13}C/(^{12}C+^{13}C)] of background, which was assumed to be 0.0108, according to Metges et al. (1990).

Data Analysis

The data were analyzed using PROC GLIMMIX in SAS version 9.3 (SAS Institute Inc.). Liquid pH and gaseous and aqueous production of H_2 and CH_4 were analyzed using a mixed-effects model with fermentor within treatment as repeated measures and a heterogeneous autoregressive (1) covariance structure. The model included treatment, hour after feeding, and their interactions as fixed effects, and fermentor and period as random effects. Nutrient digestibilities, cumulative gaseous H_2 and CH_4 emissions, VFA net and total production rates, interconversions among VFA, and proportions of microbial C sequestrated from VFA were analyzed with treatment as a fixed effect and fermentor and period as random effects. Multiple comparison tests were performed using the Tukey-Kramer method. Significance was declared at $P < 0.05$.

Microbial community relative abundances were analyzed using R (package lsmeans) with the fixed effect of treatment and random effects of fermentor and period. After a Kenward-Roger correction for degrees

Table 2. Effects of acetate, propionate, and pH on nutrient digestibility, VFA concentrations, and aqueous concentration and cumulative gaseous emission of H_2 and CH_4 in continuous culture[1]

Item	Con	InfAc	InfPr	LowpH	SEM	*P*-value
Digestibility (%)						
Apparent OM	23.1[b]	24.3[b]	17.7[b]	39.5[a]	3.9	<0.001
NDF	44.5	48.2	48.2	52.4	4.5	0.34
ADF	38.5	42.1	40.7	41.5	3.9	0.51
Starch	78.2	80.6	81.2	82.4	1.8	0.28
VFA (m*M*)						
Acetate	39.4[ab]	43.4[a]	37.9[ab]	36.5[b]	4.9	0.02
Propionate	13.7	13.7	15.4	14.8	1.7	0.13
Butyrate	6.0[a]	5.8[ab]	5.9[a]	5.3[b]	0.5	0.005
Acetate:propionate	2.7[b]	2.6[b,c]	3.0[a]	2.3[c]	0.12	<0.01
Gas emission and concentration						
Aqueous H_2 (μ*M*)	2.3	1.7	2.0	1.5	0.3	0.34
Headspace H_2 (μmol/d)	277[b]	605[a]	325[b]	80[c]	128	<0.001
Aqueous CH_4 (μ*M*)	120[c]	130[bc]	176[ab]	187[a]	18	0.02
Headspace CH_4 (mmol/d)	41.5[a]	47.4[a]	42.2[a]	24.8[b]	9.5	<0.001

[a–c]Means with different superscripts differ at $P \leq 0.05$.

[1]Con = buffer infusion with average pH of 6.75 (control); InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con.

of freedom was applied, significance was declared at $P < 0.05$ and trends at $0.05 \leq P < 0.10$. The model and pairwise comparisons of means were as described previously. Relative abundances were not transformed because various transformation attempts from the same sequencing run previously failed to improve data normalization (Faulkner et al., 2017). Diversity of microbial communities (α and β) was analyzed using R (package vegan) with cutoffs for significant factors of $P < 0.05$.

RESULTS

As expected, pH for LowpH was lower than that of other treatments throughout the day ($P < 0.01$; Supplemental Figure S1; https://figshare.com/articles/journal_contribution/Supplement_docx/20542719; Li, 2022). No significant treatment effect on pH was observed among Con, InfPr, and InfAc treatments ($P > 0.05$). The 24-h integrated pH in the LowpH group ranged from 6.06 to 6.38, whereas it ranged from 6.63 to 6.94 in other treatments. There was a consistent pH trend over time for all treatments, where pH declined gradually after feeding for 10 to 12 h, followed by a steady increase, returning to near the original pH at 22 h after feeding.

The 24-h nutrient digestibility results are presented in Table 2. The LowpH treatment increased apparent OM digestibility relative to other treatments ($P < 0.001$). However, the digestibility of NDF, ADF, and starch was not affected by treatments ($P > 0.05$). On average, 48, 41, and 81% of NDF, ADF, and starch, respectively, was digested across all treatments in the culture fermentors.

Volatile fatty acid concentrations relative to feeding time are displayed in Figure 1. We detected no treatment × time interactions ($P > 0.05$) for any of the 3 VFA, but there was a significant time effect ($P < 0.0001$), which was similar for all 3 VFA, with concentrations increasing after the meal to a plateau 6 to 8 h after feeding, and then gradually decreasing thereafter. The overall treatment effects on VFA concentrations are listed in Table 2. The LowpH treatment induced lower acetate concentrations than InfAc ($P < 0.05$), and lower butyrate concentrations than the Con and InfAc treatments ($P < 0.05$). No other significant differences were observed among treatments on VFA concentrations.

Aqueous concentrations and headspace emissions of H_2 are displayed in Figure 2. There was not a significant treatment × time interaction or treatment effect on H_2(aq) concentrations ($P > 0.05$). H_2(aq) concentrations fluctuated over time ($P = 0.08$), increasing after feeding for all treatments, peaking around 2 h postfeeding, and declining thereafter. Significant time effects were also observed for headspace gaseous H_2 [$\mathbf{H_2(g)}$] emission rates ($P < 0.0001$). The H_2(g) emission pattern was consistent with H_2(aq) concentrations, with emission rates dramatically increasing within 3 h of feeding and decreasing thereafter. There was no significant treatment × time interaction or overall treatment effect on H_2(g) emissions ($P > 0.05$). However, LowpH decreased H_2(g) emissions relative to other treatments 2, 3, and 4 h after feeding; InfAc caused greater H_2

