Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. II: Relation with solid passage rate and pH on neutral detergent fiber degradation and microbial function in continuous culture

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ABSTRACT

To support improving genetic potential for increased milk production, intake of digestible carbohydrate must also increase to provide digestible energy and microbial protein synthesis. We hypothesized that the provision of exogenous branched-chain volatile fatty acids (BCVFA) would improve both neutral detergent fiber (NDF) degradability and efficiency of microbial protein synthesis. However, BCVFA should be more beneficial with increasing efficiency of bacterial protein synthesis associated with increasing passage rate (k_p). We also hypothesized that decreasing pH would increase the need for isobutyrate over 2-methylbutyrate. To study these effects independent from other sources of variation in vivo, we evaluated continuous cultures without (control) versus with BCVFA (0 vs. 2 mmol/d each of isobutyrate, isovalerate, and 2-methylbutyrate), low versus high k_p of the particulate phase (2.5 vs. 5.0%/h), and high versus low pH (ranging from 6.3 to 6.8 diurnally vs. 5.7 to 6.2) in a $2 \times 2 \times 2$ factorial arrangement of treatments. Diets were 50% forage pellets and 50% grain pellets administered twice daily. Without an interaction, NDF degradability tended to increase from 29.7 to 35.0% for main effects of control compared with BCVFA treatments. Provision of BCVFA increased methanogenesis, presumably resulting from improved NDF degradability. Decreasing pH decreased methane production. Total volatile fatty acid (VFA) and acetate production were decreased with increasing k_p , even though true organic matter degradability and bacterial nitrogen flow were not affected by treatments. Decreasing

pH decreased acetate but increased propionate and valerate production, probably resulting from a shift in bacterial taxa and associated VFA stoichiometry. Decreasing pH decreased isobutyrate and isovalerate production while increasing 2-methylbutyrate production on a net basis (subtracting doses). Supplementing BCVFA improved NDF degradability in continuous cultures administered moderate (15.4%) crude protein diets (excluding urea in buffer) without major interactions with culture pH and k_p .

Key words: branched-chain volatile fatty acid, fiber degradation, rumen pH, continuous culture

INTRODUCTION

Rumen-degraded carbohydrate and protein need to be provided in appropriate amounts to optimize the efficiency of microbial protein synthesis in the rumen and passage to the duodenum to optimize metabolizable protein supply while minimizing environmental nitrogen excretion (Firkins et al., 2007). Those authors explained that increasing ruminal passage rate, which is likely with increasing DMI by high-producing cows, should improve efficiency of microbial protein synthesis. To achieve this potential improvement, adequate supply of dietary preformed AA helps to increase the proportion of ATP derived from fermentation that is used for growth versus nongrowth ATP expenditure (Hackmann and Firkins, 2015a). Roman-Garcia et al. (2016) noted an important relationship between either isovalerate or isobutyrate with efficiency of microbial protein synthesis in a meta-analysis of rumen microbial outflow studies in dairy cattle; however, these effects need to be re-evaluated directly, because most studies used a procedure that allows isovalerate and 2-methylbutyrate to coelute. Therefore, our preliminary study evaluated different combinations of branched-chain AA (BCAA) or their deaminated and decarboxyl-

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ated products (i.e., branched-chain volatile fatty acids, **BCVFA**) in batch cultures of mixed rumen microbes (Roman-Garcia et al., 2021a). The BCAA were primarily converted to BCVFA, but the latter was more extensively assimilated into bacterial branched-chain fatty acids. The BCVFA are required growth factors for numerous strains of cellulolytic bacteria (Stewart et al., 1997), which are key to initiating colonization and optimizing degradability of fiber in the rumen (Gruninger et al., 2019).

Numerous dietary factors could influence ruminal BCVFA concentration (Firkins et al., 2006). Dosing BCVFA has increased efficiency of bacterial growth and substrate degradability in batch culture (Cummins and Papas, 1985; Gorosito et al., 1985; Mir et al., 1986). Individual or groups of BCVFA increased NDF degradability at 24 h of incubation in batch cultures of mixed rumen microbes, especially when orchardgrass was forage (Roman-Garcia et al., 2021a). Therein, we noted that either isovalerate or isobutyrate plus 2-methylbutyrate consistently improved NDF degradability. Continuous culture can derive total VFA production while measuring NDF degradability and microbial protein synthesis values that are more reflective of ruminal conditions than batch culture. Kone et al. (1989) reported that acetate and total VFA concentration increased when supplementing BCVFA in continuous cultures fed timothy hay and urea, but those authors did not assess NDF degradability or evaluate addition of starch as would be typical with dairy rations. Few other data are available on BCVFA supplementation of continuous cultures.

Decreasing pH below 6.0 might depress attachment by fibrolytic bacteria that are thought to benefit most from BCVFA (Hoover, 1986), although the pH probably is more detrimental to initial attachment (Mouriño et al., 2001). Decreased pH needs to be differentiated from pH-independent differences related to decreasing forage-to-concentrate ratio (Firkins, 2010). Decreasing pH shifted VFA stoichiometry and decreased protein degradability (Calsamiglia et al., 2008; Wenner et al., 2017) and decreased efficiency of microbial protein synthesis (Wenner et al., 2017).