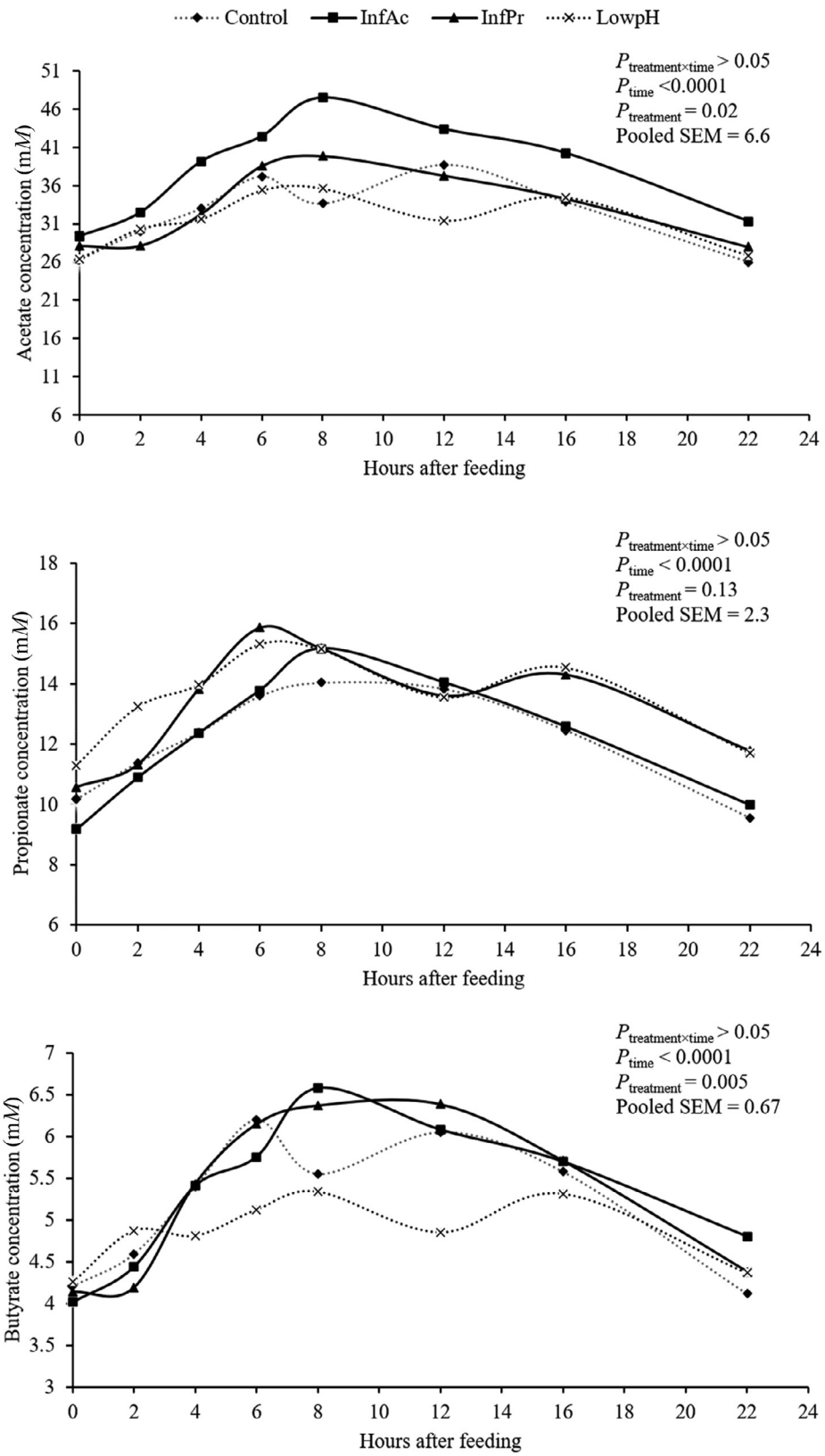

Figure 1. Effects of acetate, propionate, and pH on VFA concentrations over time in continuous culture fermentors (n = 4). Control (Con) = buffer infusion with average pH of 6.75; InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con.

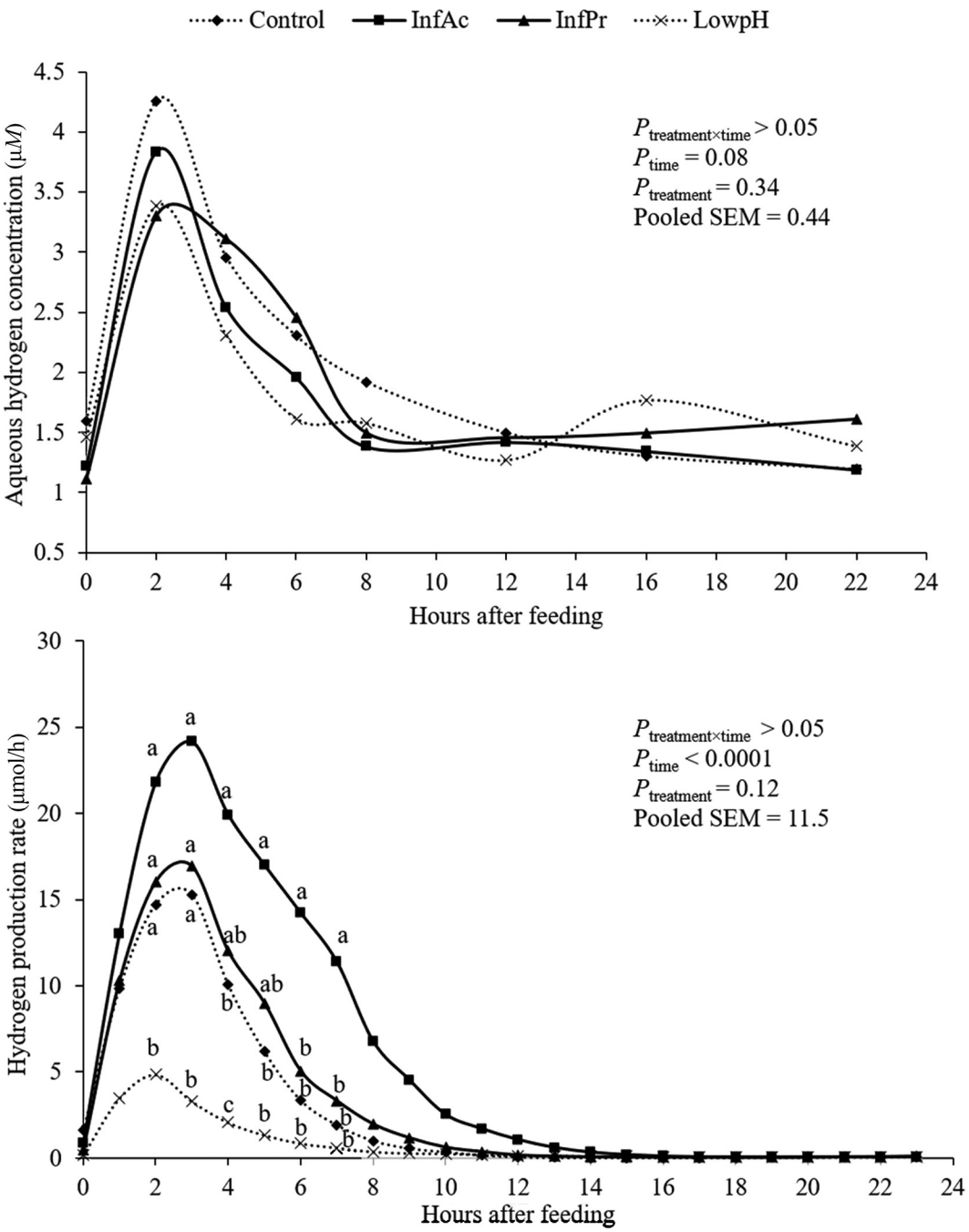

Figure 2. Effects of acetate, propionate, and pH on aqueous H concentration and headspace H_2 emission over time in continuous culture fermentors (n = 4). Control (Con) = buffer infusion with average pH of 6.75; InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con. Different letters (a–c) indicate significantly different means at each time point ($P \leq 0.05$).

emission rates than the other treatments 6 and 7 h after feeding. Cumulative H_2(g) emissions (μmol/d) are presented in Table 2. Compared with Con, InfAc increased cumulative H_2(g) emissions, whereas LowpH decreased cumulative H_2(g) emissions. No significant differences were observed between Con and InfPr.