Because BCVFA are precursors for bacterial branched-chain fatty acids in membranes and BCAA in protein, BCVFA uptake likely increases with increasing bacterial growth rate. The efficiency of bacterial growth was increased by increasing particulate phase passage rate (k_p) in continuous culture (Qiu et al., 2004; Wenner et al., 2017). In situations of high k_p when bacterial growth rate might increase to maintain populations, BCVFA were hypothesized to assimilate more extensively into bacterial constituents; thus, the availability of primers and their concentration to com-

pete with straight-chain primers for fatty acid synthesis might be limiting because of faster washout. Although an increased BCVFA requirement with increasing DMI has been speculated (Hoover, 1986), data are limiting to explain how ruminal k_p influences BCVFA requirement by cellulolytics.

The objective of this study was to test for interactions in how decreasing pH and increasing k_p affect the potential for exogenous BCVFA to improve NDF degradability and efficiency of bacterial protein synthesis. We hypothesized that increasing growth rate with faster k_p would increase NDF degradability and efficiency of microbial protein synthesis, and BCVFA addition would help fibrolytic bacteria compete with more acid-tolerant bacteria to withstand periodic depressions in pH.

MATERIALS AND METHODS

Continuous Culture Operation

Experimental Design and Treatments. In this study, 8 dual-flow continuous culture fermentors were used in an incomplete block design with 4 experimental periods. The 12-d period comprised 8 d of adjustment followed by 4 d of sampling. We tested a $2 \times 2 \times 2$ factorial arrangement of 8 treatment combinations. Treatment factors were (1) control or BCVFA supplementation, (2) high pH (ranging from 6.3 to 6.8) or low pH (ranging from 5.7 to 6.2), and (3) low (2.5%/h) or high (5.0%/h) solids k_p . The BCVFA treatment included 2 mmol each of isovalerate, isobutyrate, and 2-methylbutyrate dosed per day. Based on prior results (Roman-Garcia et al., 2021a), valerate was deemed unnecessary and not dosed.

Dose Estimation. When scaled to the largest fermentor volume (1,920 mL), our individual BCVFA doses from batch culture (Roman-Garcia et al., 2021a) would equal 1.9 mmol/d. However, dose should be related to amount of substrate fed and VFA produced, not to a concentration that could be diluted simply by changing the volume of the buffer. Fowler et al. (2015) used similar conditions and noted treatment shifts in BCVFA production. Those authors reported an average of 180 mmol/d of total VFA in continuous cultures; total BCVFA averaged 4.7 mmol/d and had a maximum production of 5.6 mmol/d. With the goal of at least doubling the amount of BCVFA to test our objectives, we dose 2 mmol/d of each BCVFA (i.e., 6.0 mmol/d total).

Continuous Culture Operation. At the beginning of each period, 8 L of rumen contents were manually sampled from 4 ruminally cannulated lactating Jersey cows fed a standard lactation diet averaging 35% corn

silage, 16% alfalfa hay, 22% ground corn, 8.8% wet brewers grains, 8.5% AminoPlus (AGP Ag Processing Inc.), 2.7% soybean meal, 2.1% soybean hulls, 1.4% Megalac (Church and Dwight), 0.5% dried molasses, and 3.0% of the remainder containing vitamins, minerals, and additives (no ionophores). Diet formulations averaged 17.1% CP and 10.2% RDP according to Spartan Dairy Ration Evaluator (Michigan State University, Lansing). Cows were housed according to Institutional Animal Care and Use Committee standards at the Waterman Dairy in Columbus, Ohio. Ruminal contents were rapidly squeezed through 2 layers of cheesecloth, and then liquid was placed in prewarmed, insulated containers maintained at 39°C. Rumen fluid was pooled and inoculated into fermentors at 50% of fermentor working volume, with the remainder being filled with buffer previously bubbled with O₂-free CO₂ and maintained at 39°C. Clarified rumen fluid (centrifuged at 15,000 × g, 4°C, 15 min, and autoclaved) was used in a 1:20 dilution of buffer for the first day of each period (Wenner et al., 2017).

The liquid (buffer) infusion was maintained at a constant 10.0%/h. Buffer was made according to Weller and Pilgrim (1974), with the addition of 0.1 g/L of urea predicted to maintain NH₃-N concentration in the fermentor above 5 mg/dL. Adding H₃PO₄ or NaOH as necessary to that buffer, the low pH buffer was maintained between 6.3 and 6.4, but high pH buffer was maintained between 6.7 and 6.8 before infusion into the fermentors. Buffer was continuously bubbled with O₂-free CO₂ for 1 d before use and while being infused into the fermentors. Before each period, the fermentor seals were tested with a manual pressure pump (Wenner et al., 2020), and any leaks were repaired.

Fermentors were fed 50 g (as-fed basis) of a 50:50 concentrate:forage diet twice daily (93.5 g/d of DM; 0800 and 2000 h) as a single meal through a port that was opened only at feeding. The diet consisted of a mixture of 17% alfalfa hay pellets, 33% orchard grass hay pellets, and 50% concentrate pellet. Concentrate pellet was composed of 68.0% corn grain, 6.4% soybean hulls, 2.0% corn oil, 20.0% soybean meal (48% CP), 0.40% dicalcium phosphate, 1.0% trace mineral supplement, 0.8% limestone, 0.2% magnesium oxide, and 0.3% Se supplement (200 mg/kg supplied as Na₂SeO₃). On a dry matter basis, the mixed diet had 23.7% starch, 15.4% CP, and 32.6% NDF (measured by wet chemistry methods at Cumberland Valley Analytical Services, Waynesboro, PA). Dosing of BCVFA started on d 2 (1 mmol of each of the 3 BCVFA at each of 2 daily feedings), allowing for 7 d of adaptation to the treatment before sampling. A priming dose of 50 mg of 10% ¹⁵N-enriched (NH₄)₂SO₄ (Isotec Stable Isotopes) was

bolus-dosed into each fermentor on d 5 and added to the buffer at 22.5 mg/L for a desired enrichment of 0.2 atom percentage excess. Agitation was set to 50 rpm to ensure thorough mixing of substrate in the fermentor, and temperature was kept at 39°C. The protozoa were primarily effluxed through a filter with a 300-μm pore size; their counts inside each fermentor were assessed at d 1, 3, 5, and 7 after inoculation and were below detection by d 7 (data not shown).

Sample Collection, Processing, and Analyses

Daily Samples. Daily effluent from the fermentors was collected twice a day over 12 h after feeding in outflow containers on ice. Liquid and solid effluent were weighed separately. A 15% (by weight) subsample of 12-h effluent was aspirated with a 6.35-mm diameter tube while stirring to prevent sampling bias against larger particles. Effluent subsamples were stored at −20°C until the end of the period, after which liquid and solids were reconstituted per collection, which were subsequently pooled by fermentor within period and dried at 55°C. A subsample of effluent was dried at 105°C overnight to determine DM, then ashed at 550°C overnight to determine OM (AOAC, 1990). Residual NDF in the subsample was determined by reflux units with use of heat-stable amylase (Ankom Technology, 2017) and sodium sulfite (Van Soest et al., 1991). Dried effluent nitrogen was determined by the Kjeldahl method (AOAC, 1990). About 1 g of effluent sample was dissolved in 10 mL of distilled H₂O; NH₃-N was analyzed (Chaney and Marbach, 1962) and determined to be negligible. Starch in the effluent was sent to be analyzed in a commercial laboratory (Cumberland Valley Analytical Services, Waynesboro, PA).

At the time of effluent collection, a 10-mL sample was obtained using the same aspiration method described previously, mixed with 2 mL of 25% metaphosphoric acid, and stored frozen for subsequent VFA analysis. A separate 30-mL sample was frozen immediately and stored at −80°C for subsequent DNA extraction (Roman-Garcia et al., submitted).

Two samples of 250 mL each were taken from effluent for bacterial pellet collection; the pH was adjusted to 2.0 (monitored with pH meter) by adding 5 N HCl dropwise (about 8 mL) and then stored at 4°C until particle-associated bacteria were harvested as described (Roman-Garcia et al., 2021a). From the supernatant of bacterial pellet collection, samples were collected for NH₃-N detection by colorimetry (Chaney and Marbach, 1962; Roman-Garcia et al., 2021a) and ammonia diffusion into filter disks (Hristov et al., 2001). The 2 pellets from both 12-h samples per day

were combined, resuspended, washed twice with saline, and then divided into 2 replicate pellets per day per fermentor. One of the pellets was used for fatty acid analysis (Roman-Garcia et al., 2021a). The other pellet was analyzed for ^{15}N and ^{13}C enrichments and N and C percentages in an elemental analyzer (Roman-Garcia et al., 2021a) and for OM (AOAC, 1990). Microbial ^{15}N enrichment was analyzed as described by Noftsker et al. (2003). Background samples were taken before dosing to correct enrichments to atom percentage excess. The VFA sample was processed and analyzed according to Roman-Garcia et al. (2021a) except that, after the valerate peak eluted, the oven temperature was increased to 195°C at a rate of 25°C per min for another 15 min until elution of the caproate peak. Pivalic acid was used as an internal standard. Daily production of VFA was calculated by measuring concentration in the effluent times the effluent volume; net production was derived by subtracting the daily dose of BCVFA.

Hourly Gas and Filtrate Sampling. On d 2 to 8 of the adaptation phase, pH and oxidation reduction potential (ORP) measurements were taken at each hour after the morning feeding. Measurement of CH_4 and H_2 emission were recorded on 4 of the 8 fermentors on d 9 to 12 via a Micro-Oxymax Respirometer (Columbus Instruments Inc.) as described by Wenner et al. (2017). Those fermentors were balanced for treatment ($n = 2$). Fermentors were opened only for feedings; gas data during that time were discarded. Hydrogen balance was calculated as derived by Ungerfeld et al. (2019).

All hourly samples were obtained in the last 4 d of the period for all fermentors. Using a y-valve septum inserted into the liquid effluent line, 5 mL of filtrate was removed from the fermentors at 0, 0.5, 1, 2, 4, 6, 8, and 12 h after feeding, mixed with 1 mL of 25% metaphosphoric acid, and frozen to be processed later for VFA analysis as described previously. To measure aqueous H_2 [hereafter referred to as $\text{H}_2(\text{aq})$], a 20-mL sample was removed using the y-valve septum at 0, 1, 2, 4, 6, 8, and 12 h after feeding and injected into a 60-mL glass culture bottle (Wheaton Glass) that was previously dosed with 5 mL of 5 M H_3PO_4 before being flushed with N_2 and sealed with a butyl rubber stopper (Wenner et al., 2017).