As displayed in Figure 3 and Table 2, there were no treatment × time interactions or time effects on CH_4(aq) concentrations, but significant treatment effects were observed ($P < 0.05$). Both InfPr and LowpH increased CH_4(aq) concentration relative to Con, whereas there was no significant difference in CH_4(aq) concentration

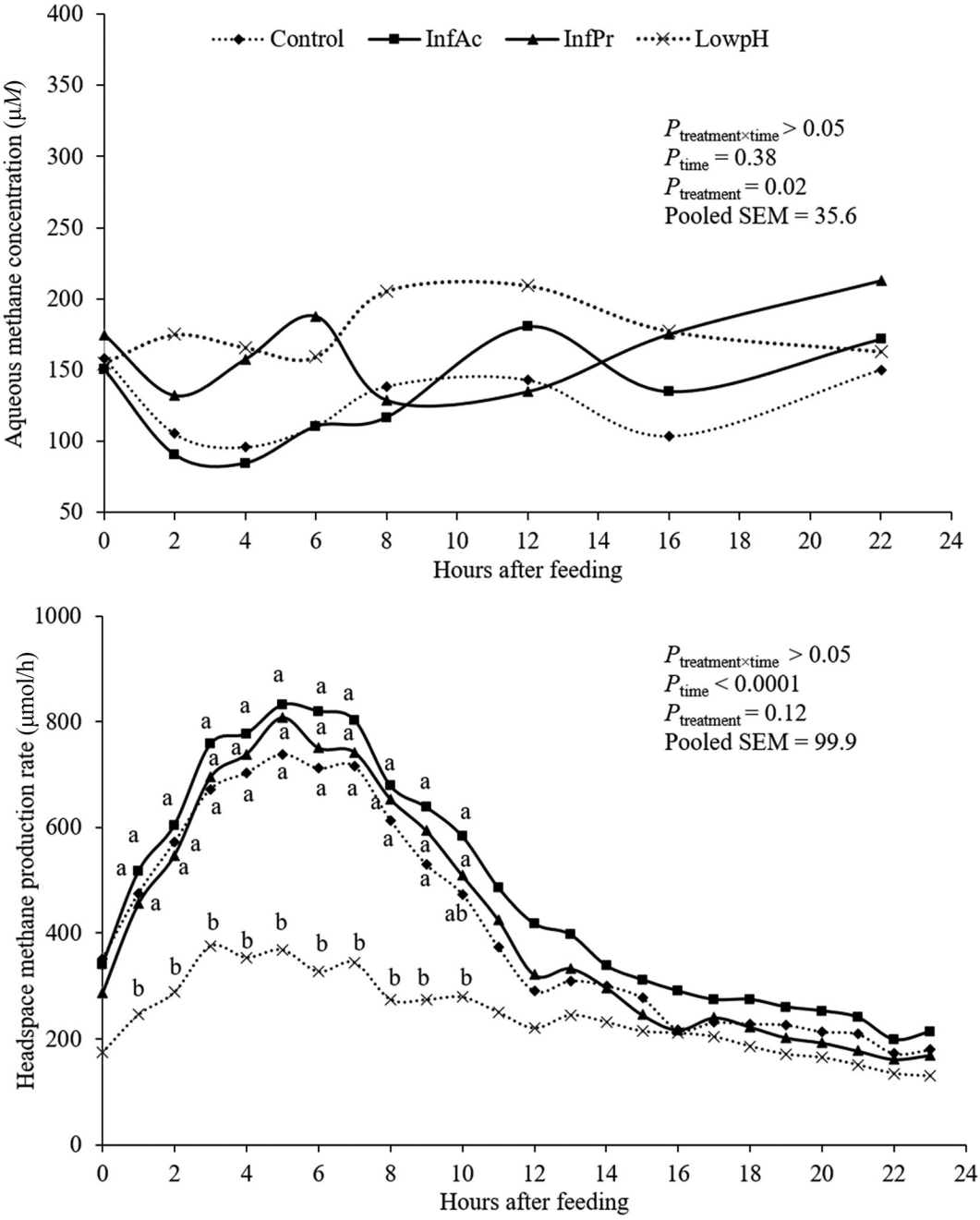

Figure 3. Effects of acetate, propionate, and pH on aqueous H concentration and headspace CH_4 emission over time in continuous culture fermentors (n = 4). Control (Con) = buffer infusion with average pH of 6.75; InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con. Different letters (a, b) indicate significantly different means at each time point ($P \leq 0.05$).

between Con and InfAc. A significant time effect was observed on CH_4(g) emissions postfeeding. The CH_4(g) emissions increased gradually and peaked at 6 to 8 h after feeding and then decreased slowly thereafter. There was no treatment × time interaction or overall treatment effect on CH_4(g) emissions ($P > 0.05$). However, CH_4(g) emissions were lower with LowpH than with other treatments from 2 to 9 h after feeding ($P < 0.05$). Similar treatment effects were also observed for cumulative CH_4(g) emissions ($P < 0.001$). LowpH significantly decreased cumulative CH_4(g) emissions compared with the other treatments, and no significant differences were observed among Con, InfAc, and InfPr (Table 2).