Statistical Analysis

Daily VFA production, digestibility, and nitrogen flows were analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc.) according to this model:

$$Y_{ijkl} = \mu + B_i + H_j + K_s + (B \times H)_{ij} + (H \times K)_{js} + (B \times K)_{is} + (B \times H \times K)_{ijs} + f_l + p_k + \varepsilon_{ijkl}$$

where Y_{ijkl} is the dependent variable; μ is the overall population mean; B_i is the fixed effect of BCVFA dose ($i = \text{control, BCVFA}$); H_j is the fixed effect of pH treatment ($j = \text{high, low}$); K_s is the fixed effect of k_p ($s = \text{high, low}$); $(B \times H)_{ij}$, $(H \times K)_{js}$, $(B \times K)_{is}$, and $(B \times H \times K)_{ijs}$ are respective interactions; f_l is the fixed effect of fermentor ($l = 1-8$); p_k is the random effect of k th period ($k = 1-4$); and ε_{ijkl} is the random error. For gas measurement, only 4 fermentors per period ($l = 1-4$) were used ($n = 2$ rather than 4). Hourly data were analyzed using the REPEATED (repeated measures) statement added to the previous model. The first-order autoregressive (for hourly CH_4 and H_2 emission), the heterogeneous first order autoregressive (for hourly pH and ORP), or the spatial power [for unequally spaced hourly VFA and $\text{H}_2(\text{aq})$] covariate structures were used based on the lowest Bayesian information criterion. When any of the treatments interacted with time ($P \leq 0.05$), the effect of the treatments was contrasted over time, and means per hour were reported. Differences were declared at $P \leq 0.05$, and trends were declared at $0.05 < P \leq 0.10$.

RESULTS

Apparent and true OM degradability were not affected by any of the treatments; nor was an interaction detectable among the treatments (Table 1). Dosing BCVFA tended ($P = 0.09$; main effect) to increase NDF degradability. Contrary to our hypotheses, the low pH main effect only approached significance ($P = 0.11$) for decreasing NDF degradability compared with high pH. We found no treatment by time interaction, but hour had an effect ($P < 0.01$) on pH, so hourly means are presented in Figure 1A. Lines were parallel between high and low pH treatments, reached a pH nadir at about 4 h after feeding, and then increased until the next feeding.

No treatment by time interaction occurred for ORP (Table 1), which was affected ($P < 0.05$) by time (Figure 1B). Curves were generally similar compared with high and low pH treatments, with higher pH inherently related to lower ORP measures (Stumm and James, 1970). Lowest (most negative) ORP were recorded about 1 h after feeding. Then, ORP increased up to 7 h following feeding, after which it decreased until the next feeding. We detected an interaction ($P < 0.01$) between pH and k_p treatments such that, when pH was low, increasing k_p decreased ORP; however, when pH was high, increasing k_p increased ORP. Hydrogen balance was not affected by any of the treatments (Supplemental Table S1; <https://doi.org/10.3168/jds.2021-20335>).

Dosing BCVFA increased ($P < 0.01$; main effect) daily CH_4 emission (Table 1). No interactions between

Table 1. Nutrient degradabilities and means for pH, redox, aqueous H₂, and gas emission in continuous cultures that were dosed without or with branched-chain VFA (BCVFA) and maintained at either high or low pH and either low or high solids passage rate (k_p)¹

Item ²	Control				BCVFA								P-value ³		
	High pH		Low pH		High pH		Low pH		High pH		Low pH				
	Low k _p	High k _p	Low k _p	High k _p	Low k _p	High k _p	Low k _p	High k _p	Low k _p	High k _p	Low k _p	High k _p	BCVFA pH × pH	BCVFA k _p × k _p	pH × k _p
Apparent OMD, %	44.3	43.8	42.4	45.0	44.2	48.1	42.4	44.2	44.2	48.1	42.4	44.2	NS	NS	NS
True OMD, %	58.0	55.8	57.3	59.2	57.3	59.3	56.2	58.5	58.5	59.3	56.2	58.5	NS	NS	NS
NDFD, %	30.7	31.4	30.2	26.8	37.4	38.9	30.4	33.2	33.2	38.9	30.4	33.2	NS	NS	NS
True N degraded, ⁴ %	80.8	71.0	75.7	73.4	75.3	77.6	73.0	71.9	71.9	77.6	73.0	71.9	NS	NS	NS
Apparent starch degraded, %	99.6	99.7	99.6	99.8	99.4	99.5	99.6	99.6	99.6	99.5	99.6	99.6	0.13	NS	NS
pH	6.55	6.62	5.96	5.98	6.48	6.57	5.89	5.98	5.98	6.57	5.89	5.98	0.20	NS	NS
ORP, mV	-304	-297	-268	-272	-297	-291	-264	-270	-270	-291	-264	-270	0.16	NS	NS
CH ₄ emission, mmol/d	71.0	68.9	41.3	52.1	91.9	71.9	63.4	61.8	61.8	71.9	63.4	61.8	<0.01	NS	0.07
H ₂ emission, ⁵ μmol/d	73.3	238.1	219.6	264.5	182.0	167.3	180.4	268.5	268.5	167.3	180.4	268.5	<0.01	NS	0.15
													NS	NS	NS

¹High pH treatment ranged from 6.8 to 6.3; low pH, 6.2 to 5.7; low k_p, 2.5%/h; high k_p, 5.0%/h.²OMD = OM degraded; NDFD = NDF degraded; ORP = oxidation reduction potential.³P-values reported for the main effects of BCVFA dose, pH, k_p, and their 2-way interactions; ^{NS}P > 0.20.⁴Percent of dietary N intake only; does not include N from buffer.⁵BCVFA × pH × k_p was P < 0.01.

BCVFA, k_p, or pH treatments and no main effect of k_p were detected for CH₄ emissions. Decreasing pH decreased ($P < 0.01$) CH₄ emission. We observed similar but opposite patterns for daily H₂ emission relative to CH₄ emission but no effects ($P > 0.15$) of treatment on daily H₂ emissions.