Table 3. Effects of acetate, propionate, and pH on net production rates and interconversions among VFA in continuous culture[1]

Item[2]	Con	InfAc	InfPr	LowpH	SEM	*P*-value
Acetate						
Net production (mmol/d)	93.9[b]	112.3[a]	94.7[b]	88.1[b]	4.3	0.01
Total production (mmol/d)	152	172	162	176	15.1	0.78
Acetate to propionate (mmol/d)	5.1	5.4	6.0	8.5	1.1	0.24
% of total production	3.4	3.1	3.6	5.0	0.7	0.4
Acetate to butyrate (mmol/d)	11.9	13.6	12.4	12.8	1.2	0.7
% of total production	7.8	7.9	7.6	7.5	0.5	0.91
Propionate						
Net production (mmol/d)	34.3[b]	35.7[ab]	38.4[a]	38.2[a]	1.5	0.04
Total production (mmol/d)	66.5	57.5	64.2	66.6	6.6	0.6
Propionate to acetate (mmol/d)	1.1	1.3	1.4	1.1	0.1	0.1
% of total production	1.8	2.3	2.2	1.6	0.2	0.28
Propionate to butyrate (mmol/d)	0.1	0.2	0.3	0.2	0.07	0.22
% of total production	0.2	0.4	0.5	0.4	0.08	0.32
Butyrate						
Net production (mmol/d)	15.4	15.9	15.9	13.9	0.8	0.14
Total production (mmol/d)	22	23.4	17.1	16.1	2.5	0.26
Butyrate to acetate (mmol/d)	9.9	5.8	3.6	5.2	2.5	0.36
% of total production	43.1	28.6	21.5	32.9	10.2	0.49
Butyrate to propionate (mmol/d)	1.5	3.1	1.4	3.2	1.7	0.86
% of total production	6.0	15.4	6.9	18.9	7.9	0.65

[a,b]Means with different superscripts differ at $P \leq 0.05$.

[1]Con = buffer infusion with average pH of 6.75 (control); InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con.

[2][2-^{13}C] sodium acetate (3.51 mmol/d), [U-^{13}C] sodium propionate (2.9 mmol/d), and [1-^{13}C] sodium butyrate (0.22 mmol/d) were continuously infused for 36 h to calculate total production and interconversions among VFA. Net production is the observed flow of the acids from the fermentors.

The effects of acetate, propionate, and pH on VFA productions and interconversions among VFA are listed in Table 3. Compared with Con, InfAc increased net acetate production ($P = 0.01$), and InfPr and LowpH treatments increased net propionate production ($P = 0.04$). There was no treatment effect on net butyrate production, and no significant differences were observed among treatments for total VFA production and interconversions among the 3 VFA ($P > 0.05$). The conversion rates of acetate to butyrate and acetate to propionate were approximately 8% and 4% of total acetate production, respectively. The conversion of propionate to butyrate was below 0.5%, and that of propionate to acetate was around 2% of total propionate production. Around 32% of total butyrate was converted to acetate, and 12% was converted to propionate. On average, 30% of total acetate and 40% of total propionate were estimated to be lost into pools other than the 3 major VFA. As shown in Figure 4, no significant treatment effect was observed on proportions of microbial C captured from acetate, propionate, and butyrate ($P > 0.05$). On average, the ruminal microbial C sequestrated from acetate, propionate and butyrate accounted for 3.6, 4.3, and 3.32% of total microbial C, respectively.

Relative abundances of archaeal and bacterial populations are reported in Table 4. Community α-diversity measures were unaffected ($P > 0.10$) by treatments (Supplemental Table S1; https://figshare.com/articles/journal_contribution/Supplement_docx/20542719; Li, 2022), whereas Bray-Curtis dissimilarity was significantly different ($P < 0.01$) for the LowpH treatment (Supplemental Figure S2; https://figshare.com/articles/journal_contribution/Supplement_docx/20542719, Li, 2022). The LowpH treatment increased ($P < 0.05$) the relative sequence abundance of *Methanobrevibacter* relative to Con (Table 3). Relative abundances of many *Firmicutes* also increased ($P < 0.05$); namely, *Oribacterium*, *Ruminococcus*, uncultured *Ruminococcaceae*, and members of family *Veillonaceae*. Uncultured *RF16* and *Clostridium* decreased ($P < 0.05$) relative to Con. *Butyrivibrio* increased ($P < 0.05$) in the InfAc treatment versus Con.

DISCUSSION

Effects of Acetate, Propionate, and Low pH on Nutrient Digestibility

The diurnal pattern of pH in the fermentors was consistent with that of studies with animals fed one meal per day (Hünerberg et al., 2015). The pattern was also consistent with nutrient degradation and VFA

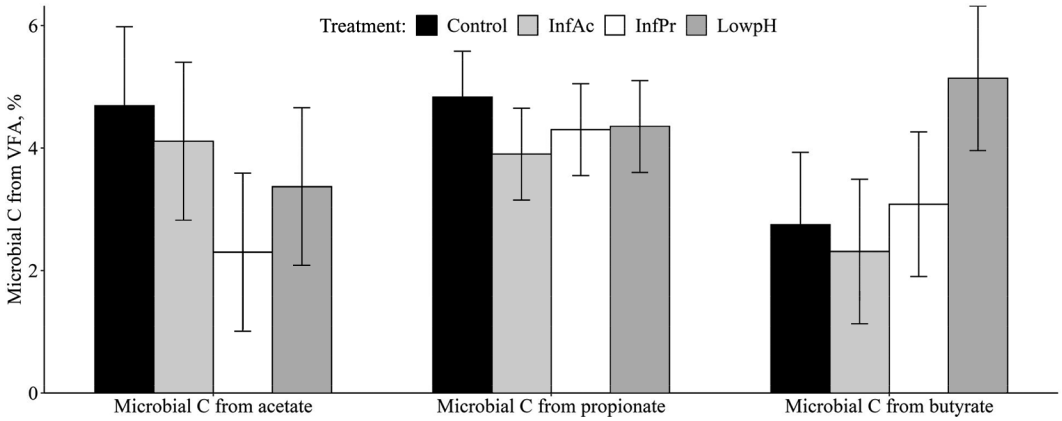

Figure 4. Effects of acetate, propionate, and pH on proportions of ruminal microbial C sequestrated from each VFA based on the isotope dilution technique (n = 4). Control (Con) = buffer infusion with average pH of 6.75; InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con. The error bars represent SEM.

production in the fermentors, the latter resulting in H^+ release. Briggs et al. (1957) observed a negative correlation between VFA concentrations and pH. Although acetate and propionate were continuously infused, we did not observe a pH reduction compared with Con, presumably due to the neutralizing effect of bicarbonate in the buffer.