Treatment by time interactions occurred ($P < 0.05$) for H₂(aq) concentration, CH₄ hourly emission rate, and H₂ hourly emission rate. Hourly H₂(aq) was decreased by low pH from 2 to 6 h after feeding and 12 h after feeding (Figure 2A). Hourly concentration of H₂(aq) was not affected by k_p or BCVFA. Low pH decreased ($P < 0.05$) hourly CH₄ emission rate from 1 to 4 h after feeding and tended ($P \leq 0.10$) to decrease hourly CH₄ emission rate up to 6 h after feeding (Figure 2B). Low pH increased ($P < 0.05$) H₂ emission rates from 0.5 to 1.5 h and 3 h after feeding (Figure 2C). From 1 to 2 h following feeding, high k_p increased H₂ emission rate. An interaction occurred between k_p and pH for 5 to 9 h after feeding such that high k_p increased H₂ emission rates only in the low pH treatment.

Total N flows were not affected ($P > 0.10$) by treatment or interactions among treatments (Table 2). Bacterial N derived from NH₃ tended to increase ($P = 0.09$) with the main effect of high k_p relative to low k_p but was not affected by any other treatment. Bacterial N flow, NAB flow, nonammonia nonbacterial N flow, and bacterial N flow per unit of true OM or unit of carbohydrate degraded were not affected ($P > 0.10$) by any treatment. Increasing k_p increased nonammonia nonbacterial N flow as a percentage of NAN flow when BCVFA were not dosed (BCVFA × k_p interaction, $P = 0.09$).

Daily net production (subtraction of BCVFA dosage from BCVFA gross production) of total VFA was not affected by BCVFA dosing, pH, or any interactions (Table 3). However, the main effect of high k_p tended to decrease ($P = 0.09$) net production of total VFA and decreased ($P = 0.05$) production of acetate. Low pH decreased ($P < 0.01$) acetate and increased ($P < 0.01$) propionate production; thus, decreasing pH also decreased ($P < 0.01$) the ratio of acetate to propionate. Butyrate and caproate productions were not affected by any treatment. Low pH decreased ($P < 0.05$) net production of isobutyrate and isovalerate but increased ($P < 0.01$) 2-methylbutyrate net production compared with high pH. Valerate recovery also increased ($P = 0.04$) with low pH.

Hour interacted with treatment ($P < 0.05$) for molar percentages of isovalerate, isobutyrate, and 2-methylbutyrate in fermentor contents. Isovalerate (Figure 3A), isobutyrate (Figure 3B), and 2-methylbutyrate (Figure 3C) molar percentages increased ($P < 0.01$) by dosing BCVFA, as planned, because these values

Figure 1. Fermentor pH (A) and redox (B) over time after feeding for continuous cultures that had treatments of either high or low pH and either low or high solids passage rate (k_p). Data are means from all 4 experimental periods taken on d 1 to 7 of the period. Buffers were adjusted to control pH. Because the branched-chain VFA (BCVFA) treatment was given in the form of acid, it lowered pH by approximately 0.06 unit. Hour did not interact with any of the treatments. The main effects and interactions of treatments were tested at each time point and are reported as follows: ** $P \leq 0.01$; * $P \leq 0.05$; ^{NS} $P > 0.20$. The SEM is pooled across treatment per time and shown for only 1 series per plot.

are not corrected for dosage of BCVFA; this increase lasted from 0.5 to 12 h after feeding. With lower pH, the molar percentages of isovalerate and isobutyrate were lower ($P < 0.01$) at all hours, whereas 2-methylbutyrate had the opposite pattern of higher molar percentages on the low versus high pH treatment.

For 2-methylbutyrate, a tendency ($P = 0.08$) for an interaction between BCVFA and pH was detected at 8 h after feeding. When BCVFA were not dosed, low pH had consistent patterns over the 12 h. In contrast, when BCVFA were dosed, the 2-methylbutyrate molar percentage on the low pH treatment remained high,

Table 3. Daily VFA net production in continuous cultures that were dosed without or with branched-chain VFA (BCVFA) and maintained at either high or low pH and either low or high solids passage rate (k_p)¹

Item	Control				BCVFA								<i>P</i> -value ¹				
	High pH		Low pH		High pH				Low pH								
	Low <i>k_p</i>	High <i>k_p</i>	Low <i>k_p</i>	High <i>k_p</i>	Low <i>k_p</i>	High <i>k_p</i>	Low <i>k_p</i>	High <i>k_p</i>	Low <i>k_p</i>	High <i>k_p</i>	Low <i>k_p</i>	High <i>k_p</i>	BCVFA × pH	pH × <i>k_p</i>	BCVFA × <i>k_p</i>	pH × <i>k_p</i>	
Total VFA, mmol/d	379	340	354	341	369	350	356	350	350	350	21	NS	NS	NS	NS	NS	NS
Individual VFA, mmol/d																	
Acetate	248	219	210	200	242	225	212	225	205	205	15	NS	<0.01	NS	0.05	NS	NS
Propionate	70.3	66.2	88.8	85.8	71.8	72.9	91.6	72.9	94.5	94.5	8.4	NS	<0.01	NS	NS	NS	NS
Butyrate	44.2	39.9	40.2	40.5	41.7	37.8	38.7	37.8	37.1	37.1	3.9	NS	NS	NS	NS	NS	NS
Isovalerate	3.24	2.58	2.22	2.26	2.74	2.30	1.78	2.30	1.76	1.76	0.39	0.01	0.01	NS	NS	NS	NS
Isobutyrate	3.64	2.88	2.41	2.67	3.29	2.36	2.09	2.36	1.95	1.95	0.65	NS	0.04	NS	NS	NS	NS
2-Methylbutyrate	2.13	1.97	2.96	2.51	1.70	1.53	2.75	1.53	2.70	2.70	0.45	NS	<0.01	NS	NS	NS	NS
Valerate	5.70	5.32	5.93	6.04	5.34	5.25	5.86	5.25	6.46	6.46	0.49	NS	0.04	NS	NS	NS	NS
Caproate	2.44	3.18	2.27	2.08	2.33	2.32	1.81	2.32	2.37	2.37	0.60	NS	0.17	NS	NS	NS	NS
Total BCVFA	9.00	7.43	7.59	7.44	7.73	6.20	6.62	6.20	6.42	6.42	1.15	0.15	NS	NS	NS	NS	NS
Acetate:propionate	3.58	3.29	2.41	2.48	3.42	3.10	2.36	3.10	2.33	2.33	0.25	NS	<0.01	NS	NS	NS	0.17