Low pH increased apparent OM digestibility, but digestibilities of NDF, ADF, and starch were not affected by low pH, in contrast to our expectations. Plaizier et al. (2001) reported that 24-h ruminal NDF digestibility decreased 20% in cows with SARA (mean pH of 5.87). Krajcarski-Hunt et al. (2002) observed reduced in situ NDF and ADF degradation rates for corn silage diets incubated in the rumens of cows experiencing SARA. Calsamiglia et al. (2007) also observed decreased NDF digestibility in continuous cultures within the pH range from 4.9 to 6.2. Depending upon the type of forages in the diets, Grant and Weidner (1992) demonstrated that the critical pH for lag in NDF digestion is in a range from 6.2 to 5.8. In the current study, the average 24-h pH for the LowpH treatment was 6.24, which is greater than the critical range of Grant and Weidner (1992), suggesting that the pH was not low enough for the LowpH treatment to suppress pH-sensitive fibrolytic bacteria and archaea. This is congruent with the previous lack of effect of transient low pH on nutrient digestibility in dual-flow continuous culture (Wenner et al., 2017).

Effects of Acetate, Propionate, and Low pH on Emissions of H_2 and CH_4

The diurnal patterns of aqueous concentrations and headspace emissions of H_2 were consistent with previously published measurements (Wenner et al., 2017). Metabolic H ([2H]) is mainly generated during fermentation of dietary fiber and starch to VFA by ruminal microorganisms (Chalupa, 1977; Baldwin and Allison, 1983) and bacterial hydrogenases recycling NADH to NAD^+ (Russell, 2002). Large amounts of H_2 released from nutrient fermentation may overload the utilization capacity of hydrogen-consuming microbes (Wang et al., 2016; Wenner et al., 2020), leading to increased H_2(aq) in the ruminal liquid and ultimately increasing H_2 escape. No significant treatment effect was observed on H_2(aq) concentrations over infusion time, suggesting that dissolved H_2(aq) reached equilibrium and was not influenced by infusion of VFA.

Acetate infusion increased cumulative H_2(g) emissions, whereas low pH decreased cumulative H_2(g) emissions compared with Con. The former is consistent with increased net acetate production due to acetate infusion; the latter is consistent with observations of decreased daily H_2 emission with reduced pH reported by Wenner et al. (2017). Considering that ruminal digestibility of NDF, ADF, and starch was not altered by treatment, differences in H_2(g) emission must be due to altered microbial community structure or metabolic pathway use. The low pH treatment increased net propionate production, which supports the hypothesis that low pH shifted metabolic pathways of VFA production, resulting in decreased H_2 emissions.

Methane is the primary end-product of H_2 and CO_2 to facilitate turnover of [2H] within the ruminant. van Zijderveld et al. (2010) reported that CH_4 production peaked 6 h after feeding and declined afterward in sheep fed once daily. This pattern of production could be explained by declining fermentable substrate over the course of the day, which has been observed previ-

Table 4. Effects of acetate, propionate, and pH on relative abundance of prokaryotic sequences listed by phyla at the genus level[1,2]

Item[3]	Con	InfAc	InfPr	LowpH	SEM	*P*-value
Archaea						
f. *Methanobacteriaceae* g. *Methanobrevibacter*	0.052[b]	0.042[b]	0.046[b]	0.155[a]	0.024	0.05
Uncultured *Vadin CA11 Gut Group*	0.043	0.036	0.048	0.016	0.013	0.37
Bacteria						
p. *Actinobacteria* f. *Bifidobacteriaceae* g. *Bifidobacterium*	0.006	0.001	0.000	0.677	0.196	0.12
p. *Bacteroidetes*, Order *Bacteroidales*						
f. *Bacteroidaceae* g. *Bacteroides*	0.285	0.175	0.242	0.203	0.074	0.70
f. Uncultured *BS11 Gut Group*	2.65	2.39	3.00	2.01	0.72	0.63
f. Uncultured Rumen *BS11 Gut Group*	2.11	1.76	2.10	2.76	0.46	0.23
f. *Prevotellaceae* g. *Prevotella*	17.8	15.7	17.8	16.9	1.0	0.34
f. Uncultured *Prevotellaceae*	7.23	7.58	7.26	7.16	0.56	0.81
f. *Prevotellaceae* g. *Xylanibacter*	0.350	0.355	0.397	0.555	0.076	0.08
Uncultured *RF16*	0.408[a]	0.465[a]	0.485[a]	0.122[b]	0.057	<0.01
Uncultured Rumen *RF16*	0.152	0.227	0.200	0.073	0.057	0.09
f. *Rikenellaceae* g. *RC9 Gut Group*	8.39	7.06	8.53	9.20	0.72	0.29
f. *Rikenellaceae* g. *SP3-e08*	0.340	0.234	0.346	0.111	0.140	0.14
Uncultured *S24–7*	1.69	1.69	1.66	1.13	0.26	0.12
Uncultured Rumen *S24–7*	5.94	5.84	5.29	6.79	1.03	0.46
Other *Bacteroidales*	1.23	0.97	0.97	1.34	0.16	0.10
Other *Candidate Division TM7*	0.078	0.064	0.070	0.219	0.073	0.44
p. *Fibrobacteres* f. *Fibrobacteraceae* g. *Fibrobacter*	1.11	1.22	1.26	1.14	0.29	0.92
p. *Firmicutes*, Order *Clostridiales*						
f. *Clostridiaceae* g. *Clostridium*	2.52[a]	1.64[ab]	1.48[ab]	0.12[b]	0.90	0.06
f. Uncultured *Christensenellaceae*	0.856	0.808	0.931	1.214	0.161	0.36
f. *Lachnospiraceae* g. *Anaerosporobacter*	0.878[ab]	0.931[ab]	1.488[a]	0.335[b]	0.497	0.10
f. *Lachnospiraceae* g. *Butyrivibrio*	6.00[b]	7.35[a]	6.25[ab]	6.96[ab]	0.66	0.08
f. *Lachnospiraceae* g. *incertae sedis*	1.32	1.50	1.31	1.24	0.40	0.64
f. *Lachnospiraceae* g. *Oribacterium*	0.159[b]	0.140[b]	0.160[b]	0.376[a]	0.043	0.01
f. *Lachnospiraceae* g. *Pseudobutyrivibrio*	2.96	4.44	3.45	3.25	0.89	0.31
f. *Lachnospiraceae* g. *Roseburia*	0.425	0.319	0.285	0.511	0.109	0.32
Uncultured *Lachnospiraceae*	3.03	3.05	2.99	2.54	0.44	0.58
Other *Lachnospiraceae*	1.08	1.21	1.22	0.81	0.15	0.19
f. *Ruminococcaceae* g. *incertae sedis*	0.343	0.326	0.480	0.364	0.110	0.41
f. *Ruminococcaceae* g. *Ruminococcus*	1.45[b]	1.70[b]	1.58[b]	5.01[a]	0.55	<0.01
f. *Ruminococcaceae* g. *Saccharofermentans*	0.338	0.348	0.423	0.493	0.073	0.26
Uncultured *Ruminococcaceae*	2.79[b]	2.55[b]	2.70[b]	4.78[a]	0.46	0.04
f. *Veillonellaceae* g. *Selenomonas*	0.303[b]	0.350[b]	0.324[b]	0.549[a]	0.081	0.04
f. *Veillonellaceae* g. *Succiniclasticum*	1.09[ab]	0.93[b]	1.06[ab]	1.57[a]	0.18	0.05
Uncultured *Veillonellaceae*	0.250[b]	0.214[b]	0.198[b]	0.560[a]	0.040	<0.01
p. *Lentisphaerae* f. Uncultured *RFP12 Gut Group*	0.284	0.298	0.325	0.346	0.078	0.63
p. *Proteobacteria*, Order *Aeromonadales*						
f. *Succinivibrionaceae* g. *Ruminobacter*	0.418	0.672	0.573	0.070	0.184	0.13
f. *Succinivibrionaceae* g. *Succinivibrio*	0.473	0.479	0.762	0.257	0.165	0.18
Uncultured *Succinivibrionaceae*	0.826	0.856	0.725	0.184	0.172	0.10
p. *Proteobacteria* o. *Pseudomonadales* f. *Moraxellaceae* g. *Acinetobacter*	0.331	0.283	0.549	0.162	0.152	0.40
Uncultured *RF3*	0.245	0.253	0.349	0.119	0.092	0.22
p. *Spirochaetes* o. *Spirochaetales* f. *Spirochaetaceae* g. *Spirochaeta*	0.830	0.721	0.959	0.874	0.196	0.65
p. *Spirochaetes* o. *Spirochaetales* f. *Spirochaetaceae* g. *Treponema*	9.94	11.73	8.05	4.86	2.73	0.19
p. *Tenericutes* o. *Anaeroplasmatales* f. *Anaeroplasmataceae* g. *Anaeroplasma*	0.800[ab]	0.839[ab]	0.894[a]	0.564[b]	0.136	0.06
Other uncultured *Verrucomicrobia OPB35 Soil Group*	0.261	0.232	0.287	0.083	0.090	0.16
Unassigned	5.15	5.39	5.40	5.20	0.46	0.94