¹High pH treatment ranged from 6.8 to 6.3; low pH, 6.2 to 5.7; low k_p , 2.5%/h; high k_p , 5.0%/h. Net production was calculated from VFA in effluent, and daily dose of BCVFA was subtracted from the respective treatments.

²P-values reported for the main effects of BCVFA dose, pH, k_p , and their 2-way interactions. All 3-way interactions were $P > 0.20$; ^{NS} $P > 0.20$.

whereas high pH decreased 2-methylbutyrate down to control BCVFA treatments by 8 h. Tabular main effect means are shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2021-20335>).

DISCUSSION

Nutrient Degradability and Methane Production

Dosing BCVFA tended to increase NDF degradability by 5.2 percentage units, probably due to cellulolytic bacterial requirements for BCVFA (Stewart et al., 1997). Results are consistent with results from mixed ruminal microbes degrading grass hay plus corn in batch culture (Roman-Garcia et al., 2021a). Low pH only approached ($P = 0.11$) a decrease in NDF degradability. Lack of difference is consistent with results of Wenner et al. (2017). Medium pH is the major determining factor in culture adaptation (Broudiscou et al., 2014). However, our cultures varied pH over the feeding cycle. The low pH treatment's initial pH being about 6.0 (Figure 1A) might have been high enough to minimize the effects of low pH on fiber degradation (Mouriño et al., 2001). Some of the negative effects of starch on NDF degradability are independent of pH. Low pH-tolerant amylolytic bacteria can outcompete for growth factors such as BCVFA to reduce NDF degradability in mixed forage and grain diets (Firkins, 2010). Even so, we detected no interactions of pH with BCVFA on NDF degradability.

Intimate contact of extracellular carbohydrate-degrading complexes to substrates is critical for fiber degradation (Terry et al., 2019). Based on an increased membrane fluidity when switching from cellobiose to cellulose as substrate (Moon and Anderson, 2001), we hypothesize that membrane fluidity is essential for translocation of these enzymes and supporting proteins extracellularly. A dynamic interchange of BCVFA between amylolytics and cellulolytics is critical for optimal fiber degradation (Morais and Mizrahi, 2019). Consequently, a higher local concentration of BCVFA by supplementing BCVFA might help cellulolytics acquire these precursors either for reductive carboxylation to BCAA or as primers for bacterial branched-chain fatty acids needed to maintain membrane fluidity required for adhesion and subsequent colonization (Roman-Garcia et al., submitted).

Measurements of ORP in aqueous solutions represent the tendency to gain (be reduced) or lose (be oxidized) electrons. The more negative the ORP, the more reduced the solution is. Because most redox reactions are coupled with protons, pH and ORP are negatively correlated (Krishtalik, 2003). Although relatively few studies have reported ORP, Huang et al. (2018) noted

Figure 3. Molar percentage of the individual branched-chain VFA (BCVFA) isovalues (A), isobutyrate (B), and 2-methylbutyrate (C) over time after feeding in continuous cultures that were dosed with or without BCVFA and maintained at either high or low pH and either low or high solids passage rate (k_p). These data are not corrected for BCVFA dose. Treatment by time interaction was detected for all of these variables. The main effects and interactions of treatments were tested at each time point and are reported as follows: ** $P \leq 0.01$; * $P \leq 0.05$; ^{NS} $P > 0.20$. Three-way interactions were all not significant. The SEM is pooled across treatment per time and shown for only 1 series per plot.

that increasing dietary forage NDF and NDF degradability in vivo were associated with decreasing ORP. However, they did not account for the likely collinearity between forage inclusion with pH; that is, both increasing forage and increasing pH should decrease ORP. In our study, BCVFA increased NDF degradability (Table 1). Also, $H_2(aq)$, a reductant, increased with high pH (Figure 2). Similarly, Huang et al. (2018) related increases in acetate production (many pathways of which release H_2) with lower ORP, which was observed in the high pH treatment.

Changing particulate k_p did not affect CH_4 emissions in our study, which is contrary to results of Wenner et al. (2017). That study retained protozoa and had a smaller filter compared with our study. The low pH treatment decreased CH_4 emission in our study, which was expected (Van Kessel and Russell, 1996); thus, its precursor, $H_2(aq)$ accumulated early after feeding. Assessment of H balance did not reveal any treatment differences. Hence, our low pH treatment might not have extended long enough postprandially to shift the rate of H_2 emission long enough over the entire day to suppress daily H_2 emission.

Deamination or decarboxylation of BCAA to BCVFA reduces cofactors, which are typically referred to as reducing equivalents and abbreviated as [2H]. Because inhibition of methanogenesis disrupts deamination of BCAA (Hino and Russell, 1985), we expected that reductive carboxylation (BCVFA converted to BCAA) would consume [2H] and compete with methanogenesis. More recently, a role for [2H] in AA metabolism and recovered in microbial protein and lipid sinks was discussed, but its role still is not clear (Ungerfeld, 2015; Martinez-Fernandez et al., 2016). In our companion report (Roman-Garcia et al., 2021b), BCVFA tended to increase FA as a percentage of OM in bacterial cells. Methanogens also require BCVFA as a growth factor (Stewart et al., 1997). The relative 16S rRNA gene sequence abundance of archaea did not increase with BCVFA treatment (Roman-Garcia et al., 2021b), but we did not measure total 16S rRNA gene abundance. Even so, abundance of methanogens is not necessarily indicative of methanogenesis (Firkins et al., 2015). Thus, the most likely reason for increased methanogenesis after dosing BCVFA is a result of increased NDF degradation increasing the [2H] pool from a shift toward pathways that liberate more H_2 or formate per unit of acetate produced (Janssen, 2010). Increased relative abundance of *Fibrobacter* (Roman-Garcia et al., 2021b) would not initially support this hypothesis because they do not produce H_2 , but they do increase cross-feeding by H_2 -producing bacteria (Martínez-Álvaro et al., 2020). However, inferences are limited because only

half of our fermentors measured gas emission (i.e., $n = 2$ per treatment).

We expected that lower pH and thus lower methane production would increase concentration of $H_2(aq)$, but we actually observed a decrease from 2 to 6 h after feeding. However, we also found higher H_2 emission with lower pH in the early hours following feeding. Increased H_2 escape into the headspace can explain the lower concentration in the aqueous phase with low pH. A similar decrease in $H_2(aq)$ concentrations with decreasing pH was observed by Wenner et al. (2017), who suggested that production of reduced VFA was a sink for the extra [2H] in low pH treatments. Those authors reported higher isovalerate molar proportion with decreasing pH, which was likely a result of coeluted 2-methylbutyrate, which increased in our study and will be further explained in our companion study (Roman-Garcia et al., 2021b). Because methanogenesis is an important sink for $H_2(aq)$, the high pH treatment was expected to lower H_2 emission rates, but this decrease was not consistent at all hours in our study.

Bacterial Protein Synthesis

The lack of response in protein degradability, bacterial nitrogen flows, and gas emissions to k_p treatment contrasted with results from other studies (Qiu et al., 2004; Wenner et al., 2017), probably because our k_p treatments did not alter the passage of all the solids (i.e., ground corn) as much as we intended. Supported by our very high observed starch degradability, the grain particles apparently settled below the overflow port and did not pass more quickly into the effluent with increasing k_p . The trends for lower total VFA and acetate production with increasing k_p and decreasing pH (Table 3) suggests diversion of degraded carbohydrate into cellular matter. Bacterial nitrogen flow was not affected, but bacterial carbon flow tended to increase with decreasing pH (Table 2). Because the increased carbon was not from FA (Roman-Garcia et al., 2021b), increased glycogen is the most likely explanation. Although glycogen storage is lower in bacteria than protozoa (Hackmann and Firkins, 2015b), glycogen can increase considerably when growth rate is slowed, as by decreasing k_p , as shown for *Prevotella ruminicola* (Lou et al., 1997). Because starch granules likely were completely degraded, available starch would be very high, and the k_p for bacteria associated with starch was effectively very slow; moreover, relative abundance of *Prevotella* almost doubled with decreasing pH (Roman-Garcia et al., 2021b).

Although BCVFA did not improve microbial nitrogen production or efficiency of microbial protein syn-

thesis in our study, Russell and Sniffen (1984) noted improved protein synthesis in batch cultures of mixed rumen microbes after adding BCFVA. Similarly, total bacteria and targeted cellulolytic bacteria (using real-time PCR) increased linearly with the addition of isovalerate (Liu et al., 2014), isobutyrate (Wang et al., 2015), or 2-methylbutyrate (Zhang et al., 2015) in steers. By contrast, Cummins and Papas (1985) detected no increase in microbial nitrogen accumulating after incubation with BCFVA in batch cultures when cottonseed meal was the main substrate at 16% CP and when corn gluten meal was the substrate at 13% CP; they still noted increased degradability of wheat straw (much lower CP). Considerable interchange appears to occur between BCFVA and BCAA in mixed ruminal microbes (Atasoglu and Guliyev, 2004). Whether or not supplemental BCFVA stimulates efficiency of microbial protein synthesis probably depends on the relative limitation of cytosolic free BCAA concentrations (Firkins et al., 2015) under those specific growth conditions and on possible imbalances of the individual BCAA precursors (Kajikawa et al., 2005). Addition of BCFVA did improve amount and efficiency of microbial protein synthesis in continuous cultures under similar conditions to the current study (Mitchell et al., 2020). Increasing k_p should wash out potentially degradable protein and increase nonammonia nonbacterial N flow in effluent, but we have no explanation for why this result was noted only in treatments without BCFVA (interaction of BCFVA with k_p). The BCAA influence pleiotropic control over genes involved in AA synthesis in bacteria (Kaiser and Heinrichs, 2018). Transcriptional regulation of proteolytic enzymes is not well characterized in ruminal bacteria.