[a,b]Means with different superscripts differ at $P \leq 0.05$.

[1]Con = buffer infusion with average pH of 6.75 (control); InfAc = Con plus continuous infusion of 20 mmol/d acetate; InfPr = Con plus continuous infusion of 7 mmol/d propionate; LowpH = buffer infusion with pH reduced by 0.5 units compared with Con.

[2]Operational taxonomic units are included in this table only if one measurement ≥0.5% relative abundance; the remainder are summed as Unassigned.

[3]p. = phylum; o. = order; f. = family; g. = genus.

ously in continuous culture (Wenner et al., 2020). However, increased CH_4(aq) and decreased CH_4 headspace emissions with the LowpH treatment were unexpected. Van Kessel and Russell (1996) found methanogenesis to be sensitive to low pH. Hook et al. (2011) reported that induction of SARA altered methanogen diversity and community structure as well as protozoal density within the rumen. Given the fact that the low pH treatment increased the relative abundance of the dominant genus *Methanobrevibacter*, it is unlikely that the LowpH

treatment decreased CH_4 emissions by changing the archaeal community structure. We can speculate that the decreased CH_4 emissions were likely caused by the reduced H_2 supply for methanogenesis.

Treatment Effects on VFA Production and Interconversions

As noted by Ungerfeld and Kohn (2006), all catalyzed chemical reactions are bidirectional, and the enzymes participating in metabolic pathways of VFA formation facilitate the reactions by reducing the activation energy required in both forward and reverse directions, with the net difference depending on the thermodynamic state. Kohn and Boston (2000) demonstrated the thermodynamic control of several metabolic pathways and the effects on final VFA concentrations. Thus, if concentrations of VFA end-products increase, a shift in equilibrium will occur, which may alter the rate of transfer or prevent the normal direction of flow, based on the second law of thermodynamics. In the current study, infusion of acetate and propionate increased net acetate and propionate production but not their concentrations. Given the latter, it is perhaps not surprising that total VFA production rates and interconversions among VFA were not affected, and our hypothesis was not well tested. Ungerfeld (2015) demonstrated that thermodynamics, enzyme kinetics, and substrate kinetics could all participate in control of the rate of biochemical pathways. Considering that adding fermentation end-products did not change VFA interconversions and total production, we could conclude that thermodynamics might not be a primary driving factor for VFA metabolic pathway flux in the rumen; however, it is more likely the changes in concentration associated with acetate and propionate infusions were inadequate to elicit a change in product concentrations. This suggests that acetate and propionate formation might have been primarily under enzyme and substrate kinetic control.

A solution for the 3-pool model with full interconversions is presented in Figure 5. About 8% of total acetate production was converted to butyrate and 4% to propionate. This indicates that 39 to 46% of net butyrate production was derived from acetate. The results align with data reported in sheep by Leng and Brett (1966) and Weller et al. (1967). The former study estimated that 40 to 50% of butyrate was derived from acetate, and the latter estimated it to be 50%. Kristensen (2001) reported a slightly larger conversion rate for acetate to butyrate (16% of total acetate production) in lactating cows, but the flux from acetate to propionate was estimated to be about 4 to 5%, which

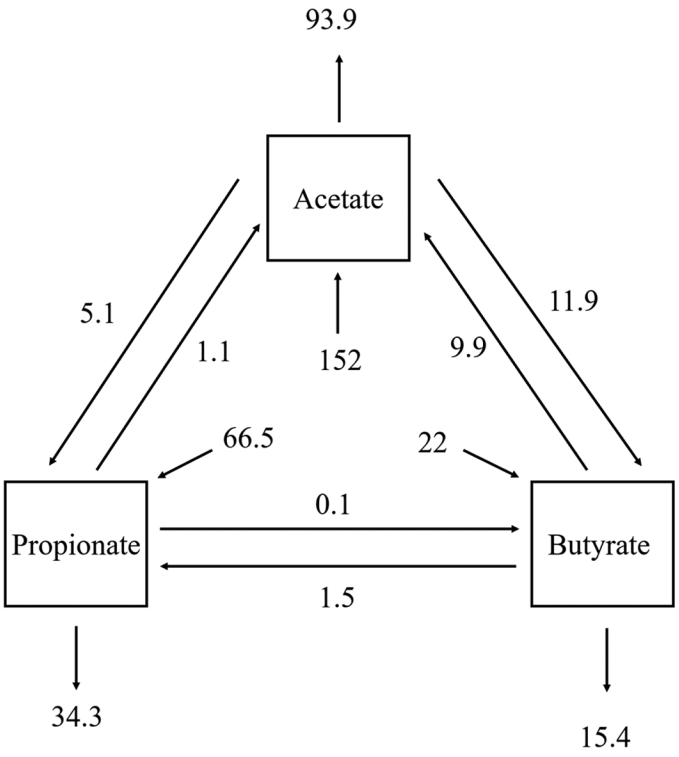

Figure 5. Total production and interconversions (mmol/d) among VFA at an average pH of 6.75 (control treatment) in continuous culture.