VFA Production

We expected a decrease in acetate net production with low pH as a result of acetate being a primary end product of fibrolytic (particularly cellulolytic) bacteria that would be inhibited by low pH (Stewart et al., 1997). At the same time, low pH decreased methanogenesis, which can result in intracellular [2H] buildup and increased production of propionate or else condensation of acetyl-CoA with exogenous VFA to produce butyrate, valerate, or caproate while decreasing H_2 -producing acetyl-CoA fermentation pathways (Janssen, 2010). High k_p also decreased acetate net production compared with low k_p in our study, which has been noted previously (Janssen, 2010). Increased propionate production in the low pH treatment (Table 3) is likely due to a greater tolerance toward low pH by amylolytics (Stewart et al., 1997).

We expected addition of BCFVA to decrease net production of individual BCFVA in our study because dosing isobutyrate decreased net production of itself but increased net production of isovalerate in batch cultures (Roman-Garcia et al., 2021a). Other such interactions were noted therein. However, dosing BCFVA in this study only approached significance ($P \leq 0.15$) to decrease net production of isobutyrate and total BCFVA by 16.7 and 14.4%, respectively. Increased actual (i.e., not net) concentration could increase feedback inhibition of deamination or decarboxylation, increase competition of these BCFVA for primers in FA synthesis, or increase catabolism of BCFVA all while balancing [2H]. Both BCAA deamination and BCFVA concentrations have had mixed associations with inhibition of methanogenesis that are not yet explained (Martinez-Fernandez et al., 2016; Ungerfeld, 2018; Wenner et al., 2020). Isovaleryl-CoA was inferred as the main feedback inhibitor in Leu synthesis by *P. ruminicola* (Allison et al., 1984). Hino and Russell (1985) noted that BCAA were metabolized by Stickland reactions but also by mechanisms that those authors did not explain and that remain unexplained for ruminal bacteria. The branched-chain keto acid dehydrogenase in *Enterococcus faecalis* (phylum *Firmicutes*) uses $NAD^+/NADH$ as a cofactor and is a main component of the *bkd* operon that produces 0.5 ATP per BCAA via substrate-level phosphorylation (Ward et al., 2000). This gene, which is annotated as K00166-7 in Kyoto Encyclopedia of Genes and Genomes orthology (<https://www.genome.jp/kegg/>), is poorly represented in literature with major ruminal bacteria. However, a similar reaction must be occurring in ruminal bacteria because BCAA deamination and decarboxylation can be critically linked to disposal of [2H] via methanogenesis in mixed ruminal microbes (Hino and Russell, 1985). Moreover, BCFVA and BCAA interchange considerably (Atasoglu and Guliyev, 2004), which also would require cycling of [2H].

Rather than the branched-chain ketoacid dehydrogenase (with $NAD^+/NADH$ cofactor) described for some model nonrumen bacteria, BCAA and BCFVA interconversion in rumen bacteria has long been reasoned to involve ferredoxin (Allison et al., 1984). Addition of oxidized methylviologen (typically used as a substitute for ferredoxin) to mixed ruminal microbes in batch culture increased BCFVA concentration 9-fold; it generally decreased moles of VFA but increased elongation (using [2H]) to caproate 3-fold (Nerdahl and Weimer, 2015). Long-chain FA were not measured in their study but also could be elongated as a result of buildup of [2H] (Ungerfeld, 2015). Only oxidized methylviologen (not NAD^+ or $NADP^+$) greatly increased the activity of branched-chain 2-ketoacid oxidoreductase

(2-ketoisovalerate oxidoreductase, *vor*) in 2 *Prevotella* strains from the human oral community (Takahashi and Yamada, 2000). Those authors explained that these 2 strains are closely related to the ruminal *P. ruminicola*. They also suggested that *vor* is closely related to or perhaps the same enzyme as pyruvate ferredoxin oxidoreductase (*por*, a complex well known to use ferredoxin as a cofactor). The *vor* gene is poorly described, and its activity is likely (but not yet biochemically characterized) to result from evolution of *por* or 2-ketoacid oxidoreductase (*kor*) in bacteria (Gibson et al., 2016). Both *nifJ* (K03737, closely related to *por*) and *korABCD* (K00174-7) are widespread in the genomes of prevotellas using the Kyoto Encyclopedia of Genes and Genomes orthology database in the Hungate 1000 collection of ruminal prokaryotes (<https://img.jgi.doe.gov/>). Although apparently not in a *bkd* operon, an acyl-CoA dehydrogenase (Clusters of Orthologous Groups database, COG1960) is flanked by *etfAB* genes (electron transfer using ferredoxin or flavodoxin as a cofactor) in the genomes of those prevotellas and likely has activity similar to that of isovaleryl-CoA dehydrogenase, which would be the first step in catabolism of BCVFA-CoA. Unfortunately, annotations are limited, and very few biochemical assays for these reactions are published for ruminal bacteria because of the strictly anaerobic conditions needed, but research is critically needed to explain how bacteria such as the dominant *Prevotella* group coordinate the anabolic and catabolic pathways intersecting through the BCVFA.

CONCLUSIONS

Dosing BCVFA increased NDF degradability in continuous cultures but did not increase bacterial protein synthesis. We expected that BCVFA would be highly required with faster bacterial growth (increased efficiency), and we expected to increase this efficiency with faster k_p . However, the lack of response in NDF degradability, bacterial efficiency, and gas emissions to changes in k_p suggest that we did not fully achieve our goal to effectively increase k_p of dense feed particles. Contrary to expectations, dosing BCVFA increased methane emission, likely due to increased NDF degradation. As expected, low pH decreased methane emissions, but the expected increase in H_2 emission and $H_2(aq)$ was not apparent in this study, and $[2H]$ was most likely redirected to production of reduced VFA such as propionate and valerate. Changes in pH affected the net production of BCVFA in continuous culture, with resulting mechanisms to be further explored in our companion paper.

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