was similar to this study. Lesser exchange of propionate C to other VFA was reported in various other isotopic studies in animals where the exchange was usually less than 5% (Bergman et al., 1965; Esdale et al., 1968; Armentano and Young, 1983). The conversion of butyrate to propionate in this study was about 2% of total butyrate production, but it was estimated with high error. Similarly, the average flux of propionate to butyrate was 0.3% of total propionate production. Acetyl-CoA is a common intermediate of acetate and butyrate formation, and thus the number of reactions required for the interchange is relatively small compared with exchanges involving pyruvate, which likely explains the greater interconversions between acetate and butyrate. Consistent with this hypothesis, fluxes between acetate and propionate and between propionate and butyrate were less than 1%. Similar results were also reported in Bergman et al. (1965) and Esdale et al. (1968), suggesting that the interconversions between acetate and propionate and fluxes between propionate and butyrate are relatively low.

In the current study, acetate, propionate, and butyrate net production based on fermentor outflow was 94, 34, and 15 mmol/d, respectively, for the Con treatment (Figure 5). From the stoichiometric parameters for

high-roughage diets reported by Murphy et al. (1982), acetate, propionate, and butyrate production were predicted to be 94, 32, and 33 mmol/d. Acetate and propionate production was close to the model-derived values whereas butyrate production was greater. The reason for the greater rate for butyrate production from Murphy et al. (1982) suggests differences in the relationships between production and absorption among the VFA, with acetate and propionate concentrations more directly reflecting production. The extensive conversion of acetate to butyrate may contribute to the butyrate divergence because 25 to 40% of net butyrate was derived from acetate. The C transfer from acetate to butyrate represents approximately 7.5% of acetate production and thus is proportionally less influential.

Total ^{13}C recoveries in the 3 measured VFA when labeled acetate or propionate was infused averaged 70 and 60%, respectively. Acetate recovery herein was almost identical to the 72% recovery estimated by Kristensen (2001). Omission of consideration of effluxes from the primary labeled pools results in overestimates of the interchanges when using the fully exchanging balance model, but it should not affect estimates of total production rates given that they are derived without assumptions of use.

McInerney and Bryant (1981) observed conversion of acetate to CH_4 and CO_2 in anaerobic cultures. Kristensen (2001) found that about 28% of the acetate produced in the rumen exited the rumen in metabolites other than acetate. In the present study, the amount of total VFA converted to other metabolites (including those not recovered) was estimated to be 41% for acetate and 43% for propionate. Peripheral tissue oxidation of acetate in vivo results in some labeled CO_2 that circulates back to the rumen, thus increasing isotope recovery (Leng and Leonard, 1965); however, that was not the case in the fermentors. When total production is large, especially right after feeding, acetate and propionate degradation is thermodynamically more favorable. Kohn et al. (2015) also contend that at equilibrium, the ΔG for acetate synthesis and degradation of acetate is near zero, and that changes in acetate concentration could shift the reaction in either direction. However, if degradation of acetate occurred in the present work, it did not result in synthesis of propionate or butyrate. Regardless of the reason, significant label conversion to metabolites other than the 3 VFA considered herein violates the conservation of mass assumptions underpinning the model, which was also noted by Kristensen (2001).

It is not uncommon for biological systems to discriminate between labeled and unlabeled metabolites because of differences in molecular weight, and this problem is generally of greater concern for deuterium (^2H) due to the large relative difference in mass (Leng and Leonard, 1965). Such discrimination has also been observed for CH_4 production (Klevenhusen et al., 2009), which results in depleted CH_4 enrichment and increased enrichment of CO_2. The latter would result in an underestimate of propionate formation from acetate due to capture of $^{13}CO_2$ in propionate. However, it is highly unlikely that discrimination could explain the apparent loss of label from the system.

A portion of the C loss is explained by microbial sequestration of VFA. Ruminal microbes can use VFA to synthesize essential nutrients for microbial growth. Kristensen (2001) reported that the atomic fraction of $[^{13}C/(^{13}C + {}^{12}C)]$ in several different fatty acids and AA isolated from ruminal microbes were increased after intraruminal infusion of [2-^{13}C] acetate, and the duodenal flow of labeled C in fatty acid and AA could account for 0.2 and 2.2% of the infused [2-^{13}C] acetate. Although no significant treatment effect was observed in the current study, we observed that approximately 10 to 15% of microbial C originated from the C of acetate, propionate, and butyrate. The loss of labeled C into microbial biomass and potential loss into valerate, isovalerate, and isobutyrate are major violations of the assumptions underpinning the fully exchanging model commonly used for the interpretation of isotopic enrichment data (France et al., 1991). Net synthesis estimates should be minimally affected, but de novo synthesis and interconversion estimates would be affected as the model was developed based on conservation of mass principles, assuming that entering C only exchanged among the 3 pools. If net synthesis rates are correct and if synthesis is proportional to concentrations across VFA, then the stoichiometric constants of Murphy et al. (1982) and Bannink et al. (2006) should not be biased on average, but they may still have low precision if the alternative uses vary by diet, physiological state, or management.

Ruminal Microbes Altered VFA Metabolic Pathways in Response to Low pH

Reduced culture pH is known to exert community-wide shifts in the ruminal microbiome of dairy cows (Hook et al., 2011; Plaizier et al., 2017; Li et al., 2021), similar to those observed in the present study. The microbial community in vivo is fairly resilient (Weimer et al., 2010), whereas, in continuous culture, species removed from the microbiome have no potential to return. However, core members of the microbiome (Henderson et al., 2015) prevailed in all treatments, and community redundancy (Weimer et al., 2010; Firkins

and Yu, 2015) likely contributed to the lack of treatment effects on nutrient digestibility despite several shifts in relative abundance with LowpH. Caution is advised in interpreting relative abundance increases in bacterial composition, especially with LowpH, as this could just as easily reflect constant states for some OTU, and reduced abundance for others, because there was no quantification of total bacteria.

The LowpH treatment was expected to decrease the relative abundance of 16S rRNA gene sequences recovered from archaea (Van Kessel and Russell, 1996), but the dominant hydrogenotrophic genus *Methanobrevibacter* actually increased. Most likely, the pH was not low enough (never below 6.06; Supplemental Figure S1; https://figshare.com/articles/journal_contribution/Supplement_docx/20542719; Li, 2022) to inhibit the relative abundance of methanogens recovered by our sequencing approach. Perhaps these *Methanobrevibacter* members were able to adjust to a lower pH by coupling proton-linked ATPase (McMillan et al., 2011) to maintain their relative abundance even though CH_4 production decreased (Table 2). Ruminal methanogen members likely distribute based on their ATP yields in response to thermodynamic control of substrate and other ecological conditions (Lynch et al., 2019).

Alfalfa and soybean hulls made up 70% of the diet and are well known to have considerable pectin compared with most other feeds. *Oribacterium* is a generalist bacterium probably degrading pectin (Kelly et al., 2019). Those authors explained that pectin decomposition might rely on a chain of microbes, including an uncharacterized member of the *Mollicutes* class, ultimately yielding methyl compounds for the methyltrophic archaea *Methanosphaera* and the order *Methanomassiliicoccales*. Unfortunately, those archaea were not well represented in our sequencing approach. However, interrupted methanogenesis from methyl groups might contribute to the suppression of methanogenesis seen with the LowpH treatment.

Ruminococcus and other *Ruminococcaceae* are diverse beyond the well-characterized cellulolytic species *Ruminococcus albus* and *Ruminococcus flavefaciens* (La Reau and Suen, 2018), including the active amylolytic *Ruminococcus bromii* (Mukhopadhya et al., 2018), which presumably would be less inhibited by a moderately depressed pH and thus displacing other taxa in the LowpH treatment. The ruminococci are well known for their active hydrogenase activity and resulting methane-promoting capacity. However, the LowpH treatment might have decreased H_2 production by *R. albus* (Greening et al., 2019) and potentially other related ruminococci. Because decreasing pH is linked with increasing redox, the LowpH treatment could have influenced the redox sensor that is linked to hydrogenase transcription (Zheng et al., 2014). One potential taxon that was likely displaced by the ruminococci is the genus *Clostridium*, members of which are highly diverse but known for H_2 and butyrate production (Calusinska et al., 2010). If ruminococci that increased in the LowpH treatment were more flexible with sensing redox and disposing of reducing equivalents other than via H_2, a likely fermentation end-product would be succinate (La Reau and Suen, 2018).

Characterized members of the *Veillonaceae* are often discussed as important lactate consumers (Nagaraja and Titgemeyer, 2007), but they also are important succinate consumers, particularly cross-feeding with succinate-producing cellulolytics, including *Ruminococcus* (Koike and Kobayashi, 2009). Using metagenomics, CH_4 suppression increased succinate production linked to *Fibrobacter* and succinate uptake linked to the propionate producers *Prevotella* and *Selenomonas* (Denman et al., 2015). Neither of the first 2 taxa were affected in the present study. *Selenomonas* is recognized to consume H_2(aq) (or perhaps formate) to reduce fumarate to succinate before propionate formation (Greening et al., 2019), but fumarate (or its precursor, malate) should not be different among treatments fed the same diet. In selenomonads, succinate decarboxylation to propionate might involve production of a Na^+ gradient, which could spare ATP (Ricke et al., 1996). Succinate transport, which uses symport with protons, might have been stimulated by the LowpH treatment. However, these mechanisms do not satisfactorily explain the succinate decarboxylation niche. After inspection of *Selenomonas* genomes, Hackmann et al. (2017) revealed a unique mechanism by which succinate could be charged (converted to succinyl-CoA) using succinyl-CoA:acetate CoA transferase without ATP expenditure from a CoA synthetase while coupling another reaction to make ATP (Hackmann et al., 2017). Exogenous succinate would be carboxylated to propionate while potentially yielding ATP. Therefore, the lower H_2 production and lower acetate and butyrate concentrations for the LowpH treatment (Table 2) support redirection of reducing equivalents from the typical hydrogenotrophic methanogenesis pathway toward propionate using either lactate or, more likely, succinate as intermediate (Koike and Kobayashi, 2009; Denman et al., 2015).

Various CoA transferases could charge exogenous VFA, but one of the best characterized is a common mechanism linked to butyrate. The relative abundance of *Butyrivibrio* was increased by the InfAc treatment, perhaps because of stimulation of the clade that takes up exogenous acetate to produce butyrate via butyryl -CoA:acetate CoA transferase (Diez-Gonzalez et al., 1999). Because exogenous acetate is used but regener-

ated in a cycle, this route for butyrate formation does not actually incorporate net acetate C into butyrate C, but the ^{13}C-label of acetate transferred to acetyl-CoA could mix with acetyl-CoA used in other anabolic pathways. How many other ruminal bacteria produce butyrate this way is uncertain, but other *Clostridiales* (as is *Butyrivibrio*) in human feces readily take up acetate or even depend on it for butyrate production (Duncan et al., 2004). Acetate is a recognized source of C for total AA synthesis in mixed ruminal bacteria (Sauer et al., 1975) and is either required for or stimulates growth of numerous strains of *Methanobrevibacter* (Rea et al., 2007; Leahy et al., 2010). Thus, recovery of ^{13}C into long-chain fatty acids or AA would be expected in mixed ruminal microbes (Kristensen, 2001), whereas only some of the C would be used on a net basis. Thus, complete recovery of the ^{13}C-acetate dose in VFA would not be expected given these reactions. The disposal of reducing equivalents in microbial biomass remains poorly understood but is potentially important in regulating acetate relative to the 2 main reducing sinks, propionate and CH_4, but also to the reductive elongation of acetate to butyrate (Ungerfeld, 2015).

CONCLUSIONS

The digestibility of NDF, ADF, and starch in continuous cultures was not affected by low pH or acetate and propionate infusions. Reducing fermentor pH by 0.5 units increased net propionate production and decreased emissions of H_2 and CH_4, primarily due to shifts in metabolic pathways of VFA formation in favor of the succinate pathway by changing bacterial community structure. The acetate and propionate infusions increased net acetate and propionate production in continuous culture, and VFA interconversions were not affected by treatment, suggesting that thermodynamics might not be the primary factor influencing VFA metabolic pathways. High interconversions between acetate and butyrate were observed, whereas interconversions between propionate and butyrate and between propionate and acetate were relatively small. A fuller understanding of underlying mechanisms and how they are governed by thermodynamics in the ruminal environment and their more accurate representation in mathematical models should lead to tighter predictions in VFA production.

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